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Evaluation of the neuroprotective effect of parkin in a α-synuclein-based rat model of Parkinson's disease

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To my parents and to Dario

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List of abbreviations

3-MT	3-methoxytyramine
5-HIAA	5-hydroxyindolacetic acid
5-HT	Serotonin
αSp22	α-syn O-glycosylated form
α-Syn	α-synuclein
AA	Ascorbic acid
AADC	L-amino acid decarboxylase
aCSF	Artificial cerebrospinal fluid
AR-JP	Autosomal recessive juvenile parkinsonism
CDCrel-1	Cell division control-related protein
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
COMT	Catechol-o-methyl-transferase
CPu	Caudate-putamen
DA	Dopamine
DAT	Dopamine transporter
DOPAC	Dihydroxyphenylacetic acid
Dx	Right
GFP	Green fluorescent protein
GPe	Globus pallidus, external part
GPi	Globus pallidus, internal part
MAO	Monoamine oxidase
HA	Hemagglutinin
HVA	Homovanillic acid
L-DOPA	L-dihydroxyphenylalanine
LBs	Lewy body
MPP^+	1-methyl-4-phenylpyridinium

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSNs	Medium spiny neurons
NE	Norepinephrine
Pael-R	Parkin-associated endothelin receptor-like receptor
PBS	Phosphate buffered saline
PD	Parkinson’s disease
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
Str	Striatum
Sx	Left
TH	Tyrosine hydroxylase
UA	Uric acid
UPS	Ubiquitin proteasome system

Abstract

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, characterized by progressive loss of nigrostriatal dopaminergic neurons and a reduction in striatal dopamine concentration. Different causal genes linked to rare familial forms of PD have been identified, while late-onset idiopathic PD is thought to result from the interplay between predisposing genes and environmental factors. Two missense mutations (A53T and A30P) in the *α-synuclein* (α-syn) gene have received great attention with the discovery that abnormal metabolism and accumulation of α-syn in dopaminergic neurons leads to both forms of PD. Parkin functions as an E3 ubiquitin ligase involved in the cellular machinery that tags proteins with ubiquitin, thereby targeting them for degradation by the proteasome: loss of its activity seems to cause an autosomal recessive form of PD. The ubiquitin-proteasome system plays a major role in many vital cellular processes, and its dysfunction has been implicated in the pathogenesis of neurodegenerative disorders. A number of observations suggest that functional interaction between α-syn and parkin may involve the proteasome: α-syn interacts with and may be degraded by the proteasome, over-expression of α-syn inhibits the proteasome and mutant α-syn increases the sensitivity of cells to proteasome inhibition. We described previously a hemi-parkinsonian rat model based on the stereotaxic injection of TAT-α-syn-A30P in the right *substantia nigra pars compacta* (SNpc). The protein transduction domain derived from the human immunodeficiency virus-1 TAT (transactivator) protein sequence allows diffusion of the fusion protein across the neuronal plasma membrane and results in a localized dopaminergic cell loss.

Here we examined possible neuroprotective effects of TAT-parkin against α-syn-induced neurotoxicity. Rats were stereotaxically injected with TAT-α-syn A30P, TAT-parkin, or both. At different times animals were subjected to different behavioral tests and the brains processed for immunohistochemical analysis. The behavioral tests showed a impairment in motor function after TAT-α-syn A30P, TAT-parkin or their combination, and postural asymmetry in all treated groups compared to control animals. Immuohistochemical analysis showed that the most severe depletion occurs in TAT-α-syn A30P + TAT-parkin treated animals, compared to control animals. Next, animals received an intra-nigral injection of different doses of TAT-parkin to evaluate a possible dose-dependent toxicity of TAT-parkin. Damage was again assessed using two behavioral tests, and showed variable impairments in motor function indicating that only the group receiving the lowest TAT-parkin dose is devoid of a toxic effect. All animals were subjected also to *in vivo* microdialysis to monitor striatal

dopamine release; microdialysis samples were analyzed by HPLC and showed an increase in dopamine release after stimulation with nicotine. Immunohistochemical analysis revealed tissue damage as a significant loss of dopaminergic neurons in the SNpc: only animals receiving the lowest dose of TAT-parkin have a less severe damage compared to control animals. In the last experiment we examined the possible neuroprotective effect of a non-toxic dose of TAT-parkin against a 50% higher dose of TAT-α-syn A30P. Behavioral testing revealed motor function impairment among all treated groups, but the highest TAT-parkin dose seems to ameliorate it better than lower doses. The microdialysis experiment confirms that the two doses of TAT-parkin stimulate the release of DA, and shows an increase in dopamine level even in groups with TAT-α-syn A30P lesion, especially with the lowest dose of TAT-parkin. Immunohistochemical analysis revealed an increase in dopaminergic fiber optical density when TAT-parkin is used with a higher dose of TAT-α-syn A30P. The α-syn-parkin-based model reproduces the pathophysiology of PD and could be of utility to understand the mechanisms leading to dopaminergic neurodegeneration, thereby helping to identify disease-modifying strategies as opposed to therapies which provide only symptomatic relief.

Riassunto

Il morbo di Parkinson è una patologia neurodegenerative del sistema nervoso centrale dovuta alla progressiva perdita dei neuroni dopaminergici della sub stantia nigra pars compacta (SNpc), e dalla presenza di inclusioni intracellulari, denominate corpi di Lewy, nei restanti neuroni. La diagnosi clinica di morbo di Parkinson viene effettuata in presenza di rigidità muscolare, bradicinesia e tremore a riposo, a cui vengono aggiunti una serie di sintomi secondari, quali l'alterazione della postura, disfunzioni cognitive (demenza, psicosi) ed alterazioni dell'umore (depressione, ansia, anedonia). L'eziopatogenesi rimane tuttora sconosciuta, anche se evidenze sperimentali suggeriscono che molteplici fattori, soprattutto di tipo genetico ed ambientale, possono contribuire al suo sviluppo. Nonostante sia solo una ristretta minoranza di persone ad essere colpita da forme di Parkinson familiare, si stanno studiando i meccanismi genetici che stanno alla base di questi casi, nella speranza di individuare i meccanismi patogenetici responsabili di tutte le forme di Parkinson, questo perché le più recenti evidenze scientifiche hanno attribuito alla α-sinucleina (α-syn) un ruolo chiave nell'insorgenza e nel progressivo peggioramento della malattia, sia di tipo idiopatico che genetico. Due mutazioni puntiformi (A30P e A53T) di questa proteina, infatti, portano a due varianti ereditarie di Parkinson di tipo autosomico dominante, inoltre l'α-syn è altamente espressa nei corpi di Lewy, tipicamente riscontrati nei pazienti affetti da Parkinson idiopatico. L'α-syn è una proteina presinaptica la cui funzione fisiologica è ancora poco chiara. Le ipotesi più accreditate suggeriscono un suo ruolo nel coinvolgimento nei processi di plasticità sinaptica, accumulo e rilascio di neurotrasmettitori in vescicole. Per quanto concerne la sua capacità di promuovere la neurodegenerazione, è ormai accertato che diversi fattori possono indurre una sua modifica conformazionale portandola da uno stato solubile ad uno fibrillare insolubile, quest'ultimo responsabile della tossicità. 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 o a stress ossidativo, sembra costruire il meccanismo patogenetico del Parkinson giovanile, portando ad un accumulo delle proteine ed alla disregolazione del metabolismo della dopamina. In questo contesto è rilevante notare che recenti studi hanno evidenziato che l'α-syn potrebbe essere un substrato della parkina e che quest'ultima possa avere un ruolo protettivo nella sopravvivenza dei neuroni dopaminergici nigrali. Alla luce di queste ultime evidenze, il progetto di ricerca ha avuto lo scopo di

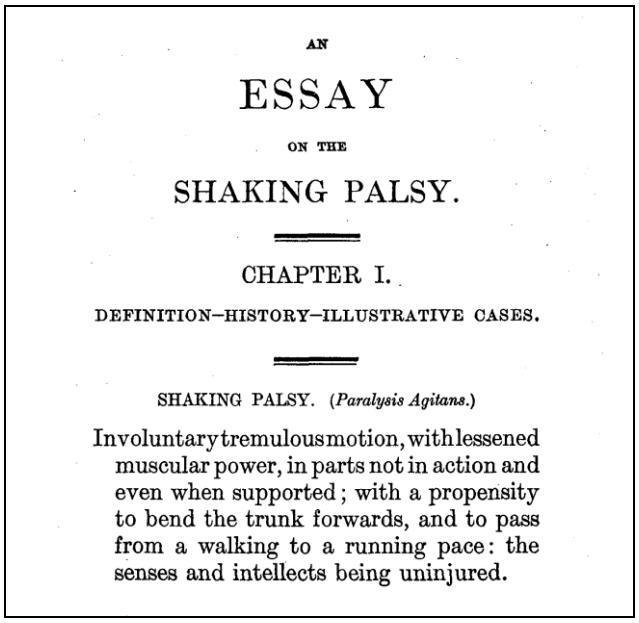
indagare il potenziale effetto neuro protettivo della parkina nel modello animale di Parkinson basato sull'iniezione intra nigrale unilaterale dell'α-syn A30P attaccata ad una sequenza di traslocazione cellulare TAT in grado di favorire la diffusione di macromolecole attraverso la membrana cellulare. Dopo la purificazione di TAT-α-syn A30P e di TAT-parkina, un primo gruppo di ratti ha ricevuto l'iniezione intra nigrale delle proteine singole o di entrambe. Il test comportamentale del RotaRod ha evidenziato una significativa riduzione della capacità motoria non solo nei due gruppi di animali che avevano ricevuto le due proteine singolarmente ma anche, contrariamente a quanto ipotizzato, anche nel gruppo che le aveva ricevute entrambe. Questo dato è stato in seguito confermato anche dall'analisi immunoistochimica condotta contro l'enzima tirosina idrossilasi (TH). Da questo primo esperimento è stato possibile notare come, l'effetto della TAT-parkina non risulta essere protettivo nei confronti del danno causato da TAT-α-syn A30P. Questo potrebbe essere causato dalla somministrazione di una dose eccessiva di TAT-parkina e a tal proposito è stato progettato un esperimento per testare le condizioni di somministrazione e le concentrazioni più opportune di TAT-parkina al fine di ottenere un effetto neuroprotettivo e non tossico. Sia il test comportamentale del RotaRod che l'analisi immunoistochimica hanno evidenziato una dipendenza della tossicità dalla dose, da cui emerge che la dose più bassa di TAT-parkina testata (0.0375 μg) abbia un effetto tossico inferiore rispetto alle altre dosi testate. Gli animali sono stati inoltre sottoposti a microdialisi cerebrale e dall'analisi dei dati è possibile notare come, a seguito della stimolazione con nicotina, gli animali iniettati con la TAT-parkina abbiano un progressivo aumento di rilascio di DA al diminuire della dose di proteina iniettata rispetto agli animali di controllo. Da questi risultati sembra che la dose più bassa di TAT-parkina abbia un effetto tossico inferiore rispetto alle altre dosi testate. A tal proposito è stato progettato un ulteriore esperimento per valutare un possibile effetto neuroprotettivo della TAT-parkina alla dose di 0.0375 μg nei confronti del danno causato da una dose di TAT-α-syn A30P del 50% superiore rispetto a quella usata nel primo esperimento. Gli animali sono stati divisi in 6 gruppi di trattamento ed hanno ricevuto o una dose di TAT-α-syn A30P del 50% superiore rispetto a quella testata precedentemente, o TAT-parkina (0.15 e 0.0375 μg) singolarmente o la combinazione di entrambe. I dati comportamentali sono risultati in accordo con quanto visto negli esperimenti precedenti, ovvero le proteine singolarmente hanno entrambe effetti tossici, mentre la capacità motoria migliora leggermente quando vengono entrambe iniettate. Dall'analisi dei dati di micro dialisi è emerso che, a seguito della stimolazione con nicotina, gli animali iniettati con la TAT-parkina evidenziano, in accordo con i risultati trovati in

precedenza, un aumento di rilascio di DA al diminuire della dose di proteina iniettata rispetto agli animali di controllo. Gli animali lesionati con TAT-α-syn A30P sono caratterizzati da una diminuzione nel rilascio di DA, mentre gli animali che hanno ricevuto entrambe le proteine hanno due rilasci intermedi rispetto ai controlli ed ai trattati con TAT-parkina singolarmente. L'analisi immunoistochimica ha confermato la diminuzione della positività dopaminergica alla TH, nei gruppi di animali iniettati con le proteine singole, mentre ha messo in luce un aumento della stessa negli animali che avevano ricevuto entrambe le proteine. Complessivamente i dati ottenuti suggeriscono che vi possa essere una correlazione tra l'attività della parkina ed il grado di lesione causato da α-syn. Le caratteristiche di questo modello sperimentale potrebbero pertanto contribuire allo studio ed alla comprensione delle fasi iniziali della malattia di Parkinson ed allo sviluppo di nuove strategie terapeutiche.

1. Introduction

1.1 Parkinson's disease

The history of Parkinson's disease (PD) began in 1817, when British apothecary James Parkinson (1755-1824) published "An Essay on the Shaking Palsy", where he first described the medical history of six individuals by observing their daily walks. The purpose of this observation 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, 2000).



AN

ESSAY

ON THE

SHAKING PALSY.

CHAPTER I.

DEFINITION—HISTORY—ILLUSTRATIVE CASES.

SHAKING PALSY. (*Paralysis Agitans.*)

Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured.

Fig. 1: First page of James Parkinson's "Essay on the Shaking Palsy" (1817).

The term "Parkinson's disease" was later coined by two French neurologists, Jean Martin Charcot and Alfred Vulpian (1861-1862); they described the mask face, some forms of contraction of hands and feet, akathisia and rigidity as PD features (Lees, 2007).

Prior to Parkinson's descriptions, others had already described features of the disease that would bear his name. Indeed, several early sources describe symptoms resembling those of PD. A 12th century b.C. Egyptian papyrus mentions a king drooling with age and the Bible contains a number of references to tremor (Lees, 2007). An ayurvedic medical treatise from the 10th century b.C. describes a disease that evolves with tremor, lack of movement, drooling and other symptoms of PD. Moreover, this disease was treated with remedies derived from the macuna family, which is rich in L-DOPA (Garcià Ruiz, 2004). Galen wrote about a disease that almost certainly was PD, describing tremors that occur only at rest,

postural changes and paralysis (Lanska, 2010). After Galen there are no references unambiguously related to PD until the 17th century. In this and the following century several authors wrote about elements of the disease, preceding the description by Parkinson. Franciscus Sylvius, like Galen, distinguished tremor at rest from other tremors, while Johannes Baptiste Sagar and Hieronymus David Gaubius described festination, a term for the gait abnormalities characteristic of PD (Koehler and Keyser, 1997). John Hunter provided a thorough description of the disease, which may have given Parkinson the idea of collecting and describing patients with "paralysis agitans" (Currier, 1996). Finally, Auguste François Chomel in his pathology treatise, which was contemporary to Parkinson's essay, included several descriptions of abnormal movements and rigidity matching those seen in PD (Garcia Ruiz, 2004). The coming of the 20th century greatly improved knowledge of the disease and its treatments and PD was then known as “paralysis agitans” (shaking palsy).

Parkinson’s disease occurs with a prevalence of 0.1% and mean onset between 50 to 60 years of age, although earlier onset may occur (Tanner and Goldman, 1996; Langston, 1998). The main clinical features defined by Parkinson are akinesia (absence of movements), bradykinesia (slowness of movement execution), hypokinesia (reduced movements), rigidity, tremor, and postural imbalance. Symptoms gradually develop over a period of years and often go unnoticed (reviewed in Marsden 1994; Nutt 2001; Sian, 1999). Many parkinsonian patients also display non-motor symptoms such as depression, hypovolemic voice (whispering), anxiety, autonomic dysfunction and cognitive impairments that resemble frontal lobe dementia (Marsden 1994; Martignoni, 1995; Owen, 2004; Owen, 1992). Also micrographia, shrinkage of handwriting and mask-like expression of the face are frequent symptoms (Olanow, 2004).

The etiology of PD is still largely unknown. Genetic factors are believed to play a role, and mutations in the *parkin* and *α-synuclein* genes have been identified in about 10% of all PD patients (Kitada, 1998). Environmental factors may also be important. Pathogenic mechanisms are multiple and include abnormal protein processing, inflammation, oxidative stress and mitochondrial dysfunction. The main neuropathological feature underlying PD is the progressive degeneration of midbrain dopaminergic neurons in the *substantia nigra pars compacta* (SNpc). The death of nigral neurons leads to dopamine (DA) deficiency in the striatum, to which the nigral neurons project. The total number of midbrain dopaminergic neurons is estimated to be approximately 550,000 (Pakkenberg, 1991). These neurons are lost during normal ageing at a rate of approximately 5% per decade (Fearnley and Less, 1991). In

PD the progression of dopaminergic cell death in the midbrain and the concomitant loss of dopaminergic fibers in the caudate-putamen (CPu) is accelerated 10-fold compared to healthy controls. The first symptoms of PD usually appear when 50-60% of the dopaminergic neurons have degenerated and 70-80% of striatal DA level is lost (Marsden 1990). Thereafter, the extent of nigral cell loss is correlated to the severity of the disease symptoms (Riederer and Wuketich, 1976; Fearnley and Less, 1991; Foley and Riederer, 1999). Neurodegeneration in PD extends beyond the dopaminergic system; the noradrenergic, serotonergic and cholinergic systems are also affected (Jellinger, 1990). PD thereby also affects the cerebral cortex, the olfactory bulb and the autonomic nervous system.

1.2 Dopamine and dopaminergic synapses

Dopamine is a catecholamine neurotransmitter present in a wide variety of vertebrates and invertebrates. DA functions as a neurotransmitter and is produced in several brain areas, including the *substantia nigra* (SN) and the *ventral tegmental area* (Benes, 2001). DA is implicated not only in PD (Serra, 2003a) but also in cognitive functions (O'Neill, 2005) and in the reward system (Wightman and Robinson, 2002).

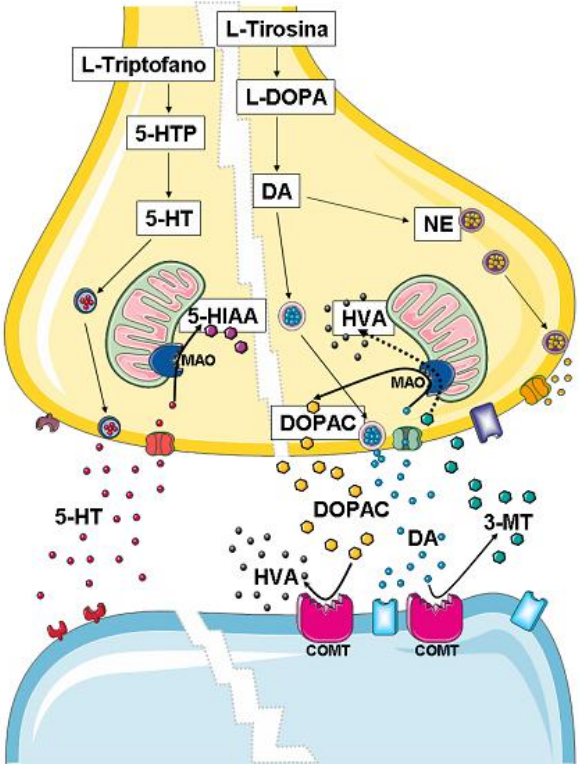


Fig. 2: Schematic representation of the striatal dopaminergic synapse and DA metabolism.

Dopaminergic neurons in the SN express the gene for tyrosine hydroxylase (TH), which converts tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). In striatal axon terminals L-DOPA is converted into DA by aromatic L-amino acid decarboxylase (AADC).
From here L-DOPA is concentrated in presynaptic vesicles and released in response to an action potential. DA released into the synaptic cleft binds to postsynaptic striatal D1 and D2 receptors to exert its action. Excess synaptic DA is taken up into the presynaptic terminal via the DA transporter (DAT) and re-stored in vesicles. Free DA in the presynaptic terminal is inactivated by monoamine oxidase (MAO) with intracellular production of 3,4-dihydroxyphenylacetic acid (DOPAC), which then diffuses into the synaptic cleft. Catechol-O-methyl-transferase (COMT) transforms DA into 3-methoxytyramine (3-MT) and DOPAC to homovanillic acid (HVA). The 3-MT thus produced enters the presynaptic neuron where MAO converts this to HVA - the final product of DA metabolism (Fig. 2). Under conditions of oxidative stress, a significant amount of extracellular DA is converted to a DA-ortochinone by auto-oxidation

1.3 Functional organization of the basal ganglia

The motor signs and symptoms of PD are attributed to dysfunction of the basal ganglia circuitry. The basal ganglia is a group of nuclei in the brain interconnected with the cerebral cortex, thalamus and brainstem. They are involved in a number of separate functions including motor, cognitive and limbic processes (Mink and Thach, 1993). The basal ganglia nuclei receive major input from the cerebral cortex and thalamus and send their output back to the cortex (via the thalamus) and to the brain stem (Mahlon R. Delong, 2000). They consist of four parts that play a major role in normal voluntary movements: the striatum divided into *caudate* and *putamen* (CPu in rodents), the *globus pallidus*, external and internal part (GPe, GPi) (GP and EP respectively in rodents), the *subthalamic nucleus* (STN) and the SN. The SN consists of a DA-producing structure, the SNpc, and a γ-aminobutyric acid (GABA)-ergic part, the *substantia nigra pars reticulata* (SNpr) (Fig. 3). Signals between these nuclei regulate motor output from the cortex in a complex manner. The *striatum* (Str) is the major input nucleus that receives excitatory (glutamatergic) input from the cerebral cortex and the thalamus. The Str is composed of one principal neuron cell type, the Medium Spiny Neurons (MSNs). MSNs are the major input target and the major projection afferents of the Str,

organized into two distinct pathways: the so-called direct and indirect pathways (Gerfen, 1992b).

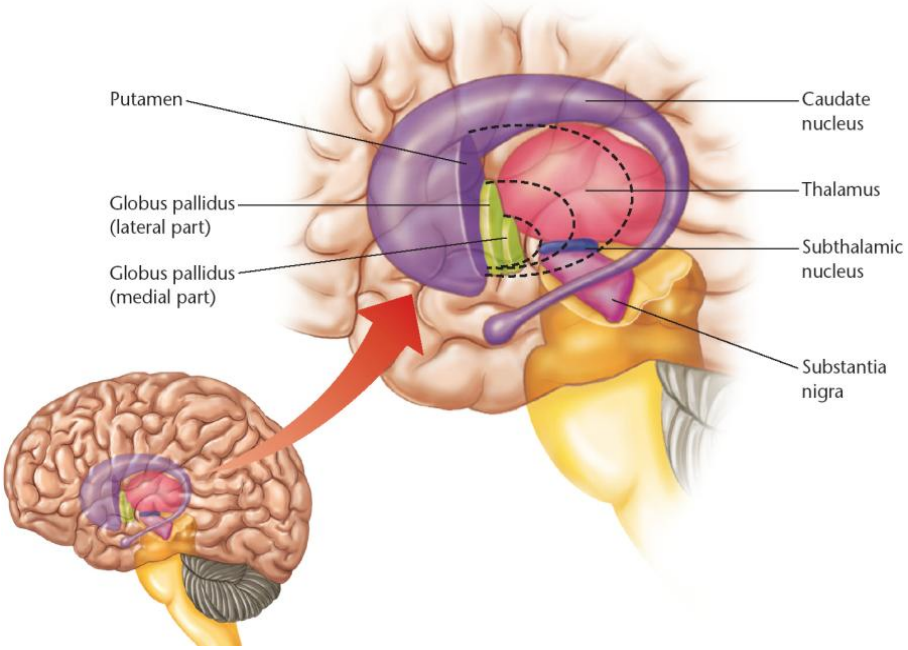


Fig. 3: Basal ganglia circuitry (http://cti.itc.virginia.edu).

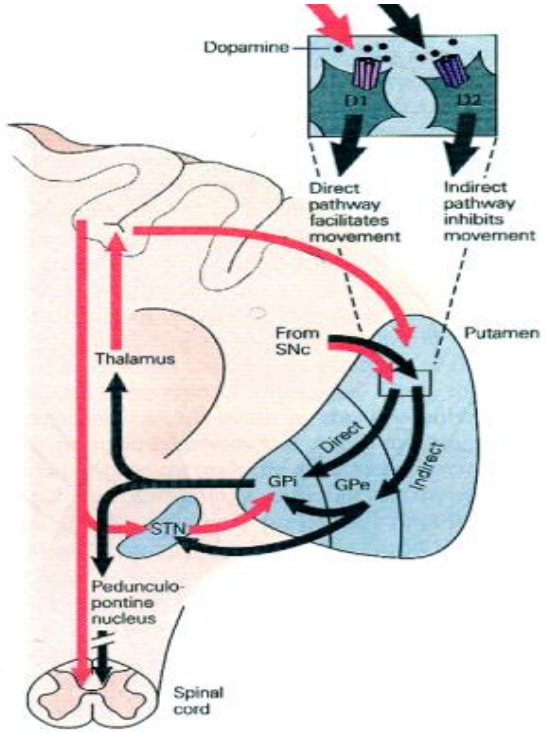


Fig. 4: Simplified model of the basal ganglia circuitry (modified from Kandel, Schwartz, Jessell, 2000). Deficiency of DA in PD therefore results in akinesia, hypokinesia, or bradykinesia (Prasad,

1998) (Fig. 5). L-DOPA uptake and metabolism are altered in the brain after severe DA denervation. In the intact dopaminergic system the processing of exogenous L-DOPA occurs primarily in nigrostriatal neurons, where AADC converts L-DOPA to DA. The latter is then loaded into synaptic vesicles by vesicular monoamine transporter-2, and released into the extracellular space by physiological stimuli. Clearance of DA from the extracellular space is ensured by DAT, a highly efficient reuptake transporter present on the plasma membrane of nigrostriatal neurons (Raevskii, 2002). After its reuptake, cytosolic DA is subjected to enzymatic breakdown by MAO and COMT.

About one-half of MSNs project directly to the GPi and SNpr, which are the output stations of the basal ganglia communicating with the rest of the brain. This direct pathway provides a direct inhibitory control over the basal ganglia output nuclei, and ultimately leads to disinhibition of the thalamus. The other half of MSNs creates the indirect pathway. Here the MSNs project to the GPe, that in turns projects to the STN. The STN provides an excitatory transmission to the basal ganglia output nuclei (GPe and SNpr) that ultimately leads to inhibition of the thalamus (Rouse, 2000). Activation of the direct pathway results in net *excitation* of the thalamocortical circuits and facilitation of movements. Activation of the indirect pathway increases excitatory drive to the basal ganglia output nuclei, resulting in *inhibition* of the thalamocortical circuit and impairment of movement (Fig. 4).

A: PHYSIOLOGICAL B: PARKINSON'S DISEASE

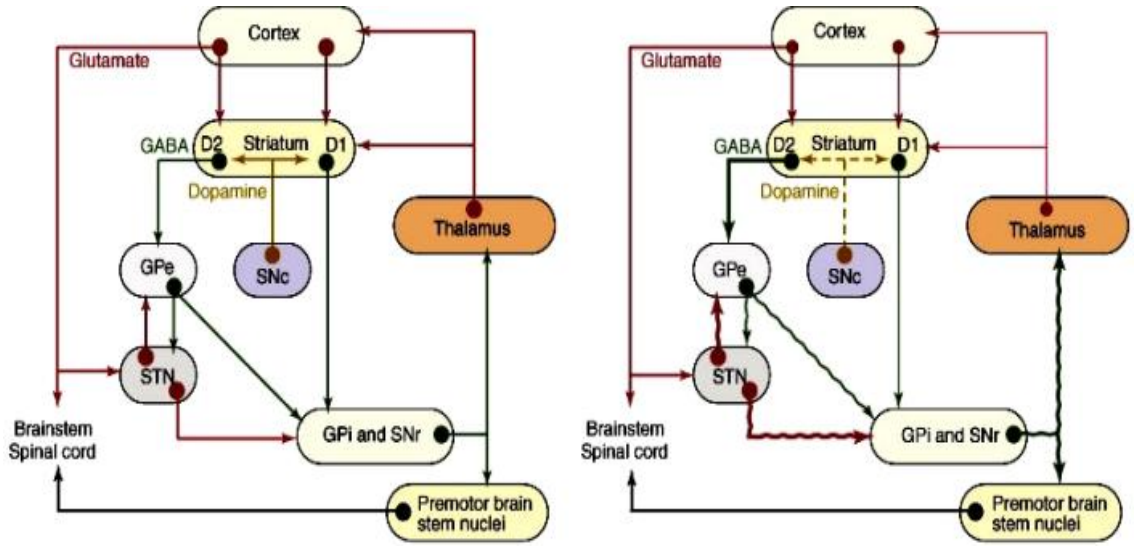


Fig. 5: Schematic overview of basal ganglia pathways. In the physiological state (A), glutamate input from the motor and premotor cortex projects in the direct pathway via Str to GPi/SNpr. Alternatively it projects in the indirect pathway to GPe and STN. In this state DA regulates the two different pathways. However, in the parkinsonian state (B), the loss of nigral DA causes a pronounced

activation of indirect pathway that causes an excessive inhibition of movement (modified from Cenci, 2007)

DA is involved in the modulation of both pathways in order to permit normal sequences of movement to occur. The role of DA-containing nigrostriatal afferents is to enhance transmission through the direct pathway and suppress transmission through the indirect pathway. The net effect of DA, then, is to enhance positive feedback to the cortical motor areas and facilitate volitional movements (Fig. 5).

1.4 Treatments

Since the late 1960s, when Arvid Carlsson first discovered that L-DOPA could improve motor symptoms caused by DA depletion in animals, this drug has been the gold standard treatment for PD (Carlsson, 1957). L-DOPA is produced from tyrosine by TH. It is the precursor molecule for the catecholinergic neurotransmitters DA, norepinephrine and the hormone epinephrine (adrenaline). L-DOPA is able to cross the blood-brain barrier whereas DA itself cannot. Once L-DOPA has entered the central nervous system (CNS), it is metabolized to DA by AADC, elevating striatal levels and relieving the motor symptoms of PD. However, conversion to DA also occurs in peripheral tissues, causing adverse effects and decreasing the available DA to the CNS. It is therefore standard practice to co-administer a peripheral inhibitor of the breakdown enzyme to restrict the conversion of L-DOPA to the brain (Olanow, 2004). The treatment is generally very good initially. However, over a time-course of 4-6 years of treatment with L-DOPA, approximately 30% of patients develop therapy-related complications known as dyskinesia. Motor fluctuation and dyskinesia eventually affect about 90% of PD patients (Marsden, 1990; Lang and Lozano, 1998: Quinn, 1998). Motor fluctuations are seen as abrupt changes between “on-periods” when the drug provides a good anti-parkinsonian treatment and “off-periods” when the response “wears off” and the PD symptoms worsen.

Dyskinesia denotes the presence of hyperkinetic and dystonic abnormal involuntary movements and postures, and was first described by Cotias in 1967 (reviewed extensively in Nutt, 1990; Nutt, 2001; Obeso, 2000a; Obeso, 2000c). In many patients dyskinesia becomes more severe as drug treatment continues, and eventually overshadows the beneficial effects of L-DOPA pharmacotherapy (Nutt, 1990). Apart from debilitating motor side effects, L-DOPA

treatment may also induce psychiatric disturbances such as psychosis and mania in PD patients (Schrag, 2004; Wolters and Berendse, 2001). Treatment options for the complicated stages of PD are surgical interventions, either by implanting stimulation electrodes (deep brain stimulation) or by making small lesions in parts of the basal ganglia network. Only patients that do not respond to medical therapy are generally considered for surgery; on the other hand, this treatment is contraindicated in patients with severe psychiatric or cognitive problems.

A surgical treatment of choice is high frequency stimulation of the STN (Ashkan, 2004). Transplantation of immature fetal DA neurons has been clinically tested since the mid 1980s (Backlund, 1985; Bjorklund, 2003). The procedure is unfortunately hampered by ethical issues and limited by the small supply of donor tissue. Furthermore, several patients developed severe dyskinesia after transplantation. Transplantation may represent a treatment option in the future, provided that functional and safe DA-producing stem cells can be generated and that graft-induced dyskinesia can be prevented or managed (Lindvall and Hagell, 2000; Mendez, 2002). As of today, no intervention to arrest, reverse or slow down the disease progression exists, although cigarette smoking, coffee consumption and the use of non-steroidal anti-inflammatory drugs are proposed to protect against its development (Korell, 2005). However, several treatment interventions, both pharmacological and surgical, are available for symptomatic relief.

In Sweden there have been clinical trials aiming at replacing the lost DA cell with transplantation of fetal DA-producing cells into the striatum. Both in animal models and clinical studies such transplantations have shown major improvement in many motor deficits (Lindvall, 1994; Winkler, 1999). However problems such as large inter-individual variability in the clinical outcome, and the occurrence of post-operative dyskinesias, i.e. Off-L-DOPA limit this approach.

Many patients are symptomatically relieved by surgical intervention with either deep brain stimulation or surgical lesioning of, e.g. GPe or STN. Unfortunately, surgical therapy is costly and invasive and not suitable for patients with psychological deficits or at an advanced age (Baba, 2005). The medical management of PD has seen much effort directed to improving DA therapies as well as identifying non-DA drugs. Among the latter, anti-cholinergic drugs (that have actually been used decades before the introduction of L-DOPA), adenosine A2a antagonists (Fuxe, 2001) and N-methyl-D-aspartate receptor antagonists (mainly as adjunct to L-DOPA pharmacotherapy) are used (Schwarzschildn, 2006). Since the early 1960s, DA replacement therapies have been extensively used with predictable effects (and side-effects).

Dopamine agonists (mainly activating D2 and D3 receptors) are currently in use, as are inhibitors of DA metabolism such as MAO and COMT. A longer duration of action may account for the lower incidence of motor complications with DA agonists (Nutt et al., 2000). To date, none of the synthetic DA agonists have surpassed the efficacy of L-DOPA, which remains a cornerstone of anti-parkinsonian therapy. Despite its effective control of motor symptoms, L-DOPA causes high levels of motor complications, particularly involuntary movements, i.e. dyskinesia.

1.5 Etiology of PD

The etiology of PD is unknown, although the role of environmental and genetic factors in disease onset has received much attention. The hypothesis of environmental factors has predominated in the last century, mainly due to the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism and the examples of post-encephalic PD. The discovery of genes implicated in PD (1980) has renewed interest in genetic susceptibility factors. Both factors are likely to play a role in triggering PD. Another possibility, which does not relate to either genetic or environmental factors, is that endogenous neurotoxins are responsible for neurodegeneration in PD. Alteration of normal metabolism can create toxic substances as a result of exposure to environmental hazards or altered metabolic pathways (Dauer and Przedborski, 2003).
Whatever is the insult that triggers neurodegeneration, studies of models of PD suggest two main hypotheses for the pathogenesis of the disease:

- Misfolding and aggregation of proteins
- Mitochondrial dysfunction and oxidative stress

1.5.1 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 (Dauer and Przedborski, 2003). All genes implicated in PD such as PARK1 (α-synuclein (α-syn)), PARK2 (parkin), PARK7 (DJ-1), and

PARK5 (ubiquitin C-terminal hydrolase L-1) are more or less directly involved in altering the ubiquitin proteasome system. Under physiological conditions, the complex ubiquitin proteasome system is a deputy to the degradation of mutant, damaged or misfolded proteins (McNaught and Olanow, 2003). Its failure results in the accumulation of abnormal proteins that cannot be removed; their subsequent aggregation into insoluble inclusions determines the alteration of homeostasis and cell integrity (Forno, 1996). Genetic causes account for 2-3% of late-onset cases and ~50% of early-onset forms of PD (Farrer, 2006). Typical, late-onset PD with Lewy body (LBs) pathology is linked to mutations in three genes: SNCA encoding for α-syn, (Polymeropoulos, 1997), LRRK2/*dardarin* encoding for leucine-rich repeat kinase 2 (LRRK2) (Zimprich, 2004) and EIF4GI encoding for the elongation initiation factor 4G1. Gain-of-function mutations in these genes lead to an autosomal dominant form of PD, resulting in the clinical manifestation of parkinsonism. Additional mutations linked to early-onset PD are found in affected individuals under the age of 45 and account for about 1% cases of all types of PD. They are autosomal recessive loss-of-function mutations in both alleles of the genes encoding parkin (Kitada, 1998), DJ-1 (Bonifati, 2003) and PIK1 (Bonifati, 2005), resulting in the clinical manifestation of parkinsonism.

1.5.2 Environmental factors

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). Research has concentrated on environmental susceptibility factors such as viruses (*Encephalitis Lethargica*), toxins (e.g. MPTP), other agents like herbicides, insecticides and pesticides (e.g. paraquat and rotenone), exposure to heavy metals, well-water ingestion, head injury and lack of exercise (Bower, 2003; Elbaz and Tranchant, 2007, Thacker, 2008).

The idea that a deficiency in oxidative phosphorylation could play a role in the pathogenesis of PD arose when it was discovered that MPTP inhibited complex I of the mitochondrial electron transport chain (Nicklas, 1987). Later studies identified abnormalities in the activity of complex I in PD patients (Greenamyre, 2001). Inhibition of complex I increases production of $O_2^{\cdot -}$, which can lead to the formation of hydroxyl radicals or react with nitric oxide to form peroxynitrite. These radicals cause cellular damage by reacting with nucleic acids, proteins and lipids. One of the targets of these molecules seems to be the same

electron transport chain (Cohen, 2000), which causes further mitochondrial dysfunction and production of reactive oxygen species.

1.6 α-Synuclein

α-Syn was initially isolated from *Torpedo* cholinergic synaptic vesicles (Maroteaux, 1988), and later as the non-amyloid component of plaques from Alzheimer's disease brains (Ueda, 1993). This small soluble protein of 14-19 kDa expressed primarily in presynaptic terminals in the CNS is phylogenetically conserved and abundant with widespread expression throughout the CNS (Maroteaux, 1988). α-Syn belongs to the synuclein family that includes β-syn and γ-syn, which so far have been described only in vertebrates (George, 2002). α-Syn and β-syn are predominantly expressed in brain at presynaptic terminals, particularly in the neocortex, hippocampus, Str, thalamus and cerebellum (Iwai, 1995). γ-Syn is highly expressed in various areas of the brain, particularly in the SNpc and is over-expressed in some breast and ovarian tumors (Lavedan, 1998). The α-syn, β-syn and γ-syn genes have been mapped to human chromosomes 4q21, 5q35 and 10q23, respectively (Lavedan, 1998; Spillantini, 1995). The sequences of all synucleins are similar (George, 2002), although only α-syn is implicated in disease. Human α-syn is an abundant presynaptic protein with a perinuclear localization first identified as the precursor of a peptide, called the non-Aβ component, present in extracellular amyloid plaques in some forms of Alzheimer's disease patients (Iwai, 1995). This protein lacks a secondary structure in solution and therefore exists in a random coil conformation with a propensity to fold into a α-helical configuration upon interaction with lipid membranes (Chandra, 2003). Further structural changes to a β-sheet conformation readily occur when α-syn is exposed to conditions of molecular crowding, changes in pH and interactions with highly reactive molecules such as DA (Kowall, 2000). β-Sheet formation leads to toxic oligomers and protofibrils, which are seen in a variety of synucleopathies including PD. The normal function of α-syn remains elusive and no one specific role has been ascribed to this protein.

Interest in α-syn has intensified following the discovery that two missense mutations, A53T and A30P in the α-syn gene appear to account for rare cases of autosomal dominant early-onset PD in families of European origin (Polymeropoulus, 1997; Kruger, 1998). The sequence of α-syn can be subdivided into three distinct domains (Fig. 6). The highly conserved amino-terminal domain of α-syn (residues 1-65) includes six copies of an unusual 11 amino acid imperfect repeat that display variations of a KTKEGV consensus sequence and is unordered

in solution, but can shift to an α-helical conformation (George, 2002) that appears to consist of two distinct α-helices interrupted by a short break (Chandra, 2003). The amphipathic α-helix is reminiscent of the lipid binding domains of class A_2 apolipoproteins (Davidson, 1998). In agreement with these structural features, α-syn avidly binds to negatively charged phospholipids and becomes α-helical upon binding (Eliezer, 2001; Davidson, 1998), suggesting that the protein may normally be membrane-associated (Davidson, 1998). Several recent studies (Conway, 2001) have shown that lipidic environments that promote α-syn folding also accelerate α-syn aggregation, suggesting that the lipid-associated conformation of α-syn may be relevant to α-syn misfolding in neurodegenerative diseases. The α-helix forming domain hosts two independent missense mutations (Fig. 6) at position 53, changing an Ala to Thr (A53T), and at position 30, changing an Ala to Pro (A30P); these have been shown to cause autosomal dominant heritable early-onset PD (Clayton, 1999).

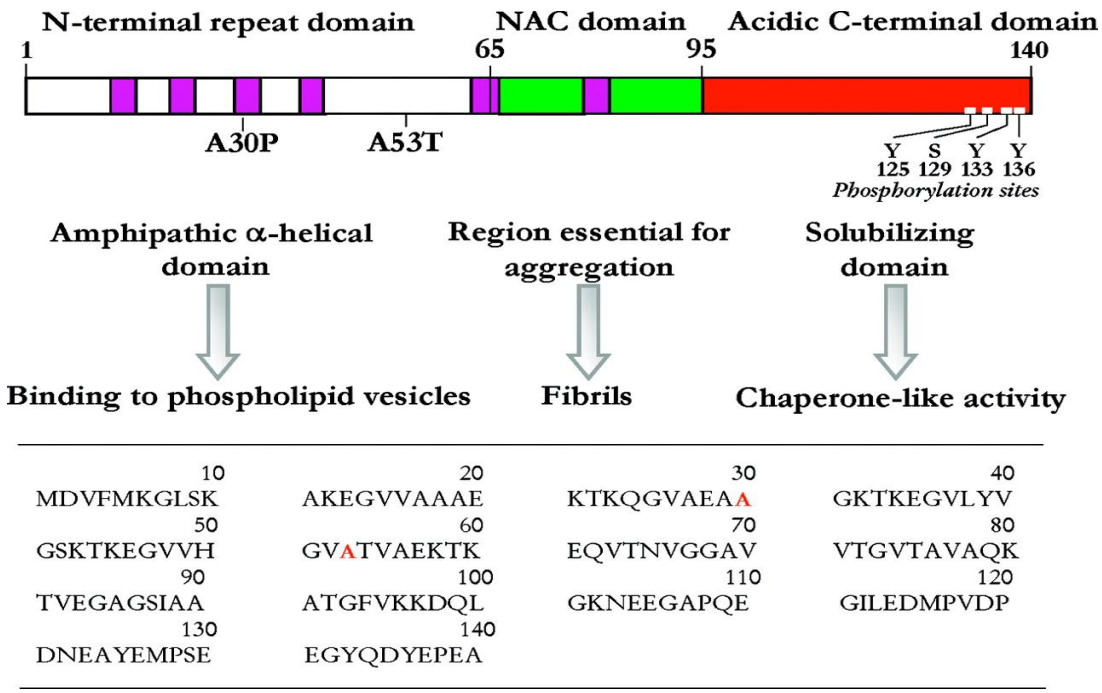


Fig. 6: Human α-syn sequence and domains. The imperfect KTKEGV repeat regions are shown in violet. Missense mutations at residues 30 (A30P) and 53 (A53T) are shown in red (Recchia, 2004).

Recently, a short triplication containing the α-syn gene plus flanking regions on chromosome 4 and a novel E46K mutation in α-syn have been identified in separate kindreds, in which individuals manifest classical PD or dementia with LBs (Singleton, 2003). The aggregation and accumulation of these abnormal α-syn proteins in dopaminergic neurons have

been postulated to be responsible for subsequent neurodegeneration (Kruger, 1998). α-Syn aggregation is present also in the classic form of PD and in other CNS disorders, called α-synuceopathies, which include Alzheimer's disease (Recchia, 2004; Goedert, 2001). α-Syn is a major component both of LBs filaments and of dystrophic Lewy neurites (Goedert, 2001; Conway, 1998). A recent study has shown that aggregated α-syn mediates dopaminergic neurotoxicity in vivo (Periquet, 2007). The mechanism of α-syn-induced toxicity is unclear but may be related to the propensity of normal α-syn and its mutated forms (A53T, A30P, E46K) to self-aggregate at higher concentrations, producing fibrils (Wood, 1999) with amyloid-like cross-beta conformation (Serpell, 2000). PD-associated α-syn is more fibrillogenic than β- and γ-syn (Biere, 2000). The protofibrillization rate of both α-syn mutants is higher than that of the wild-type protein (Conway, 2000). This complex behavior of α-syn has made difficult the development of models for studying synucleopathies in neurodegenerative disorders, particularly PD.

1.6.1 Physiological function and role in PD pathogenesis

The normal physiological role of α-syn is just beginning to be elucidated, and the prevalent presynaptic distribution may modulate synaptic vesicle function (Kahle, 2001). It is found in presynaptic nerve terminals in close association with synaptic vesicles and binds reversibly to brain vesicles and components of the vesicular trafficking machinery (Jensen, 2000) via vesicles budding or turning over (Rajagopalan, 2001). In striatal dopaminergic terminals, α-syn participates in the modulation of synaptic function, possibly by regulating the rate of cycling of the readily releasable pool (Abeliovich, 2000). Biochemical and biophysical evidence is also consistent with a role for α-syn in cellular membrane dynamics: α-syn binds to lipid membranes, changing the conformation of a protein's previously unfolded N-terminus to a stable α-helical secondary structure (Eliezer, 2001), suggesting that membrane binding elicits a functionally important alteration in the protein. Other studies point to the cellular membrane as a key site of α-syn action (Ahn, 2002). The membrane-related function may be trafficking proteins to the plasma membrane, as suggested by the demonstration that α-syn could be involved in membrane localization of the DA transporter (Lee, 2001). α-Syn knockout mice exhibit enhanced DA release at nigrostriatal terminals only in response to paired electrical stimuli, suggesting that α-syn is an activity-dependent, negative regulator of dopamenergic neurotransmission (Abeliovich, 2000). Electron microscopic observations show that depletion with antisense nucleotides of α-syn from cultured primary hippocampal

neurons decreased the distal pool of presynaptic vesicles (Murphy, 2000). It has also been suggested that α-syn functions as a chaperone protein in vivo as it appears able to interact with a variety of ligands and cellular proteins (Ostrerova, 1999; Xu, 2002), thus modifying their activities. Ostrerova (1999) reported that the N-terminal portion of α-syn shares 40% homology with molecular chaperone 14-3-3, the latter particularly abundant in brain, and suggests that the two proteins subserve the same function (Fig. 7) (Ostrerova, 1999; Boston, 1982). α-Syn could thus help the cell deal with effects of increased stress as part of an initial effort to protect itself against the accumulation of damaged proteins (Ostrerova, 1999). However, over-expression of wild-type α-syn is toxic to dividing cells, and over-expression of the mutant A53T and A30P forms results in even greater toxicity (Ostrerova, 1999), which may due to interaction of α-syn with proteins involved in signal transduction. The fact that α-syn is abundant in LBs suggests that its propensity to misfold and form amyloid fibrils may be responsible for its neurotoxicity in pathological situation such as PD, and that pathogenic mutations endow it with a toxic gain-of-function (Kruger, 1998). Many studies support this notion and link the pathogenesis of PD to other neurodegenerative diseases that involve protein aggregation (Goedert, 2001).

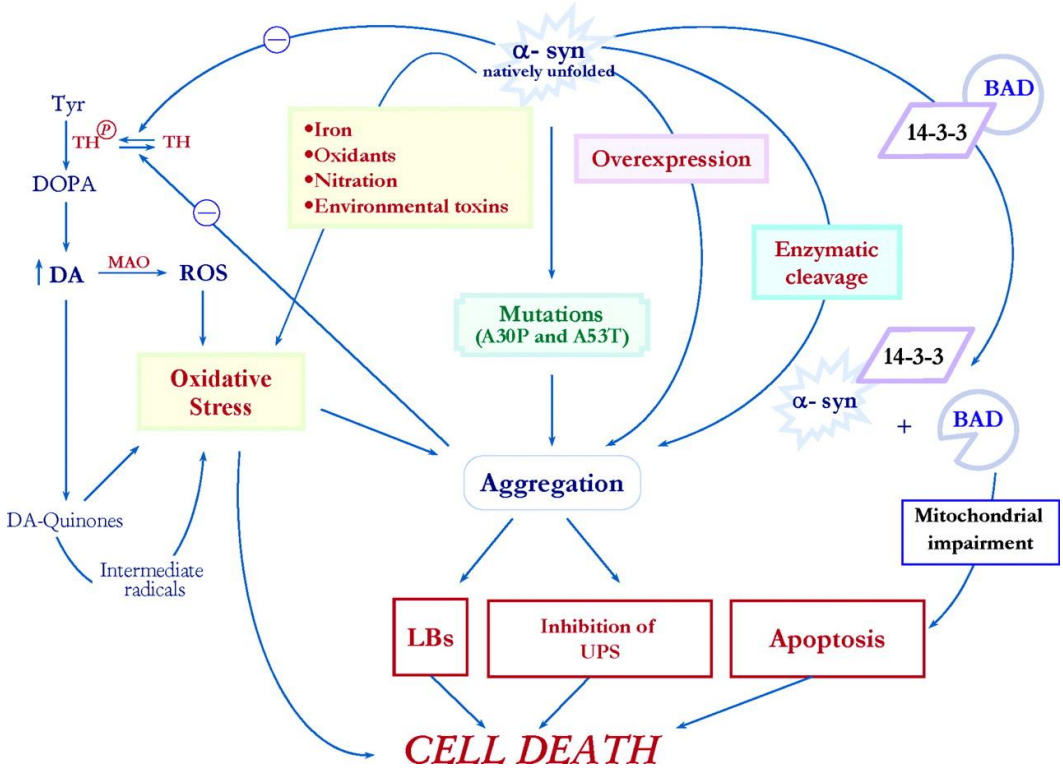


Fig. 7: α-Syn aggregation and toxic effects in dopaminergic neurons. A hypothetical scheme depicts various pathways involving aggregation of natively unfolded α-syn, oxidative stress, mitochondrial impairment, and cell death (Recchia, 2004).

Both wild-type and mutant α-syn form amyloid fibrils resembling those seen in LBs (Giasson, 1999) as well as nonfibrillary oligomers (Conway, 1998), termed "protofibrils". Since the α-syn A53T and A30P mutations promote protofibril formation (Conway, 1998), they may be the toxic species of α-syn. Consistent with this view and the association of α-syn with synaptic vesicles, protofibrils may cause toxicity by permeabilizing synaptic vesicles (Volles, 2001, Lashuel, 2002), allowing DA to leak into the cytoplasm and participate in reactions that generate oxidative stress. Furthermore, the selective vulnerability of dopaminergic neurons in PD may derive from the ability of DA itself to stabilize these noxious α-syn protofibrils (Conway, 2001). Considering the important role that α-syn may play in the pathogenesis of sporadic as well as familial PD, there is clearly a need to develop well-characterized α-syn-based animal models of PD to further clarify disease etiopathogenesis and for designing new and more efficacious therapeutics. In an attempt to better reproduce the key features of human PD, particularly the progressive nature of neurodegeneration, an alternative approach to the development of PD models has been explored which builds and the genetic and neuropathological links between α-syn and PD.

1.7 Parkin

The PARK2 gene was identified in 1998 in Japanese families as a causative gene for autosomal recessive PD, and was named *parkin* by Mizuno and colleagues (Kitada, 1998). Mutations in the *parkin* gene are a major cause of autosomal recessive early onset parkinsonism, with slow progression and additional features such as dystonia (Thomas, 2007). For instance, autosomal recessive juvenile parkinsonism (AR-JP) as a clinically defined entity has been studied for decades, primarily in Japan (Yamamura, 1973; Ishikawa, 1996). Subsequent studies suggest that parkin mutations are the most numerous case of AR-JP with an average onset before 40 years of age, but late onset cases have also been described (Winklhofler, 2007). Prior to identification of the *parkin* gene, the defining characteristics of AR-JP were: the existence of affected family members within the same generation, often born to consanguineous parents, an early onset (before 40 years old), a parkinsonian syndrome with a number of atypical clinical features, the absence of LBs in the brain (Von Coeln, 2004), foot dystonia at onset, hyperreflexia of the lower limbs, diurnal fluctuations with a marked sleep benefit, good response to L-DOPA therapy, early onset of L-DOPA-induced dyskinesias, and slow disease progression (Ishikawa, 1996). However, the full variety and complexity of this familial form of PD was only appreciated once its cause was defined at the

molecular level, thereby allowing for the accurate diagnosis, even in cases not fitting these criteria (Abbas, 1999; Klein, 2000).

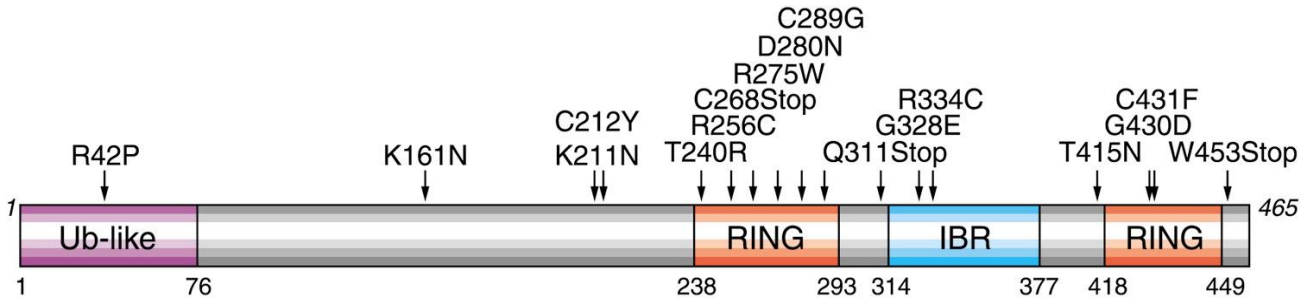


Fig. 8: Schematic representation of the parkin protein. Parkin consists of several domains. an amino-terminal ubiquitin-like domain (Ub-like) and two carboxy-terminal RING finger domains separated by an IBR. The location of disease-related point mutations resulting in amino acid substitutions or protein truncation is depicted. Additional frameshift and deletion mutants have been described (Giasson, 2001).

In 1997, the gene locus (PARK2) of AR-JP was mapped to chromosome 6q25.2-27, and spans more than 55 kbp (Matsumine, 1997). Its 12 exons, separated by extended intronic regions, encodes a 465-amino acid protein of an apparent molecular weight of 52kDa with a unique modular structure (Kitada, 1998) (Fig. 8). As a consequence, is among the largest genes in the human genome (Kitada, 1998; West, 2001). Parkin is an evolutionary conserved gene product, with orthologos in *Caenorhabditis elegans, Drosophila melanogaster*, mouse, rat, and other species (Bae, 2003). Parkin harbors a ubiquitin-like domain at the N-terminus and a RBR (RING between RING fingers) domain close to the C-terminus, consisting of two RING finger motifs separated by a cysteine-rich IBR (in between ring) domain (Winklhofer, 2007). Interestingly, the parkin promoter functions as a bidirectional promoter, regulating not only transcription of parkin but also transcription of a gene upstream of and antisense to PARK2 (West, 2003). The mutations that initially led to identification of the parkin gene were large homozygous deletions of one or five exons, respectively (Kitada, 1998). Numerous mutations have since been identified: deletions of single or multiple exons, duplications or triplications of exons, frameshift mutations, and point mutations that can be subdivided into missense, nonsense, and splice site mutations (Abbas, 1999; West, 2002). The majority of point mutations localize to the RING-IBR-RING domain in the C-terminus half of parkin and, in particular, to the first RING domain (R1), implying essential functional relevance for this region of the protein. Parkin mutations associated with AR-JP occur as either homozygous or

compound heterozygous mutations, i.e. with different mutations on both alleles (Von Coeln, 2004). Mutations in the *parkin* gene lead to a loss of parkin function through decreased catalytic activity, aberrant ubiquitination, and impaired proteasomal degradation of mutant parkin (Winklhofer, 2003). Thus, the general view is that disease-causing mutations in parkin lead to a loss of parkin function, albeit through different mechanisms. In addition, parkin is inactivated by nitrossidative stress, dopaminergic stress, and oxidative stress, which are pathogenic processes in sporadic PD (Dawson, 2010).

1.7.1 Physiological function and potential neuroprotective role in PD

Like many other proteins containing a RING domain, parkin functions as an E3 ligase (Shimura, 2000). E3 ligases are part of the cellular machinery that tags proteins with ubiquitin, thereby targeting them for degradation by the proteasome. The ubiquitin-proteasome system plays a major role in many vital cellular processes, and its dysfunction has been implicated in the pathogenesis of neurodegenerative disorders (Giasson, 2003; Ciechanover, 2003; Moore, 2003). Ubiquitin tagging of membrane-bound cytoplasmic and nuclear proteins identifies these molecules as a target for degradation by the proteasome and can also facilitate the endocytosis of plasma membrane proteins to lysosomes for degradation (Giasson, 2001). Ubiquitin is a 76-amino acid protein that is usually covalently linked to the ε amino group of Lys residues in the targeted proteins by the formation of an isopeptide with its C-terminus (Giasson, 2001). This posttranslational modification of proteins is called ubiquitination and occurs through sequential steps catalyzed by ubiquitin-activating (E1), conjugating (E2), and ligase (E3) enzymes (Fig. 9). In subsequent cycles of the same process, additional ubiquitin molecules are linked on the previously ligated ubiquitin, resulting in the formation of a polyubiquitin chain. This chain is the signal recognized by the proteasome complex (Von Colen, 2004). The ubiquitin-proteasome system is crucially involved in two tasks: the accurate and timely regulation of the level of short-lived proteins that play a key role in processes such as cell cycle progression and metabolism; protein quality control (Schubert, 2000). Furthermore, a variety of external stress factors can result in the misfolding of previously functional proteins, requiring either chaperone-mediated refolding activity or degradation of the misfolded proteins (Tanaka, 2001). Like other E3 ligases parkin can induce its own ubiquitination and degradation, and disease-associated mutations in the RING finger domains can impair these properties (Imai, 2000; Zhang, 2000). Parkin's E3 ligase and its

impairment by pathological mutations suggest that ubiquitination may be important in the etiology of PD (Giasson, 2001).

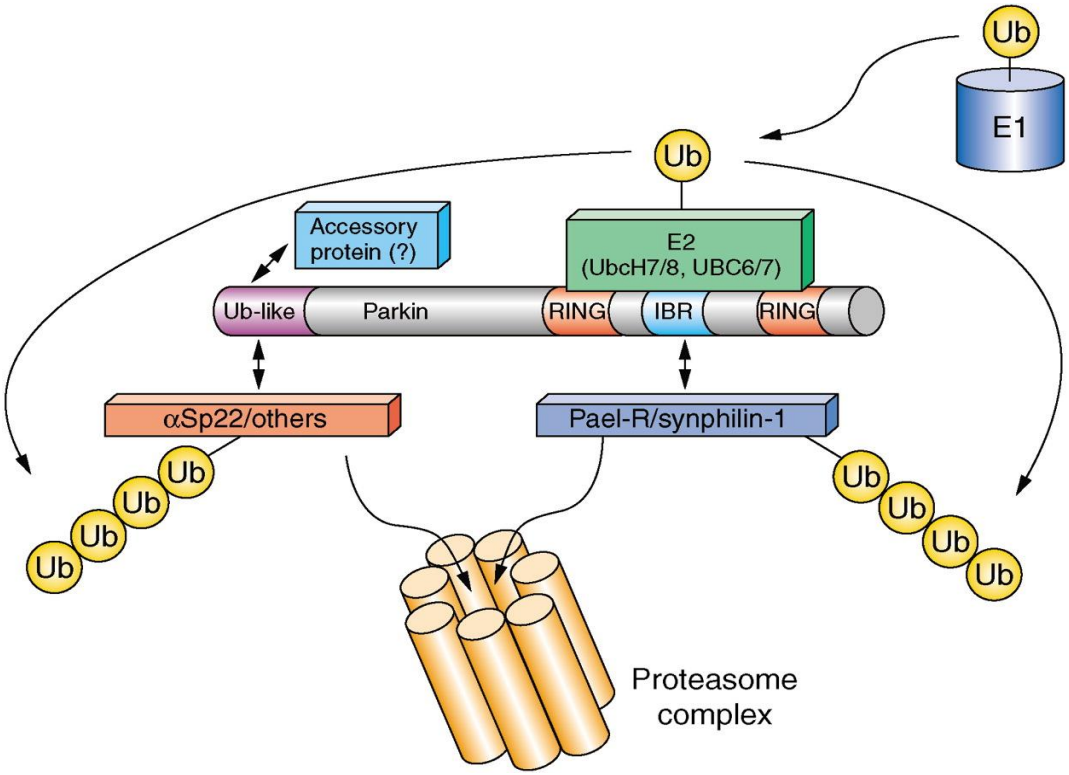


Fig. 9: Involvement of parkin in the ubiquitin-proteasome pathway. Parkin (E3) serves as a link between the substrate for ubiquitination and E2, which binds to the RING finger region. Alternate regions in Parkin may recognize different substrates. The ubiquitin-like region is either required for the recruitment of an accessory protein or the binding of some substrates that may include αSp22. Pael-R and synphilin-1 interact with the RING finger region, but synphilin-1 preferentially binds to the IBR region and the second RING finger domain. Ubiquitin moieties are transferred from E1 to E2 and finally to the substrate, which becomes a target for degradation by the proteasome (Giasson, 2001).

Different groups have recently demonstrated that parkin interacts with and promotes the ubiquitination of many substrates (Dawson, 2010). CDCrel-1 (cell division control-related protein) was the first substrate identified (Zhang, 2000) It belongs to a family of GTPases called septins and is predominantly expressed in the nervous system, where it is associated with synaptic vesicles (Beites, 1999). Synphilin-1 was originally identified as an interactor of α-syn involved in the formation of inclusion bodies in cultured cells. It interacts with and is ubiquitinated by parkin, leading to the formation of protein aggregates when over-expressed with α-syn (Engelender, 1999). The finding that α-syn is the major component of LBs, which are absent in parkin-associated PD, has fuelled the idea that parkin activity is required for the formation of LBs. α-Syn has therefore become an intensely studied candidate substrate of parkin (Chung, 2001).

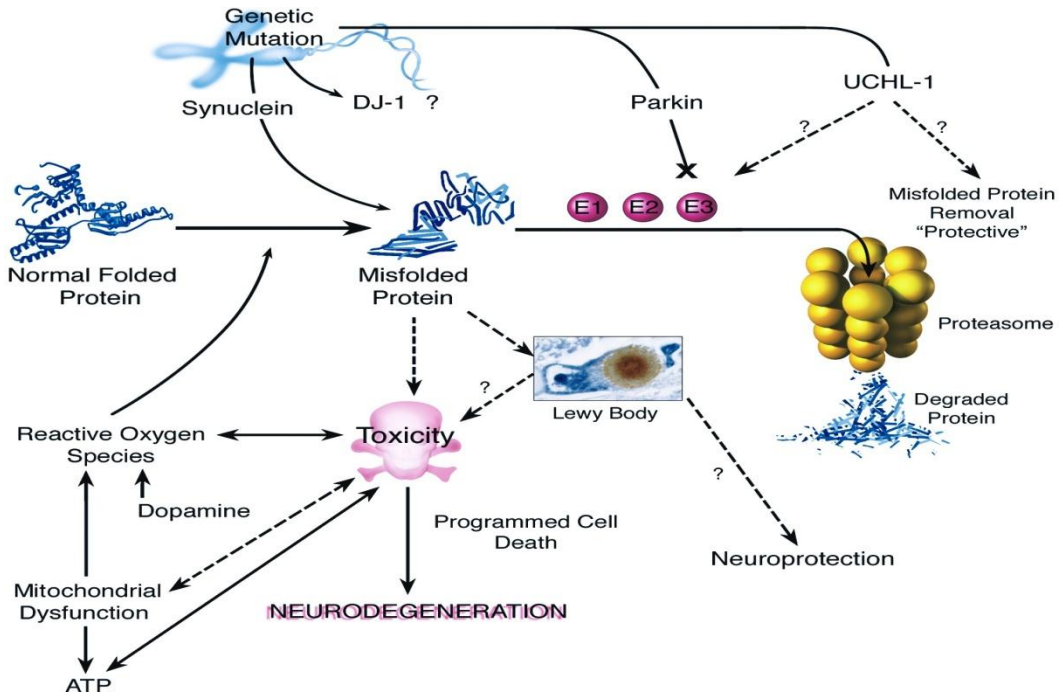


Fig. 10: Mechanisms of neurodegeneration. A growing body of evidence suggests that accumulation of misfolded proteins is likely to be a key event in PD neurodegeneration. Pathogenic mutations may directly induce abnormal protein conformations (as believed to be the case with α-syn) or damage the ability of the cellular machinery to detect and degrade misfolded proteins (Parkin, UHC-L1); the role of DJ-1 remains to be identified. Oxidative damage, linked to mitochondrial dysfunction and abnormal dopamine metabolism, may also promote misfolded protein conformations. It remains unclear whether misfolded proteins directly cause toxicity or damage cells via the formation of protein aggregates (LBs). Controversy exists regarding whether LBs promote toxicity or protect a cell from harmful effects or misfolded proteins by sequestering them in a insoluble compartment away from cellular elements. Oxidative stress, energy crisis (i.e. ATP depletion) and activation of programmed cell death machinery are also believed to be factors that trigger the death of dopaminergic neurons in PD (Dauer and Przedborski, 2003).

Three reports suggest a relationship between parkin and α-syn function (Shimura, 2001; Petrucelli, 2002) or aggregation (Chung, 2001). Notably, the E3 ligase activity of parkin modulates the sensitivity of cells to both proteasome inhibitor and mutant α-syn-dependent cell death (Petrucelli, 2002). A number of observations suggest that functional interaction between α-syn and parkin may involve the proteasome: α-syn interacts with and may be degraded by the proteasome (Ghee, 2000; Snyder, 2003), over-expression of α-syn inhibits the proteasome (Stefanis, 2001) and mutant α-syn increases the sensitivity of cells to proteasome inhibition (Tanaka, 2001a; Petrucelli, 2002) (Fig. 10).

Whereas the abundant, unmodified form of α-syn does not interact with parkin, a rare O-glycosylated form of α-syn (αSp22) has been identified that interacts with and is ubiquitinated

by parkin (Shimura, 2001). Also Pael-R (parkin-associated endothelin receptor-like receptor), a putative G-protein-coupled transmembrane protein with homology to the endothelin receptor type B, is another parkin substrate (Von Coeln, 2004). In the brain Pael-R is expressed in oligodendrocytes and in a few distinct subpopulations of neurons, including SNpc catecholaminergic neurons. When over-expressed in cultured cells, Pael-R becomes insoluble and unfolded and is a substrate of parkin ubiquitination (Imai, 2001) (Fig. 9). Recently a number of additional putative parkin substrates have been identified, including α/β-tubulin, which shows decreased stability in cells over-expressing parkin (Ren, 2003). Steady-state levels of synaptotagmin XI expression are decreased in the presence of parkin, and LBs are immunoreactive for this member of a large family of calcium-binding proteins (Huynh, 2003). SEPT5_v2/CDCrel-2, another member of the septin family and a close homolog to CDCrel-1 has been reported as a parkin substrate (Choi, 2003). Cyclin E specifically interacts with parkin in the context of complex formation of Parkin with hSel-10 and Cullin-1 (Staropoli, 2003). In primary neuronal cell cultures, knockdown of parkin increases kainate-induced accumulation of cyclin E and concomitant cell death, whereas over-expression of parkin has the opposite effect in this model of excitotoxicity (Staropoli, 2003).

Parkin can protect cells against a remarkably wide spectrum of stressors, including mitochondrial dysfunction, excitotoxicity, endoplasmic reticulum stress, proteasome inhibition and over-expression of α-syn (Moore, 2006). In line with a central role of Parkin in maintaining neuronal cell viability, *parkin* gene expression is up-regulated in various stress paradigms (Henn, 2007). In *Drosophila*, over-expression of parkin can suppress loss of dopaminergic neurons induced by α-syn or Pael-R (Yang, 2003; Haywood, 2004). Furthermore, lentiviral delivery of parkin prevents dopaminergic degeneration caused by mutant α-syn in a rat model and protects skeletal muscle cells against mitochondrial toxins (Lo Bianco, 2004; Rosen, 2006). Parkin is neuroprotective against stresses in which the direct relationship to its substrate 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, 2002; Kim, 2003; Chung, 2004). Parkin also suppresses mutant α-syn toxicity in *Drosophila* models (Yang, 2003). 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, 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, 2001) or in *Drosophila* (Yang, 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, 2001), which would not require E3 activity. There is also evidence for α-syn degradation by lysosomal proteases (Paxinou, 2001; Lee, 2004).

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 the parkin gene. Neither transgenic line shows loss of nigral neurons, although there are subtle changes in dopaminergic neurotransmission (Goldberg, 2003; Itier, 2003). Although *Drosophila* parkin knockouts did not exhibit loss of catecholaminergic neurons, they showed mitochondrial damage and apoptosis of flight muscles (Greene, 2003). Interestingly, one of the knockout mouse models reportedly had deficits in mitochondrial respiration (Palacino, 2004). The association of parkin with PD and its wide neuroprotective capacity makes parkin an attractive candidate for the development of prophylactic or therapeutic strategies. Based on knowledge of the physiology and pathophysiology of parkin, the following approaches are conceivable: increase parkin expression; prevent inactivation of parkin; modulate signaling pathways regulated by parkin (Winklhofer, 2007). It is reasonable, therefore, to hypothesize that enhancing the function of parkin might facilitate proteasomal processing in DA neurons, thereby protecting the dopaminergic nigrostriatal tract against multifactorial insults (Cookson, 2005).

1.8 Animal models of PD

In order to explore pathophysiological hypotheses of PD and test new therapies animal models are essential. There are several different animal models of PD, and differ with respect to mechanisms of dopaminergic cell death and the extent of DA-dependent motor deficits. The ideal model of PD would mimic the human condition aetiopathologically, in both the distribution of neurodegenerative damage and its temporal profile. As the cause of sporadic PD is not known, no model yet mimics all features of the disease but only particular aspects of the cell damage process, such as oxidative stress, mitochondrial dysfunction or protein aggregation (Mandel, 2003). Two types of models are commonly used in PD research: genetic models and toxin-based models (Dauer and Przdeborski, 2003). Genetic models, such as loss-of-function mutations of the *α-synuclein* and *parkin* genes in mice are used to study how deficits of the ubiquitin-proteasome pathway lead to protein aggregation with possible

neurotoxicity as a consequence. Several neurotoxins can be used to induce dopaminergic neurodegeneration of the SNpc and parkinsonian motor symptoms. More recently, the herbicide paraquat and the insecticide rotenone have received attention since it was found that animals chronically exposed to these pesticides display parkinsonian symptoms and possible degeneration of the nigrostriatal dopamine system (Betabert, 2000, McCormack, 2002). However, the two most extensively used and experimentally evaluated toxins are MPTP and 6-hydroxydopamine (6-OHDA). Both neurotoxins selectively kill midbrain cathecolaminergic (predominantly dopaminergic) neurons through oxidative damage and mitochondrial failure (Berretta, 2005; Elkon, 2004; Ferger, 2001).

Different animal models of PD are best suited for different types of applications, and model selection may condition the outcome of the study (Cenci, 2002). For example, studies of neuroprotection would require a model with slow and progressive degeneration of DA neurons. In contrast, restorative or symptomatic treatments that are meant to be applied in advanced stages of PD are better studied in animals with a severe and already established lesion. For this type of application, the mechanism of DA cell death may be less important, as long as the damage produced is stable and leads to quantifiable motor deficits (Beck, 1995; Tomac, 1995).

Lesion is most commonly peformed unilaterally, as animals receiving bilateral destruction of the DA system develop aphagia and adipsia and require extensive post-operative monitoring and care (Ungersterdt, 1971a; Zigmond and Stricker, 1972). Far from being a disadvantage, asymmetrical lesioning has been used to devise simple and objective tests for monitoring the effects of symptomatic or restorative treatments. Spontaneous rotational behavior is an expression of lesion-induced asymmetry in DA input to the basal ganglia, which can be further accentuated by the administration of amphetamine or other DA agonists (Ungerstedt, 1971b). An animal with unilateral DA depletion will turn away from the hemisphere where DA receptor stimulation is stronger, i.e. towards the side of the lesion after challenge with DA-releasing drugs, and away from the lesioned side after treatment with L-DOPA or direct DA agonists. The latter phenomenon is due to DA receptor supersensitivity on the side ipsilateral to the lesion (Creese, 1977; Marshall and Ungerstedt, 1977).The asymmetry is also an advantage in the molecular and biochemical analysis where the intact side is used as an internal control (Fig. 11).

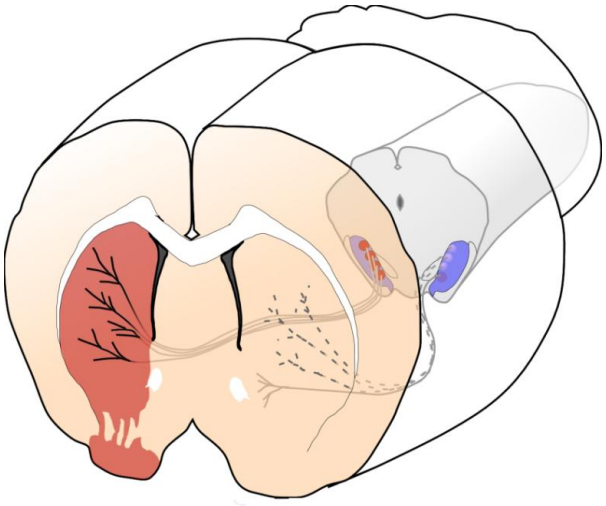


Fig. 11: Schematic representation of the unilateral lesion produced by injection of 6-OHDA into medial forebrain bundle. The injection induces an acute injury of the axon terminals of dopaminergic neurons. By using a technique of immunohistochemical staining with anti-tyrosine antibodies, one can highlight the surviving cells. On the left the dopaminergic fibers can be seen, while on the right there is an almost complete loss (>95%) striatal dopaminergic fibers.

1.8.1 6-OHDA

The unilateral 6-OHDA-model in the rat was the first animal model of PD associated with nigral dopaminergic neuronal death, introduced more than 30 years ago (Ungerstedt, 1968). This model is cost-effective and well standardized. 6-OHDA inhibits mitochondrial complex I and generate free radicals, resulting in oxidative stress (Olanow, 1993). 6-OHDA is a selective neurotoxin for catecholaminergic neurons and acts in a concentration-dependent way, entering dopaminergic and noradrenergic neurons via the DA and noradrenaline reuptake transporters. After intracerebral injection, 6-OHDA is taken up by DA neurons via DAT causing selective destruction of the dopaminergic cells. In order to obtain a near-complete lesion of the ascending nigrostriatal DA projections, 6-OHDA is injected into the medial forebrain bundle (MFB) close to the DA cell bodies in the SN. The MFB is a bundle of fibers, composed of axons of the dopaminergic, serotonergic and noradrenergic neurons, that project from the brainstem of the forebrain. The most important effect of the lesion is degeneration of the nigrostriatal pathway.

With the most commonly used procedures, reduction in striatal DA content is generally more than 97% and represents a very advanced stage of PD (Rioux, 1991b). Serotonergic and noradrenergic neurons are also affected, although the underlying mechanism is unclear (Ungerstedt, 1968; Takeuchi, 2003; Fulceri, 2006). The 6-OHDA lesion is usually performed

unilaterally, as bilateral injections induce severe aphagia and adipsia (Dunnett, 1983a). The DA depletion is completely stable within two weeks after 6-OHDA injection, producing a hemi-parkinsonian syndrome including asymmetries in body posture, locomotion, and sensorimotor deficits on the side contralateral to the lesion.

1.8.2 MPTP

MPTP neurotoxicity, discovered in 1983 in persons taking contaminated heroin, causes parkinsonism (Langston and Ballard, 1983) indistinguishable from idiopathic PD (Lau, 2005). The pro-toxin MPTP is highly lipophilic and, when systemically administered crosses the brain-blood barrier to enter the brain parenchyma where it is oxidized by MAO-B in astrocytes to 1-methyl-4-phenyl-2,3-dihydropyridinium. This intermediate then undergoes spontaneous oxidation to the active toxic molecule 1-methyl-4-phenylpyridinium (MPP^+) and is released into the extracellular space. MPP^+ enters DA neurons via the DAT and is concentrated within mitochondria, where it impairs oxidative phosphorylation by inhibiting complex I activity (Dauer and Przedborski, 2003). Alterations in energy metabolism and generation of free radicals lead to neurodegeneration of DA neurons. MPTP neurotoxicity in non-human primates was the first effective primate model of PD. Surprisingly MPTP, for reasons unknown, is unable to destroy the dopaminergic innervation in other species, except for certain strains of mice (C57 black and Swiss Webster) (Jenner, 2008). The MPTP-treated mouse, although widely used is not reproducible and robust. Stereotaxic injection of MPP- in rats has been use, but offers no advantages over the 6-OHDA rat model and is not routinely employed.

1.8.3 Rotenone

Rotenone is an odorless chemical that is used as a broad-spectrum insecticide and pesticide. It occurs naturally in the roots and stems of several plants such as the jicama vine plant. In mammals, including humans, it is linked to the development of PD (Tanner, 2011). Rotenone is highly lipophilic, diffuses rapidly in all organs (Talpade, 2000), freely crosses cell membranes and can accumulate within mitochondria. Rotenone damages mitochondrial oxidative phosphorylation by binding to and inhibiting NADH-ubiquinone reductase activity of complex I. It can also inhibit the formation of tubulin microtubules. As an excess of tubulin

monomers may be toxic to cells, this effect could be relevant in the mechanism of degeneration of dopaminergic neurons (Bové, 2005). In *in vivo* models, rotenone can be used with different routes of administration: oral administration, through the food, seems to be responsible for a reading neurotoxicity in animals (Marking, 1988); systemic administration, either intravenous or subcutaneous, often causes a dose-dependent neurotoxicity and mortality, while stereotactic administration of rotenone in the MFB causes striatal depletion of DA and serotonin (Heikkila, 1985). The intravenous infusion of low-dose rotenone causes selective degeneration of nigrostriatal dopaminergic neurons accompanied by inclusions similar to LBs immunoreactive for ubiquitin and α-syn (Betabert, 2000). From a behavioral point of view, systemic rotenone-treated animals show a reduced mobility, impaired posture and in some cases rigidity (Sherer, 2003) and catalepsy (Alam and Schmidt, 2002). In these studies it appears that motor dysfunction may be improved by administration of L-DOPA (Alam, 2004).

1.8.4 Paraquat

Paraquat is one of the most widely used herbicides in the world and is also toxic to humans and animals. For many years, experimental studies with paraquat have focused on its effects in lung, liver and kidney, probably due to the fact that death is due to acute exposure to this herbicide causing toxicity in these organs (Smith, 1988). Following systemic administration of paraquat, there is a reduction of motor activity, a dose-dependent loss of striatal dopaminergic fibers and nigral cell bodies (Brooks, 1999), and high levels of α-syn inclusions in neurons of the SN (Manning-Bog, 2002). Its toxicity is related to the formation of $O_2^{\cdot -}$ (Day, 1999), and several deaths have been reported due paraquat ingestion or exposure. Although paraquat's structure does not allow for ease of diffusion across the blood-brain barrier, brain damage has been reported in people who have died from paraquat poisoning.

1.8.5 α-Synuclein

The latest model of PD, in chronological terms, is that based on the neuropathological and genetic link between PD and α-syn. There are two animal models -transgenic and non-transgenic. In transgenic models of *Drosophila* and mice, there is over-expression of both

wild-type human and mutated α-syn (A30P and A53T). *Drosophila* models show a significant loss of DA, while mouse models do not show relevant anomalies as regards the dopaminergic neurons, although some show a synucleopathy-induced neurodegeneration. Transgenic mouse models are still an important tool in the study of α-syn toxicity *in vivo* and the causes that modulate aggregation. The non-transgenic models are based instead on the administration of α-syn, directly into the SNpc using viral vectors. Viral vectors, such as those derived from the HIV virus, permit administration in any brain area and at any time in the animal's life. They can also be used with rats, which is the preferred species for behavioral studies (Recchia, 2008). The administration of α-syn, mutant or wild-type associated to the carrier, causes selective dopaminergic neuron degeneration in the rat SNpc and the appearance of α-syn-containing cytoplasmic structures very similar to LBs (Recchia, 2008).

2. Aims of the thesis

In our laboratory we have recently developed an animal model of PD based on the intra-nigral injection of the A30P α-syn mutant fused to a TAT transduction domain. The fusion protein causes depletion of dopaminergic neurons (26.3 ± 5.0 %) and the appearance of evident motor symptoms, attributable to an early stage of PD (Recchia, 2008). A number of findings are beginning to strengthen the functional links between parkin, α-syn and proteasome function, supporting an essential role of parkin in the survival of dopaminergic neurons.

The purpose of this research project is to evaluate the possible neuroprotective effects of TAT-parkin in this animal model.

The specific aims of the different experiments are to:

- evaluate an absence of TAT-parkin dose-dependent toxicity;
- test neuroprotecion of TAT-parkin against different doses of TAT- α-synA30P.

If successful, these data may serve to encourage further studies in animal PD models to support the therapeutic potential of Parkin in this neurodegenerative disease.

3. Materials and methods

3.1 Cloning and TAT-fusion protein generation

3.1.1 Synthesis of TAT-α-synA30P

The fusion protein TAT-α-synA30P was generated as described (Albani et al., 2004). A human brain cDNA library (CLONTECH, Heidelberg, Germany) was used to amplify the human *α-syn* gene by polymerase chain reaction (PCR), using a specific primer complementary to the double-stained cDNA and based on the published sequence of the human *α-syn* gene:
primer a, 5′ATGGCTAGCATGGATGTATTCATGAAAGGAC3′;
primer b, 5′CGAAGCTTAGGCTTCAGGTTCGTAGTCTGG3′.
The purified PCR product was cut at the *NheI* and *HindIII* restriction sites and cloned directly into the bacterial expression vector pRSETB in the same restriction sites (plasmid pSyn). Site-directed mutagenesis of pSyn was then carried out to introduce the A30P α-syn mutation (plasmid pA30P-α-syn). A pTAT vector was constructed as reported for pTAT-HA (HA, hemagglutinin) (Becker- Hapak et al., 2001), and the sequence of α-synA30P was cloned after the TAT sequences between the *NheI* and *HindIII* restriction sites (plasmid pTAT-A30P-α-syn). A green fluorescent protein (GFP)-α-syn fusion protein was constructed using plasmid pGFP-CNTF (CNTF, ciliary neurotrophic factor) (Negro et al., 1997) as vector and replacing the CNTF sequence with that of α-syn. The coding sequence for GPF fused to α-syn was subcloned between the *NheI* and *HindIII* restriction sites of expression vector pTAT-HA to give pTAT-GFP-α-syn. All clonings were verified by sequence analysis at the CRIBI core sequencing facility (University of Padova).

3.1.2 Expression and purification of TAT-α-synA30P

The fusion protein expression construct pTAT-A30P-α-syn was transformed into *Escherichia coli* strain BL21 (DE3) pLysS; 2 l was grown in LB/ampicillin broth for 12 h with shaking at 37°C. Fusion protein production was induced by the addition of 500 μM isopropyl β-thiogalactoside. After 3 h of induction at 37°C, the cells were harvested. Because TAT-α-synA30P was produced as inclusion bodies, the protein product was liberated by resuspending the pelleted cells in inclusion body sonication buffer (500 mM NaCl, 20 mM Tris/HCl pH 8.0)

and heating for 10 min at 100°C. The lysate was then centrifuged at 11,000×g for 20 min and the supernatant retained. Twenty millimolar of imidazole was then added to the lysate and loaded onto a 2.5 ml Ni^{2+}-agarose column (Qiagen). After washing with 20 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl pH 8.0, the recombinant protein was eluted with 0.25 M imidazole. The recovered protein was dialysed against phosphate-buffered saline (PBS) and aliquots flash frozen in liquid nitrogen and stored at -80°C.

3.1.3 Synthesis of TAT-parkin

The pTAT-parkin plasmid was contructed essentially as described for TAT-α-syn (Albani et al., 2004), and was kindly provided by Prof. Alessandro Negro (CRIBI). A human placenta cDNA library (CA9, Clontech, Palo Alto, CA, USA) 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 *NhelI* 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-HA expression vector (Becker-Hapak et al., 2001) and the sequence of *parkin* was cloned after the TAT sequences between the *Nhel* 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).

3.1.4 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 isopropyl-1-β-

thiogalactoside. After 3 h the cells were harvested by centrifugation, washed with 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 agarose column (Quiagen, Hilden, Germany). The column was exhaustively washed with 6 M guanidinium-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 0.002% 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.

3.2 Animals

Adult male Sprague-Dawley rats (280 ± 20 g body weight at the beginning of the experiment) were obtained fron Harlan Italy (S. Pietro al Natisone, UD). Rats were housed under controlled conditions of temperature (23 ± 2°C) and illumination (12-h light; 12-h darkness; darkness: 7 p.m. – 7 a.m.) and had free access to a standard diet and water. All experiments were carried out from 10 a.m. to 5 p.m. and followed guidelines governing animal experimentation (European Union decree of 24/11/1986 (86/609) IIC)).

3.3 Lesion surgery

Rats (5 animals per group) were anesthetized intramuscularly with a solution of ketamine (87 mg/kg) plus xylazine (13 mg/kg) and secured in a Kopf stereotaxic apparatus, with the tooth bar set + 5 mm above the interaural line. Lesions were made by unilateral injection of TAT-α-synA30P (4 μg/μl), TAT-parkin (0.15 μg/μl) or both proteins into the right SNpc at the following coordinates: AP: − 5.7, LM: − 2.0: DV: − 8.7 mm from the Bregma (Paxinos and Watson, 1986). Control sham-operated animals received the same volume of vehicle (PBS 1X + glycerol 20%) at the same coordinates. Administrations were performed using a 27-gauge Hamilton syringe

connected to an infusion minipump (Harvard Compact Infusion Pump, Holliston; MT), at a rate of 0.5 µl/min. The syringe was left in place for 5 min before slowly retracting it to allow substance infusion and prevent reflux.

3.4. RotaRod test

The development and manifestation of motor deficits were monitored using the RotaRod test performed 15 and 60 days post-lesion. In this test the animals walk on a rotating drum and motor performance assessed as described (Hamm et al., 1994). The device consists of a plastic frame with a motorized, rotating assembly of rods (Geramotor, Bedine Electric Co., Chicago. IL, USA) on which the animal must walk. Sham animals were first assessed at five speeds of rotation: 8, 10, 12, 14 and 16 rpm. Each rat was allowed to remain stationary at 0 rpm for 10 s, after which the speed was suddenly increased to 8 rpm for 10 s and then again in the same manner to 10, 12 and 14 rpm for 10 s each. The highest speed, 16 rpm, was then maintained for 190 s until the 4-min test period elapsed. These rotational speeds were chosen so that sham animals would not fall off during the test. A rat was considered failing the 4-min period test if (a) it fell completely off the device before the time period elapsed or (b) it simply gripped the rungs and spun for two consecutive revolutions rather than actively walking on the rotating rods. Rats from all groups of treatment were then tested five times evry day over a 5-day period. The time average of all trials was utilized for data analysis.

3.5 Footprint test

Motor function can be assessed by analyzing footprint patterns and walking tracks (Klapdor et al., 1997). Briefly, this test was conducted in groups of rats treated with vehicle, TAT-α-synA30P or TAT-parkin after 30 days. Each rat had its hind feet dipped in food dye and was then placed on a gangway 100 cm long, 12 cm wide, and with 40 cm high side walls. The floor of the gangway was lined with ordinary paper. Rats were allowed to walk up the gangway five times without prior conditioning. The footprints were then scanned, stored as black/white bitmaps and evaluated using the ImageJ program that permits evaluation of the number of steps and % area of support legs.

3.6 Microdialysis probe construction and implantation

Single probes for striatal microdialysis were used for these experiments. The probes were contructed according to a coaxial geometry (Miele et al., 2000) using plastic-coated silica tubing (i.d. 75/ o.d. 150 μm, Scientific Glass Engineering, Milton Keynes, UK) placed in the center of a semi-permeable polyacrylonitrile dialysis fibre (molecular cut-off weight of 12 kD, Filtral 16 Hospal Industrie, France) with a final diameter of 220 μm. To complete the inlet a polyethylene tube (i.d. 580 μm, portex, Hythe, UK) was added to the silica tubing. The tips of the dialysis fibres were sealed and joined using quick-drying epoxy glue, while the other end of the fibre was introduced into another polyethylene tube that serves as outlet for sample collection. The semi-permeable membrane was coated with epoxy glue leaving an active length of 4 mm. The probe allowed us to collect a volume of dialysate enough to permit simultaneous measurement of DA, metabolites and amino acids without increasing the flow rate of perfusion or the interval sampling time.

The microdialysis probes were implanted in the right striatum, in proximity to the dopaminergic endings of nigrostriatal neurons, following the coordinates: AP: + 0.5, LM: − 2.5: DV: − 7.0 mm from the Bregma (Paxinos and Watson, 1986). Body temperature during anesthesia was manteined at 37°C by means of an isothermal heating pad.

3.7 Microdialysis and samples analysis

Following surgery, the animals were placed in large plastic bowls (50x50 cm), and mantained in a temperature- and light-controlled environment, with free access to food and water. Experiments were carried out 24 h after probe implantation with the animal in its home bowl. This arrangement allows the rats free movement. The composition of the artificial cerebrospinal fluid (aCSF) used was as follows, in mM: NaCl 147.0, KCl 4.0, $CaCl_2$ 1.2, $MgCl_2$ 1.0. A microinfusion pump (CMA/100, Microdialysis, Sweden) pumped aCSF into the probe at a flow rate of 1.5 μl/min using a syringe connected to the inlet by a length of polyethylene tubing. For intrastriatal administration, 5mM nicotine was dissolved in aCSF and infused for 1 h during the experiment. Every 20 min, 30 μl dialysate samples were collected manually from the outlet in 250 μl micro-centrifuge tubes (Alpha Laboratories, UK). After 60 min of stabilization, three

baseline dialysates were collected at 20-min intervals. Starting from 40 min, 5 mM nicotine was infused for 60 min and samples were continously recovered during drug infusion and for 60 min after nicotine discontinuation. The content of DA and its metabolites in microdialysis samples was analyzed by means of high performance liquid chromatography with electrochemical detection (HPLC-EC), using a Varian 9001 HPLC pump equipped with a Rheodyne injector (mod. 7725, Rohnert Park, CA, USA), a C18 reverse-phase column (Adsorbosphere C18, 100 mm x 4.6 mm i.d., particle size 3 μm) (Alltech, USA), an electrochemical detector BAS model LC4B, and a PC-based analog-to-digital converter system (VarianStar Chromatographic Workstation, Valnut Creek, CA, USA). The mobile phase was: KH_2PO_4 2.1 g/l, K_2HPO_4 0.7 g/l, EDTA 0.5 mM, MeOH 8.0%, and OSA 100 mg/l (pH=2.65); the flow rate was 0.8 ml/min and the potential applied was +780 mV. For the quantification of substances in the sample we used a mix of standards, whose concentrations were: AA 7.5 μM, UA 1.0 μM, NE 15 nM, L-DOPA 12.7 nM, DOPAC 0.6 μM, DA 7.0 nM, 5-HIIA 0.5 μM, HVA 0.5 μM, 3-MT 50 nM and 5-HT 5 nM.

3.8 Immunohistochemistry

Rats were sacrificed and their brains were removed, snap-frozen in a dry ice/isopentane bath and stored at -80°C. Serial coronal sections of 9 μm were cryostat cut from each SNpc. Immunohistochemistry was carried out on sections to visualize TH-positive fibers. Briefly, sections were fixed and dehydrated in methanol for 20 min at -20°C, incubated with 0.3% H_2O_2 for 20 min to inactivate endogenous peroxidase activity, followed by 20 min incubation with normal goat serum to block non-specific binding sites. Sections were then incubated with a mouse anti-TH antibody (Clone TH16, Sigma, 1:4000) for 1 h at room temperature followed by a 15 min rinse with PBS and incubation with a peroxidase-conjugated anti-mouse secondary antobody (ImmPress REAGENT Anti-Mouse IgG, Vector Laboratories, Burlingame, CA). The sections were finally treated with 3,3-diaminobenzidine to visualize the reaction product.

3.9 Image analysis

A series of regularly spaced sections across the SNpc were analyzed, starting with the first section on which the region appeared and continuing every 150 μm until the structure terminated.

Images of the right and left SNpc from each section were digitized at a primary magnification of 5X using a digital camera (Leica DC 200, Leica Microsystems GmbH, Wetzlar, Germany) mounted on a light microscope (Leica DM-R, Leica Microsystems GmbH, Wetzlar, Germany) and saved as TIFF files.

3.10 Statistical analysis

Data are expressed as the mean ± SEM, in spite of the probable non-normality of the distribution of scores. Rotarod and footprint test data were subjected to one-way nonparametric ANOVA, followed by Dunnett's and Bonferroni's Multiple Comparison Tests, respectively. HPLC data were analyzed by one-way ANOVA followed by Newman-Keul's Multiple Comparison Test. Significant differences in TH-positive cell numbers between right and left SNpc were determined using Student's t-test for paired data. The significance limit was set at $p<0.05$.

4. Results

4.1 Is TAT-parkin neuroprotective against TAT-α-synA30P?

The aim of the first experiment was to evaluate a possible neuroprotective effect of TAT-parkin in rats lesioned with a unilateral intranigral injection of TAT-α-synA30P. A total of 20 rats were subdivided into 4 different treatment groups, with the treatments described below:

- 3 µl vehicle (sham)
- 3 µl TAT-α-syn A30P, final dose of 4 µg
- 3 µl TAT-parkin, final dose of 0.15 µg
- 3 µl TAT-α-syn A30P + TAT-parkin, final dose 4 µg and 0.15 µg, respectively.

In the last treatment group both proteins were injected simultaneously. The RotaRod test, in which animals walk on a rotating drum, is widely used to assess motor status in laboratory rodents. All animals were tested, at 15 days post-surgical injury, for one trial/day of 5 sessions each for 5 consecutive days. For time analysis the time average for all sessions is considered.

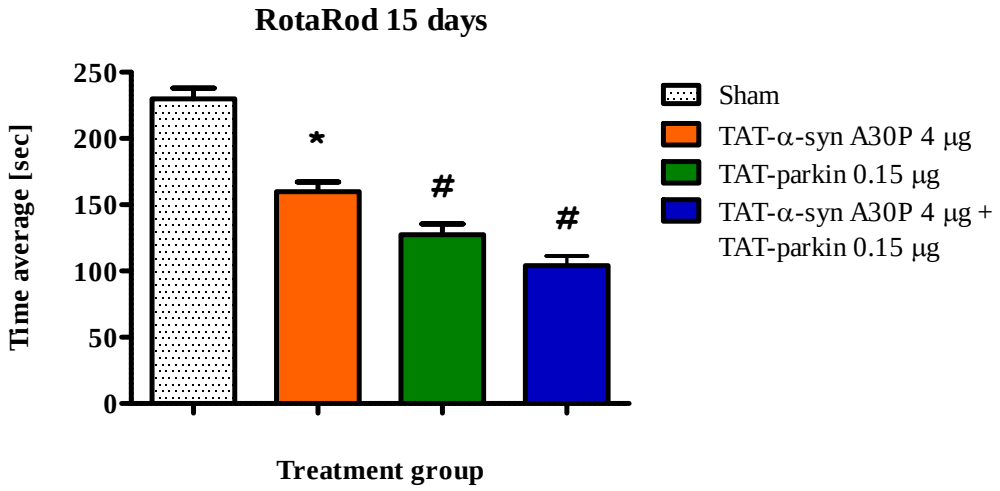


Fig. 11: RotaRod test. Running performance in rats receiving intranigral injection into the right SNpc of: vehicle, TAT-α-syn A30P, TAT-parkin or both proteins, performed 15 days post-lesion. The test reveals a impairment in motor function among the different treatment groups, especially in animals receiving TAT-α-syn A30P in combination with TAT-parkin. Data are means ± SEM. * $p<0.05$ vs all other groups, # $p<0.05$ vs TAT-α-syn A30P group (one way ANOVA followed by Newman-Keuls Comparison Test).

Sham rats have a time average of 229.8 ± 8.242 sec, and after a few sessions of learning all animals in this group can reach the end of each trial in all testing sessions. In the TAT-α-syn A30P-treated group, rats demonstrated a significative reduction in motor performance to 159.9 ± 7.737 sec compared to sham animals ($p<0.05$). Unexpectedly, the groups injected with TAT-parkin or the combination of both proteins showed a motor impairment statistically different to sham and TAT-α-syn A30P-treated animals, with an average of time spent on the rotating drum of 127.4 ± 8.121 sec and 104.0 ± 7.347 sec, respectively ($p<0.05$) (Fig. 11). The same test was repeated for all treatment groups 60 days post-lesion.

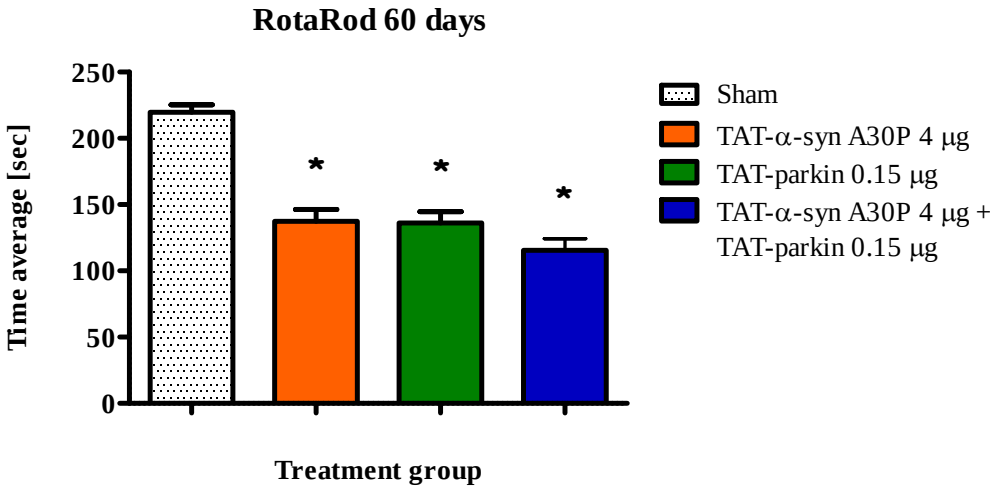


Fig. 12: RotaRod test. The evaluation of motor performance on the same animals was performed also 60 days post-lesion. Sham rats did not show any alteration in motor performance, while the deficit remains without significant variation among all other treated groups. Data are means ± SEM. * $p<0.05$ vs sham and TAT-α-syn A30P group (one way ANOVA followed by Newman-Keuls Comparison Test).

The test did not show any significant difference from the previous one (15 days), with sham animals having a normal motor performance with an average walking time of 219.6 ± 5.64 sec. Rats lesioned with TAT-α-syn A30P had a motor impairment progressively more evident with time (137.4 ± 8.901 sec). The two groups that received TAT-parkin alone or both proteins show a gradual increase in motor performance and the average of walking time increased to 136.2 ± 8.409 sec and 115.6 ± 8.671 sec, respectively (Fig. 12), confirming the motor dysfunction when compared to sham animals. After the last behavioral session all animals were sacrificed and brains processed for TH-immunostaining of SNpc dopaminergic

neurons (Fig 13).TH is the enzyme responsible for catalyzing the conversion of L-tyrosine to the DA precursor L-DOPA. This reaction is the rate-limiting step in catecholamine biosynthesis and occurs in the cytosol of all catecholamine-containing cells, making it useful to estimate dopaminergic degeneration extension. Injection of TAT-α-syn A30P or TAT-parkin into the right SNpc produced a significant decrease in TH immunoreactivity to 84.932 ± 6.818 % and 76.427 ± 10.491 % compared to SNpc dx/sx (right/left) of the sham animal group ($p<0.05$).

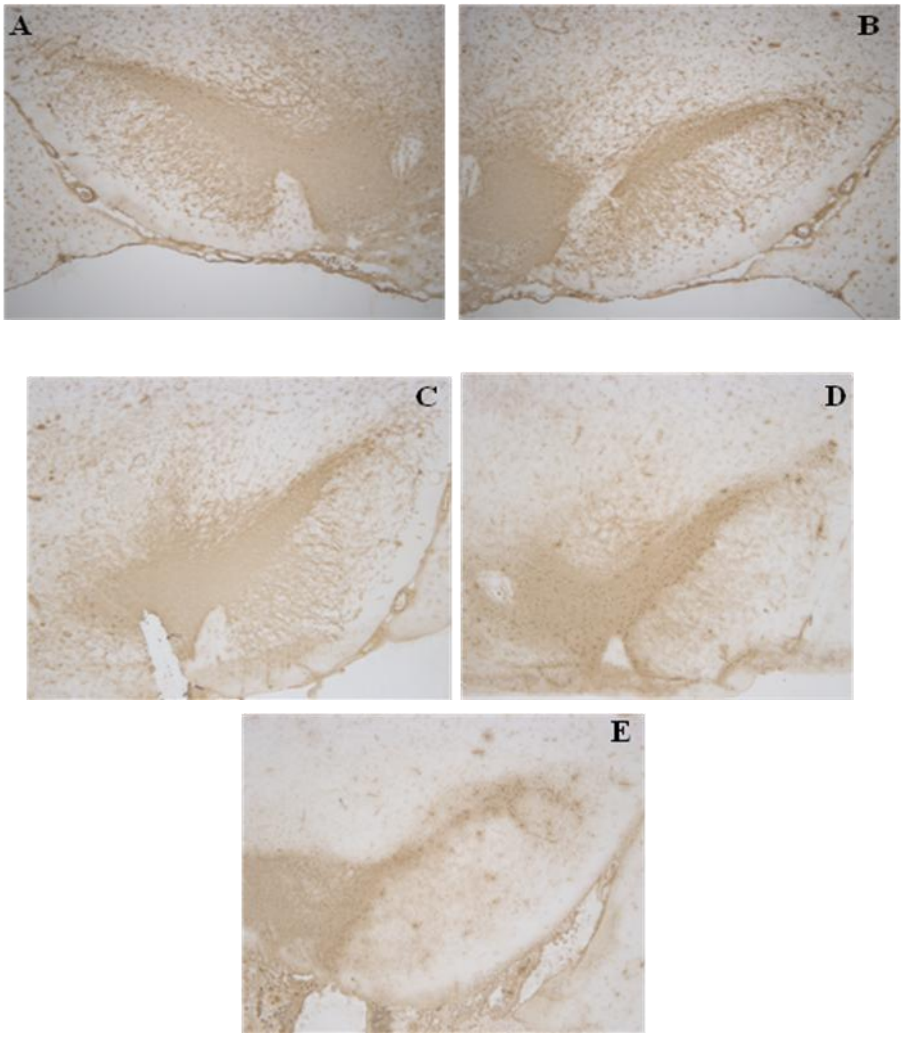


Fig. 13: Evaluation of dopaminergic damage in SNpc. (A,B) Nigral TH staining from a representative sham animal in the SNpc (A) left intact side or (B) right lesioned side. There is no significant difference between the two sides. TH staining from a representative: (C) TAT-α-syn A30P treated rat; (D) TAT-parkin treated rat; (E) TAT-α-syn A30P + TAT-parkin treated rat. Note the increasing depletion of dopaminergic terminals on right SNpc in all treated groups compared to left SNpc of sham animals. The most severe depletion occurs in animals treated with both proteins.

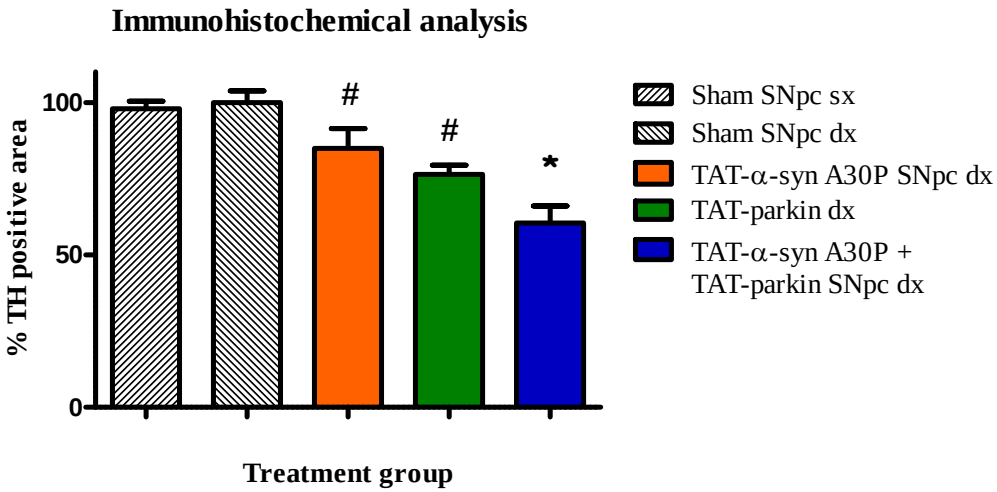


Fig. 14: Effect of intranigral injection of TAT-α-syn A30P, TAT-parkin, or both on TH cell immunoreactivity. Immunohistochemical analysis reveals a different dopaminergic degeneration in the SNpc of lesioned hemisphere compared with the contralateral/ipsilateral intact side among all treatment groups, especially in animals lesioned with both TAT-α-syn A30P and TAT-parkin. * $p<0.05$ vs all other groups; # $p<0.05$ vs sham SNpc dx and sham SNpc sx (one way ANOVA followed by Newman-Keuls Comparison Test).

The most severe depletion occurs in TAT-α-syn A30P + TAT-parkin treated animals, with a more robust decrease of TH immunoreactive neurons to 60.419 ± 4.921 % ($p<0.05$) (Fig. 14). Neither TAT-α-syn A30P nor TAT-parkin, or a combination of both, produced a significant decrease in TH immunoreactivity among controlateral sides (data not shown).Results of this first experiment confirmed our animal model based on stereotaxic injection of TAT-α-syn A30P, as previously described by Recchia et al. (2008), although a neuroprotective effect was not evidenced by combined administration of TAT-α-syn A30P and TAT-parkin. We will further investigate if this could be caused by a too high dose of TAT-parkin tested.

4.2 TAT-parkin dose-dependent toxicity

In the second experiment we wanted to evaluate if the toxic effect of TAT-parkin on dopaminergic neurons is dose-dependent. A total of 25 rats were subdivided into 5 different treatment groups, with the treatments described below:

- 3 µl vehicle (sham)
- 3 µl TAT-parkin, final dose 0.0375 µg
- 3 µl TAT-parkin, final dose 0.075 µg
- 3 µl TAT-parkin, final dose 0.15 µg
- 3 µl TAT-parkin, final dose 0.3 µg.

The RotaRod test was performed 15 days post-lesion as described above. Testing at 60 days was omitted, as rats exhibited the same behavioral patterns as at 15 days. In agreement with the first experiment there was a significative impairment in motor function after TAT-parkin injection 15 days post-lesion (which progressively decreased with increasing dose). Sham animals were able to remain on the rod for 205.4 ± 6.762 sec (Fig. 15).

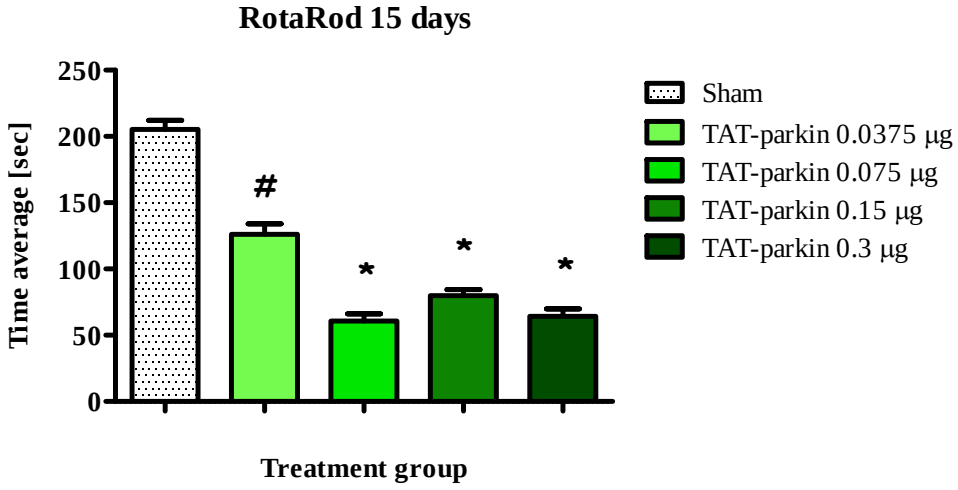


Fig. 15: RotaRod test. Running performance in rats receiving intranigral injection of increasing doses of TAT-parkin (0.0375 µg to 0.3 µg), performed 15 days post-lesion. Note significant impairment in motor function among all treatment groups. The lowest dose of 0.0375 µg seems to be less toxic compared with other treatment groups. Data are means ± SEM. * $p<0.0001$ vs sham group and 0.0375 µg TAT-parkin. # $p<0.0001$ vs sham group (one way ANOVA followed by Newman-Keuls Comparison Test).

Injection of increasing doses of TAT-parkin results in progressive decrease in motor function among all treated groups: In particular, animals receiving the highest dose (0.3 µg) displayed the lowest (and significant) value of 64.3 ± 5.685 sec. Animals receiving the two intermediate doses (0.15 µg and 0.075 µg) had an average of time spent on the rotating drum of 79.83 ± 4.646 sec and 60.770 ± 5.471 sec, respectively, while those animals receiving the lowest dose (0.0375 µg) of TAT-parkin actually increased with time to 126.1 ± 7.981 sec. All the doses tested are statistically different when compared to sham animals ($p<0.001$), but only the lowest dose seems to be less toxic than others.

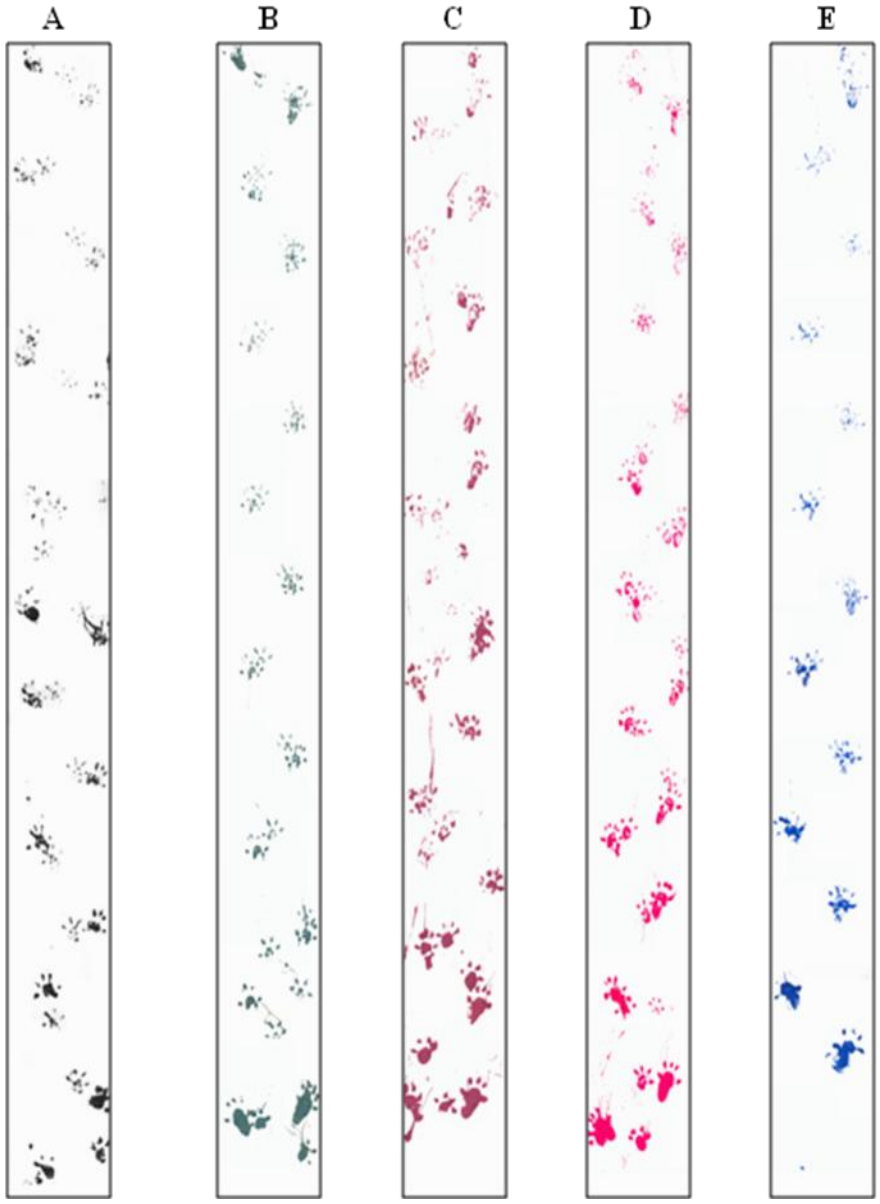


Fig. 16: Footprint test. Shown are images from a representative animal for each treatment group. Sham (A), TAT-parkin at: (B) 0.0375 µg, (C) 0.075 µg, (D) 0.15 µg, (E) 0.3 µg.

The footprint test was then performed to assess rat's motor function not influenced by external conditions (Fig. 16). Motor function can be assessed by analyzing walking patterns, by the evaluation of the % area of support legs and the total number of steps.

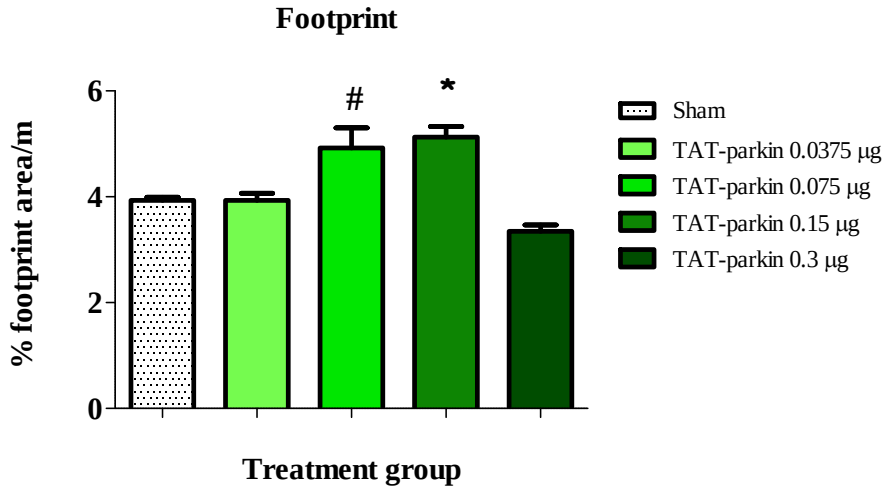


Fig. 17: Footprint test: % area of support legs. This parameter is significantly affected in animals receiving TAT-parkin (0.075 µg or 0.15 µg) compared to sham animals. Data are means ± SEM. * $p<0.05$ vs sham and 0.0375 µg TAT-parkin groups; # $p<0.05$ vs 0.0375 µg TAT-parkin group (one way ANOVA followed by Newman-Keuls Comparison Test).

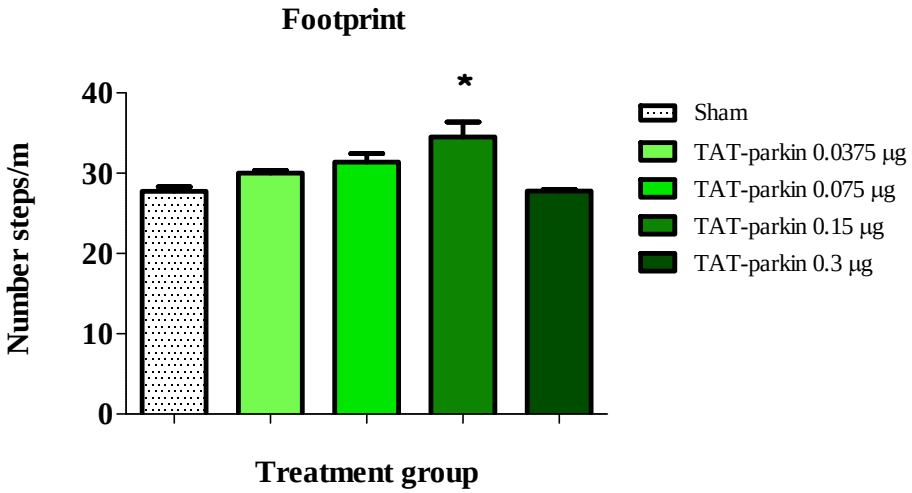


Fig. 18: Footprint test: number of steps. This parameter is significantly increased with respect to sham animals only in the group lesioned with 0.15 µg TAT-parkin. Data are means ± SEM. * $p<0.05$ vs all other groups (one way ANOVA followed by Newman-Keuls Comparison Test).

Figure 17 shows that animals receiving TAT-parkin at 0.075 μg or 0.15 μg demonstrated a significant impairment in walking behavior; in fact they needed, respectively, 4.922 ± 0.85 % and 5.129 ± 0.4 % area of support legs to balance their body weight, when compared to sham animals (3.931 ± 0.12 %). The second parameter analyzed showed significant differences between treatment and control groups, in particular the group of animals that received 0.15 μg TAT-parkin needed more steps to reach the end of the race compared to the sham group (34.5 ± 3.750 and 27.75 ± 1.11 steps, respectively, $p<0.05$).

One month after induction of the lesion dopaminergic neurochemical system behavior in TAT-parkin-treated animals was studied by cerebral microdialysis. We built and used a striatal probe to study DA release under physiological stimulation as a possible measure of dopaminergic system damage. The semi-permeable membrane of the probe allows for recovery of DA and other neurochemicals from the striatal extracellular space. Microdialysis experiments started 24 hours after stereotaxic probe implantation on awake, freely-moving animals. Samples were collected and analyzed by high performance liquid chromatography (HPLC). Baseline values of DA were recorded and a DA-releasing agent (nicotine 5 mM) was added to the microdialysis perfusion fluid. Figure 19(A) shows that baseline levels of DA remained invariant for the first hour before treatment start. Intrastriatal infusion of nicotine for 1 hour induced a rapid increase in DA concentrations in dialysates collected from all treated animals. In the sham group DA increase was of 37.21 ± 17.56 nM, while 0.0375 μg TAT-parkin, contrary to our expectations rapidlyincreased DA with a peak of 72.69 ± 11.55 nM after 60 min (Fig 19). Rats treated with the highest dose (0.3 μg) of TAT-parkin had an increase limited to 46.21 ± 2.45 nM, while groups receiving the two intermediate doses (0.075 μg and 0.15 μg TAT-parkin) had an increase limited to 60.10 ± 14.99 nM and 63.47 ±21.66 nM, respectively. No significative changes were observed between the different treatment groups, or when compared to sham group. Surprisingly, an increase was observed, rather than a reduction in DA levels in treated rats that would be indicative of stable nigrostriatal damage with lack of dopaminergic neurochemical recovery). Data shown for DOPAC+HVA (Fig. 19B) indicate that there were no changes in DA metabolism, since there are no significant differences in its metabolites in dialysate samples. At the end of the microdialysis experiment each animal was sacrificed and the brain processed for TH immunostaining. Figure 20 shows images of TH staining at the level of SNpc for a representative animal from each treatment group.

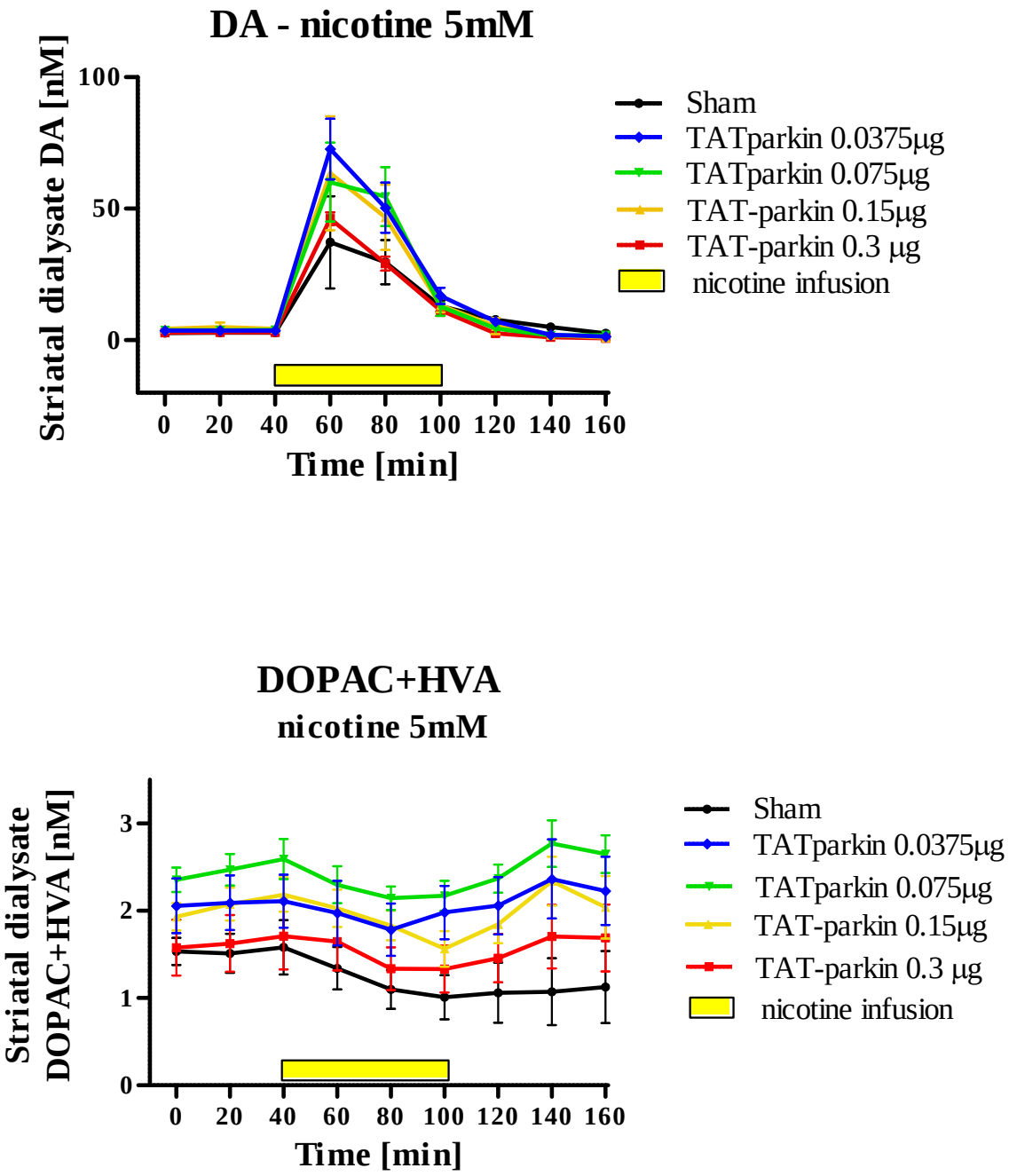


Fig. 19: DA basal levels and effect of intrastriatal infusion of nicotine (5mM) on DA concentrations in dialysates from the striatum of freely moving rats. (Upper panel) DA release; (Lower panel) DOPAC+HVA release. Microdialysis probe was inserted in the right striatum 30 days after intranigral administration of different doses of TAT-parkin. After 60 min of stabilization, three baseline dialysates were collected at 20-min intervals. Starting from 40 min nicotine was infused for 60 min (solid horizontal bar); microdialysates were continuously recovered during drug infusion and for 60 min after nicotine discontinuation. Values are mean ± SEM and refer to DA or DOPAC+HVA

concentrations in dialysates. Statistical analysis was assessed using analysis of variance (ANOVA) for differences over time determined by paired t-tests.

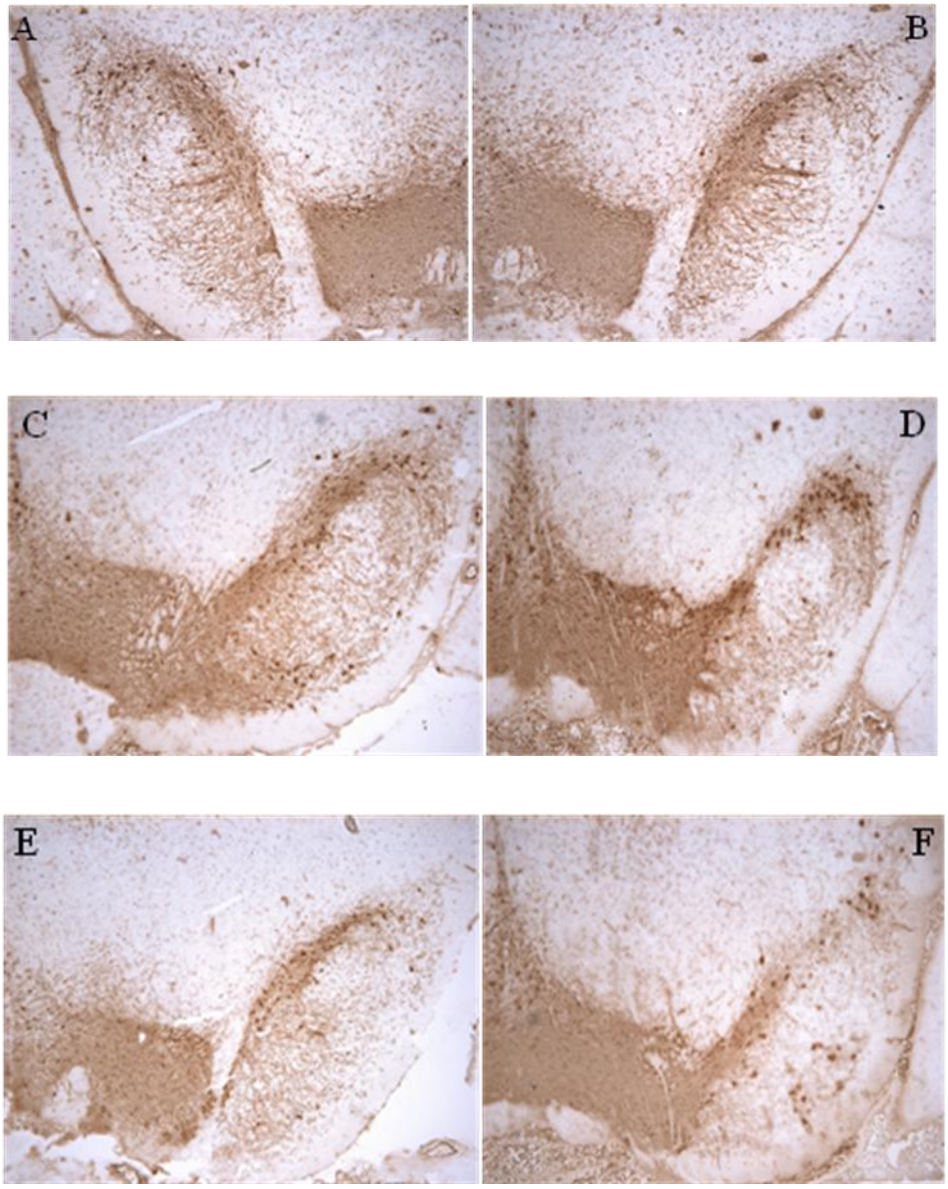


Fig. 20: Evaluation of dopaminergic damage in SNpc. (A,B) Nigral TH staining from a representative sham animal in the SNpc (A) left intact side or (B) right lesioned side. There is no significant difference between the two sides. TH staining from a representative rat treated with the following amounts of TAT-parkin: (C) 0.0375 µg, (D) 0.075 µg, (E) 0.15 µg, (F) 0.3 µg. Note the increasing depletion of dopaminergic terminals in the right SNpc in all treatment groups compared to the left SNpc of sham animals. The most severe depletion occurs in rats receiving the highest dose of TAT-parkin.

TAT-parkin injection depleted nigral TH levels in all animals as compared to sham animals, showing a direct correlation between dose and extension of the lesion. In the SNpc of rats injected with the highest dose of TAT-parkin (0.3 μg) there was a significant reduction of TH immunoreactivity to 69.06 ± 2.93% compared to the controlateral/ipsilateral side of sham animals ($p<0.05$). The two intermediate TAT-parkin groups (0.15 μg and 0.075 μg) had significant reductions to 78.74 ± 4.4% and 87.55 ± 3.73% ($p<0.05$), respectively,(Fig. 21). Total TH-positive fibers of rats treated with 0.0375 μg TAT-parkin did not differ from sham animals.

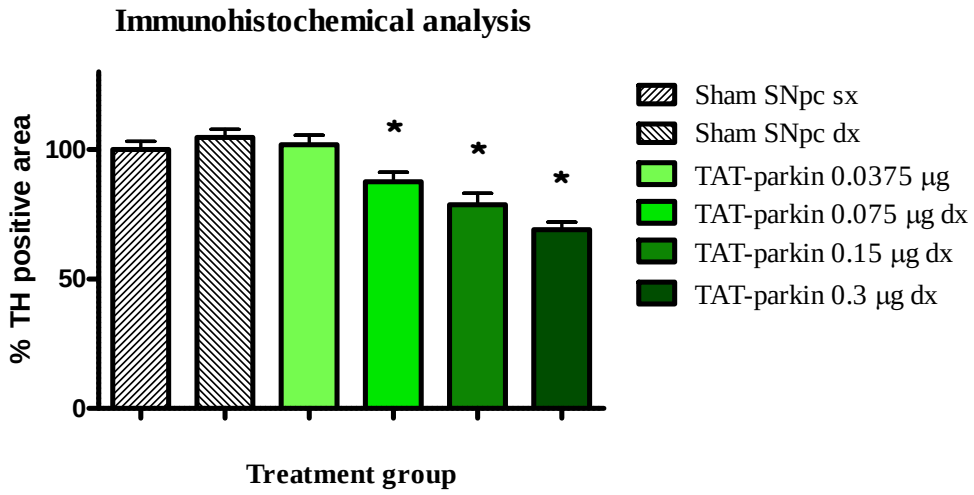


Fig. 21: Effect of intranigral injection of different doses of TAT-parkin on TH cell immunoreactivity. Immunohistochemical analysis shows dopaminergic degeneration in the SNpc of lesioned hemisphere to differ from the contralateral/ipsilateral intact side of sham animals among groups of animals lesioned with TAT-parkin at: 0.075 μg, 0.15 μg, and 0.3 μg. * $p<0.05$ vs sham SNpc sx (define), sham SNpc dx (define), and TAT-parkin 0.0375 μg (one way ANOVA followed by Newman-Keuls Comparison Test).

These experiments suggest that the lowest dose of TAT-parkin tested (0.0375μg) is devoid of a toxic effect and stimulates a massive DA release under nicotine stimulation.

4.3 Is a non-toxic dose of TAT-parkin neuroprotective?

To further analyze if this apparent least toxic dose of TAT-parkin is also neuroprotective, one more experiment was performed using a 50% higher dose of TAT-α-syn A30P in association with 0.15 µg or 0.0375 µg of TAT-parkin. A total of 30 rats were subdivided into the following 6 different treatment groups:

- 3 µl vehicle (sham)
- 3 µl TAT-α-syn A30P, final dose 6 µg.
- 3 µl TAT-parkin, final dose 0.0375 µg
- 3 µl TAT-parkin, final dose 0.15 µg
- 3 µl TAT-α-syn A30P (final dose 6 µg) + TAT-parkin (final dose 0.0375 µg)
- 3 µl TAT-α-syn A30P (final dose 6 µg) + TAT-parkin (final dose 0.15 µg).

In the two groups that received both proteins, TAT-parkin was injected 10 min before TAT-α-syn A30P injection. The RotaRod test was conducted 15 days post-lesion to assess the animals' motor deficit. Sham animals do not display any impairment in motor function among all testing sessions (224.6 ± 4.83 sec).

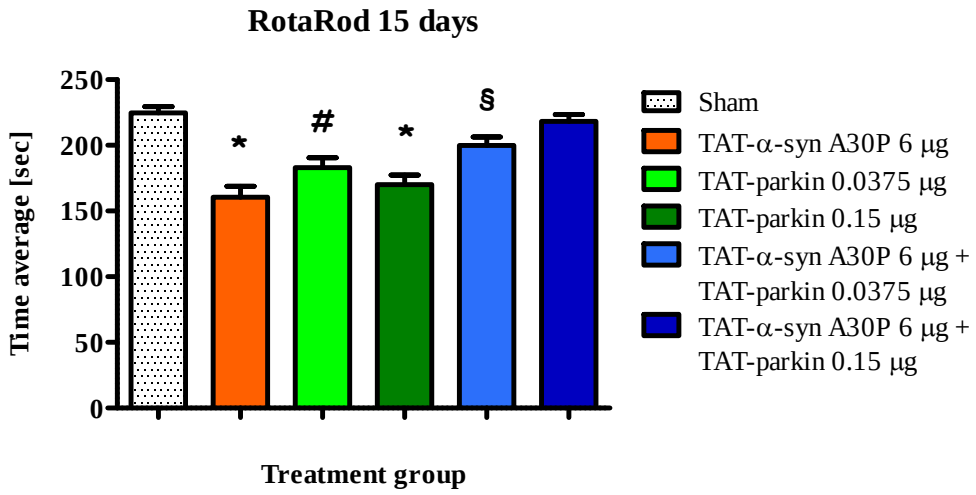


Fig. 22: RotaRod test to assess running performance in rats receiving intranigral injection of: vehicle, 50% higher dose of TAT-α-syn A30P, TAT-parkin 0.0375 µg/0.15 µg, or both combinations of the two proteins into the right SNpc, performed 15 days post-lesion. The test reveals a impairment in motor function among the different treatment groups, especially in animals receiving TAT-α-syn A30P or TAT-parkin alone. The co-administration of 6 µg TAT-α-syn A30P and 0.15 µg TAT-parkin seems to ameliorate motor performance. Data are expressed as means ± SEM. * $p<0.05$ vs sham, TAT-α-syn A30P (6 µg) + TAT-parkin (0.0375 µg), TAT-α-syn A30P (6 µg) + TAT-parkin (0.15 µg).

# p<0.05 vs sham, TAT-α-syn A30P (6 µg) + TAT-parkin (0.15 µg). § p<0.05 vs sham, TAT-α-syn A30P (6 µg) (one way ANOVA followed by Newman-Keuls Comparison Test).

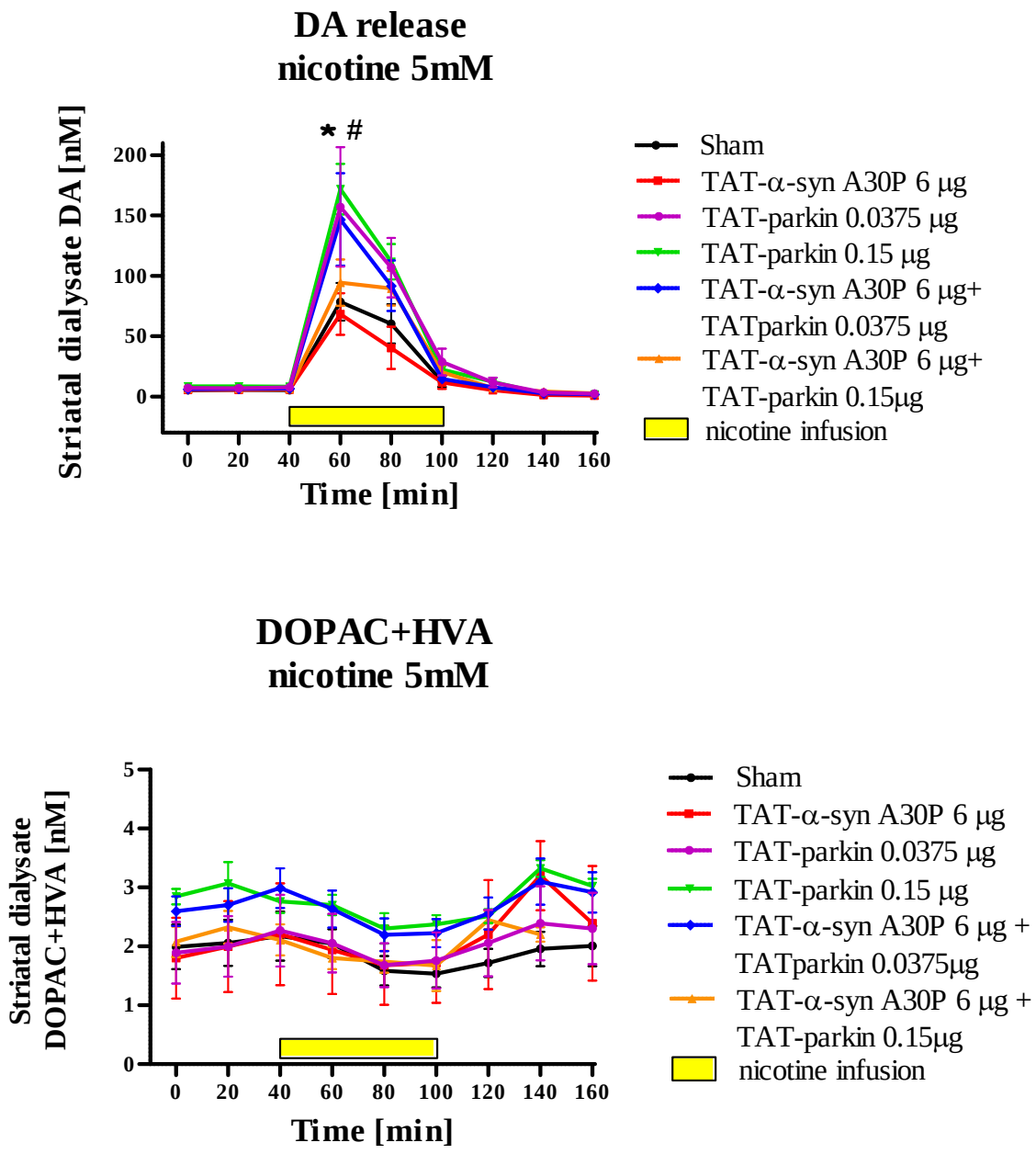


Fig. 23: DA basal levels and effect of intrastriatal infusion of nicotine (5mM) on DA concentrations in dialysates from the striatum of freely moving rats. (Upper panel) DA release; (Lower panel) DOPAC+HVA release. The microdialysis probe was inserted in the right striatum 30 days (one month??) after intranigral administration of TAT-α-syn A30P (6 µg), different doses of TAT-parkin (0.0375 µg or 0.15 µg), or combinations of both proteins. After 60 min of stabilization, three baseline dialysates were collected at 20-min intervals. Fourty minutes later nicotine was infused for 60 min (solid horizontal bar); microdialysates were continuously recovered during drug infusion and for 60 min after nicotine discontinuation. Values are mean ± SEM and refer to DA or DOPAC+HVA concentrations in dialysates. * p<0.05 vs TAT-α-syn A30P (6 µg) vs TAT-parkin

(0.0375 μg), TAT-parkin (0.15 μg), TAT-α-syn A30P (6 μg), + TAT-parkin (0.0375 μg). # $p<0.05$ sham vs TAT-parkin (0.15 μg). Statistical analysis was assessed using analysis of variance (ANOVA) for differences over time determined by paired t-tests.

Administration of a higher dose of TAT-α-syn A30P (+ 50%), TAT-parkin (0.0375 μg) or TAT-parkin (0.15 μg) produced a significant motor disability in RotaRod performance compared to sham rats, when administered alone as described in previous experiments (160.6 ± 8.28 sec; 183.1 ± 7.41sec; 170.2 ±7.16 sec, respectively) ($p<0.05$). The lowest dose of TAT-parkin tested (0.0375 μg) in combination with TAT-α-syn A30P did not significantly improve RotaRod performance, as expected (199.8 ± 6.45 sec). In this experiment rats exhibited a significant relief of motor disability when administered TAT-α-syn A30P and TAT-parkin 0.15 μg(?) (218.3 ± 5.12) (Fig. 22). Striatal microdialysis was performed 15 days post-lesion. Twenty-four hours after probe implantation, microdialysis samples were collected and analyzed by HPLC. Baseline levels of DA are not different between different treatment groups and remained invariant over the first hour of the experiment (Fig. 23).The peak reduction of DA in TAT-α-syn A30P-treated animals to 68.33 ± 17.17 nM is suggestive of a nigrostriatal lesion with lack of dopaminergic neurochemical recovery (sham group peak 78.48 ± 15.59). Animals treated with the two middle doses of TAT-parkin confirm the data obtained in the previous experiment, in which nicotine stimulation augmented the increase in DA release. In fact, both groups have increases: to 157.19 ± 49.35 nM (0.0375 μg of TAT-parkin) ($p<0.05$ TAT-α-syn A30P 6 μg vs TAT-parkin 0.0375 μg, TAT-parkin 0.15 μg, TAT-α-syn A30P 6 μg, + TAT-parkin 0.0375 μg) and to 171. 90 ± 20.97 nM (0.15 μg of TAT-parkin) ($p<0.05$ sham vs TAT-parkin 0.15 μg.). Interestingly, the two groups receiving the combination of both proteins showed an intermediate pattern of release between sham animals and TAT-parkin-treated ones, respectively: 146.75 ± 38.18 nM (TAT-α-syn A30P 6 μg, + TAT-parkin 0.0375 μg) and 94.48 ± 19.24 nM (TAT-α-syn A30P 6 μg, + TAT-parkin 0.15 μg). The microdialysis experiment confirms that the two doses of TAT-parkin stimulate the release of DA, and shows an increase in DA level even in groups with TAT-α-syn A30P lesion, especially with the lower dose of TAT-parkin. Upon conclusion of the microdialysis experiment all animals were sacrificed and brains removed for further immunohistochemical analysis (Fig. 24).

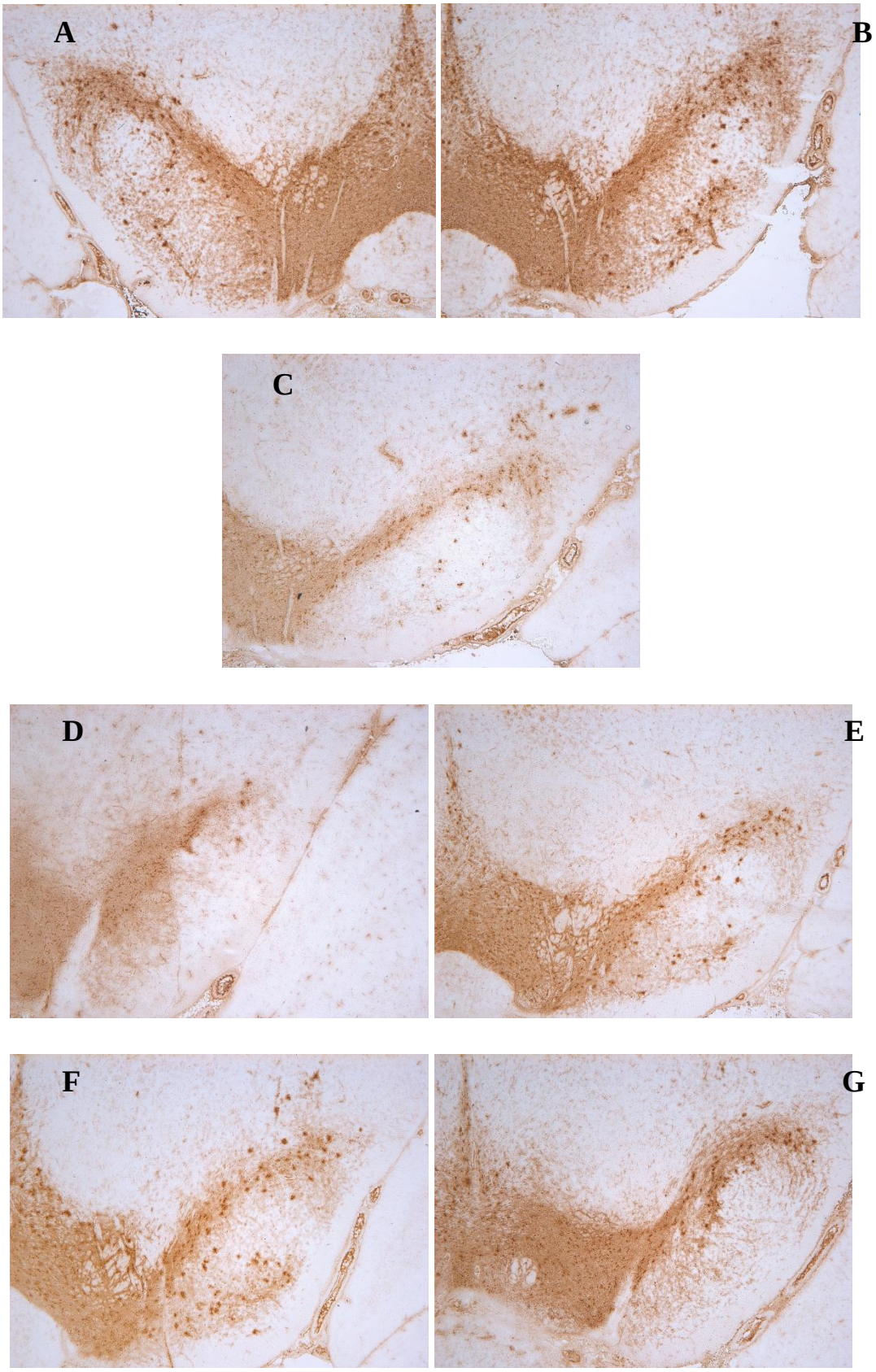


Fig. 24: Evaluation of dopaminergic damage in SNpc. (A,B) Nigral TH staining from a representative sham animal in the SNpc (A) left intact side or (B) right lesioned side. There is no

significant difference between the two sides. TH staining from a representative rat treated with the following amounts of: 6 µg TAT-α-syn A30P, (D) 0.0375 µg TAT-parkin, (E) 0.15 µg TAT-parkin, (F) 6 µg TAT-α-syn A30P 6 µg + 0.0375 µg TAT-parkin, (G) 6 µg TAT-α-syn A30P + 0.15 µg TAT-parkin 0.15 µg. Note the increasing depletion of dopaminergic terminals on right SNpc in all groups with single protein treatment compared to left/right SNpc of sham animals.

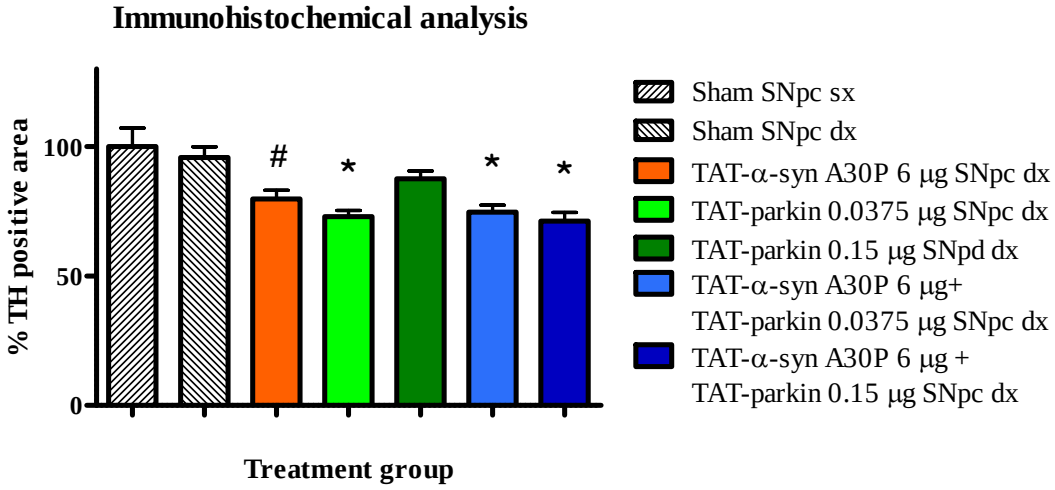


Fig. 25: Effect of intranigral injection of different doses of TAT-α-syn A30P or TAT-parkin on TH cell immunoreactivity. Immunohistochemical analysis shows dopaminergic degeneration in the SNpc of lesioned hemisphere to differ from the contralateral/ipsilateral intact side of sham animals among groups of animals lesioned with: TAT- α-syn A30P (6 µg) or TAT-parkin (0.0375 - 0.15 µg) singly, or in combination. Data are means ± SEM. * $p<0.0001$ vs sham SNpc sx, sham SNpc sx, sham SNpc dx and TAT-parkin 0.15 µg. # $p<0.0001$vs sham SNpc sx and sham SNpc dx (one way ANOVA followed by Newman-Keuls Comparison Test).

Sham-treated animals do not show any difference in dopaminergic innervation density, while animals lesioned with TAT-α-syn A30P (6 µg) have a clear and significant decrease in TH positive fibers to 79.73 ± 3.3% compared to sham animals (both sides) ($p<0.0001$).Also, the animals treated with TAT-parkin (0.0375 or 0.15 µg) confirm the previously observed toxic effect of TAT-parkin administered on its own. Indeed, the two groups showed a decrease of dopaminergic fiber density to 72.94 ± 2.39% ($p<0.001$ vs sham SNpc sx/dx) and 87.61 ± 2.98%, respectively. In the last two groups receiving 6 µg TAT-α-syn A30P together with TAT-parkin (0.0375 or 0.15 µg) showed a reduction of dopaminergic loss, suggesting a possible direct dependence not only on the dose of TAT-parkin used, but also on the initial degree of the injury. In fact the combination with the low dose of TAT-parkin had an increase

to 74.73 ± 2.61 %, and the high dose 71.24 ±3.35 %. However these increases are still statistically different from sham SNpc sx/dx ($p<0.0001$) (Fig. 25).

5. Discussion

The pathogenetic mechanism(s) underlying the nigro-striatal neurodegeneration of idiopathic and familial PD is likely to be complex and may encompass interactions among several predisposing genes and environmental factors. Mutations in the gene coding for α-syn have received a great deal of attention with the discovery that fibrillar α-syn aggregates are the major components of both Lewy neurites and characterize most familial and sporadic PD brains. Abnormal accumulation of α-syn is considered to be a key pathological event in the process leading to selective dopaminergic degeneration in α-syn-linked and sporadic PD, but the neurotoxic role of these inclusions in PD is highly debated. Although α-syn mutations are infrequent in PD, other cellular processes leading to abnormal metabolism and accumulation of this protein may play an important role in disease pathogenesis. Parkin functions as an E3 ligase, presumably targeting specific substrate proteins for degradation by the proteasome, and loss of *parkin* causes AR-JP. The finding that α-syn is the major component of LB, which are, on the other hand, absent in parkin-associated PD, has fuelled the idea that parkin activity is required for the formation of LB. Therefore, α-syn has become an intensely studied candidate substrate of parkin. Taken together, these observations suggest a model of sporadic PD, in which the absence parkin short-circuits the proper degradation of some proteins, whose subsequent accumulation, leads to cellular dysfunction and eventually the death of susceptible neurons. In our laboratory we have recently described and characterized an α-syn animal model based on the stereotaxic injection into rat right SNpc of the A30P mutated form of α-syn fused to a protein transduction domain (transactivator of transcription, TAT). The TAT sequence allows diffusion of the fusion protein across the neuronal plasma membrane and results in a localized dopaminergic loss, which is accompanied by a time-dependent impairment in motor function. The TAT- α-syn A30P model thus mimics the early stages of the human PD (Recchia, et al., 2008). Recent findings reveal that parkin has a remarkably wide neuroprotective capacity, preventing cell death under various stress conditions. The specific aim of this thesis is to examine possible neuroprotective effects of TAT-parkin in a TAT-α-syn A30P-generated hemiparkinsonian model.

In the first experiment we evaluated TAT-parkin protective potential against TAT- α-syn A30P induced toxicity in three different groups of rats. Two groups were injected with only one protein to confirm the lesion caused by TAT-α-syn A30P and to test if TAT-parkin had any intrinsic effect, while a third group received both agents simultaneously. Two weeks after administration of the proteins, all animals were subjected to the RotaRod test, which is used

frequently to assess motor deficits in rats and analyze movement in rodents with nigro-striatal lesion. During the 240 sec of each RotaRod session there is a gradual increase in the demand for muscular coordination. After a few learning sessions the sham group did not show any significant motor alteration, with an average time spent on the rotating drum of 229 ± 8.242 sec. The group lesioned with TAT-α-syn A30P showed a significant decrease of motor impairment to 159.9 ± 7.737 sec, indicating that the observed performance was indeed a consequence of the destruction of dopaminergic neurons. The TAT-parkin-injected group also displayed a decrease in motor function compared to sham animals. This indicates that the dose of protein tested could have an effect itself, with the group receiving both proteins having a more evident motor impairment compared to all other treatment groups (104. 0 ± 7.347 sec). This result was not expected, as TAT-parkin seemed to be toxic and produced no neuroprotective effect in combination with TAT-α-syn A30P; indeed, the significant decrease in motor performance probably resulted from dopaminergic cell loss. When repeated two months post-lesion this test did not show any difference compared to the first trial, confirming lesion stability with time without significant variation among all treatment groups. To further confirm the extent of the lesion all rats were sacrificed after the last behavioral session and the brains processed for TH immunostaining. TH is the rate limiting step enzyme in the conversion of L-tyrosine to L-DOPA, a precursor for DA, and is found in the cytosol of all cells containing catecholamines. TH is frequently used as a measure of the extent of dopaminergic denervation. TH immunostaining through the SNpc of all treated groups revealed a marked reduction in dopaminergic innervation on the lesion side compared both to the intact contralateral side (data not shown) and sham animals (contralateral intact/ipsilateral lesioned side). The extent of denervation was most prominent among animals that received injection of TAT-α-syn A30P and TAT-parkin. Compared to the non-lesioned side of sham animals, the optical density of TH-positive neurons was reduced to 84.932 ± 6.818% and 76.427 ± 10.491% in TAT-α-syn A30P and TAT-parkin treated animals, respectively, while in the last group only 60.419 ± 4.921% of dopaminergic neurons survived two months after TAT-α-syn A30P + TAT-parkin injection. These results confirm the behavioral (RotaRod) test results and clearly demonstrate that the tested dose of TAT-parkin may have an unknown intrinsic toxic effect, or be toxic per se because of the high dose injected. A possible deleterious effect of the TAT- sequence can be ruled out, as the literature contains numerous examples confirming its high level of efficiency for intracellular delivery of macromolecules

with molecular weights several times its own without apparent toxic effects (Lindgren, 2000; Schwarze, 2000).

To evaluate if the observed toxicity could be due, in effect to a TAT-parkin 'overdose', we decided to test four different groups of animals receiving TAT-parkin doses in the range 0.3 μg to 0.0375 μg. Two weeks post-injection all groups were subjected to the RotaRod test. A second trial after two months was omitted, as we did not find any statistical difference between the two behavioral testing sessions among any treatment group. The sham group, after a period of learning completed all testing sessions with a time average of 205.4 ± 6.672 sec. The TAT-parkin-treated groups were severely impaired and spent significantly less time on the drum compared to sham rats. Running ability decreased gradually with increasing dose of protein injected; in particular, animals receiving the highest dose (0.3 μg) had a value of 64.3 ± 5.585 sec, while the two intermediate dosed groups (0.15 and 0.075 μg) had average times of 79.83 ± 4.646 sec and 60.77 ± 5.471 sec, respectively. The group treated with the lowest dose of TAT-parkin exhibited the longest time on the drum, although still statistically different from sham animals (126.1 ± 7.981) ($p<0.0001$). These data a possible correlation between dose and toxicity induced by TAT-parkin, with the lowest dose tested (0.0375 μg) appearing to produce a less toxic effect.

In order to better analyze motor ability without the use of a testing apparatus, all animals were subjected to the footprint test. Footprints were scanned, stored as black/white bitmaps and evaluated for two parameters: % area of support legs and number of steps. Analyses revealed that both parameters were significantly affected in all treatment groups compared to sham, especially those animals injected with 0.15 μg TAT-parkin. Analysis revealed a motor dysfunction characterized by an increase in the number of steps and a greater footprint area of support legs: the first parameter indicates that lesioned animals need more steps to reach the end of the gangway, while the second demonstrates a significant impairment in motor function as animals need to touch a greater surface area to support their body weight. Analysis of such footprint patterns permits assessment of motor function in a manner distinct from the RotaRod test, in that the former does not impose on the animal a requirement for either motor ability or coordination. Taken together these behavioral data demonstrate significant deterioration in the walking pattern of rats treated with TAT-parkin and in a dose-toxicity dependent manner. After the last behavioral session all animals were subjected to cerebral microdialysis to monitor the release pattern of neurotransmitters, their metabolites and

neuromodulators in the striatum. This method is based on the diffusion of molecules through a semipermeable membrane tubing connected to a probe stereotaxically implanted into the brain. The microdialysis probe functions as an artificial blood vessel whereby the diffusion of molecules is driven by the concentration gradient that exists between extracellular space and dialysis medium. In particular, the semi-permeable membrane allows the recovery of DA and other neurochemicals from the extracellular space: DA is synthesized from L-tyrosine via TH and L-DOPA decarboxylase, then transported to the presynaptic endings where it is stored in vesicles and released as result of an action potential. MAO and COMT are involved in DA metabolism and the final product of its catabolism is HVA, derived from DOPAC and 3-MT. Microdialysis was utilized to investigate the dopaminergic neurochemical system as a lesion parameter: we measured the amount of DA release from the striatal dopaminergic endings under pharmacological stimulation. There are no differences on DA basal level among any treatment group, but 20 min after nicotine infusion DA concentrations rapidly increased in a dose-dependent fashion in samples collected from each treated animal, when compared to sham release. In sham animals DA overflow was limited to 37.21 ± 17.56 nM but, contrary to our expectations animals receiving the lowest dose (0.0375 μg) displayed the higher increase to 72.69 ± 11.55 nM. The group receiving the highest TAT-parkin dose had the lowest peak limited to 46.21 ± 2.45 nM while the other two groups had an intermediate release. Data from DOPAC+ HVA striatal release indicate that there were no changes in DA metabolism, since there are no significant differences in its metabolites. This higher DA release could be due to an increase in DA synthesis, an increase in DA uptake via DAT, or a different sensitivity and higher release after nicotine stimulation. In a new study Huynh and colleagues (you can cite their abstract from the program and include in reference list-poster presentation at Neuroscience 2011, Washington DC) demonstrated that parkin interacts *in vitro* with endogenous synaptotagmin 1 and facilitates DA uptake, suggesting that loss of parkin likely disrupts highly regulated mechanisms of DA transmission, which may contribute to degeneration of neuronal processes leading ultimately to neuronal degeneration. Histological analysis of the brains of these rats confirmed that the density of TH-positive dopaminergic neurons in the SNpc was reduced in all parkin-treated groups compared to sham, showing a direct correlation between dose and extent of lesion (except for the group treated with the lowest dose). The most severe loss of DA neurons occurred in the SNpc in all animals treated with 0.3 μg TAT-parkin, as revealed by TH immunostaining. In fact TH-positive fiber innervation, obtained by, optical density analysis showed a reduction to 69.06 ± 2.93%. Total

TH-positive fiber innervation increased to 78.74 ± 4.4% and 87.55 ± 3.73% in the two intermediate groups (0.15 and 0.075 μg, respectively), but remained significantly decrease compared to sham ($p<0.05$). In the group injected with the lowest dose of TAT-parkin dopaminergic denervation was more limited and did not differ from the sham group, suggesting that animals receiving the lowest dose (0.0375 μg) of TAT-parkin have less severe damage compared to the other three doses tested. Thus, only the lowest dose seems to be devoid of a toxic effect and stimulates a massive DA release under nicotine stimulation. Further, that there may be a direct correlation between the dose of TAT-parkin and its toxicity, as revealed by its impact on behavior, DA release and TH immunoreactivity.

Based on these observations we carried out a final experiment to identify a putative non-toxic dose of TAT-parkin (0.0375 μg) applied in combination with a 50% higher dose of TAT-α-syn A30P. In addition, the dose of TAT-parkin (0.15 μg) used in the first experiment, and which displayed a neuroprotective effect was also evaluated in combination with this higher dose of TAT-α-synA30P. The decision to utilize a 50% higher dose of TAT-α-syn A30P was based on our desire to determine if the TAT-parkin neuroprotective effect could be influenced not only by this protein but also by the initial extension of dopaminergic lesion. For co-administration, TAT-parkin was injected 10 min before TAT-α-syn A30P, since the literature reports that TAT fusion proteins need approximately 5 min to be detectable within cultured cells and that TAT fusion protein transduction increases over time to reach steady-state. Conceivably, intracellular TAT-parkin would then be in a position to exert an efficacious neuroprotective action prior to lesion induction by TAT-α-syn A30P. At two weeks post-lesion, animals injected with either TAT-parkin or TAT-α-syn A30P displayed significant impairment in locomotor activity on the RotaRod test compared to the sham group ($p<0.05$). The last data are consistent with results obtained in earlier in this study. Interestingly, an increase in motor function was observed with combination of TAT-α-syn A30P and TAT-parkin (0.0375 and 0.15 μg); moreover, the latter group was not statistically different from sham only. The behavioral results highlight that motor function could be improved when TAT-parkin is injected prior TAT-α-syn A30P.

This last experiment confirmed the earlier striatal microdialysis data (which showed an increase in DA release after nicotine infusion in all TAT-parkin-treated rats) by demonstrating the same increase in animals receiving the two intermediate doses of TAT-parkin. The significant reduction of DA release in the TAT-α-syn A30P-treated group compared to both

sham and TAT-parkin groups is suggestive of a stable nigrostriatal lesion without dopaminergic neurochemical recovery. Most interestingly in the TAT-α-syn A30P + TAT-parkin 0.0375 μg and TAT-α-syn A30P + TAT-parkin 0.15 μg treated groups, nicotine stimulates a level of DA release intermediate between sham and the two TAT-parkin-treated groups.,In particular, co-administration of 0.0375 μg TAT-parkin (instead of 0.15 μg) shows a higher DA release, suggesting an important role of TAT-parkin in regulating TAT-α-syn A30P toxicity. Indeed, analysis of TH fiber immunoreactivity clearly showed a significant nigral dopaminergic lesion in TAT-α-syn A30P-injected animals. Unexpected, the TAT-parkin toxicity data did not confirm our last observations, in that 0.15 μg TAT-parkin failed to provoke the same reduction in SNpc dopaminergic innervation as that found in the earlier experiments. While the TAT-sequence facilitates fusion protein diffusion across the neuronal plasma membrane and results in a localized dopaminergic loss, it is impossible to control the exact amount of protein entering the cell. This could lead to a toxic effect caused by a too high delivery of TAT-parkin into SNpc dopaminergic neurons, regardless of the initial dose (which can be controlled). In any case, co-administration of both proteins partially ameliorated the loss of TH immunoreactivity - especially when TAT-α-syn A30P was combined with 0.0375 μg TAT-parkin, yet, both groups still differed significantly from the sham group ($p<0.0001$).

Our *a priori* working hypothesis was that nigral co-administration of TAT-parkin and TAT-α-syn A30P would enhance proteasomal processing of TAT-α-syn A30P, thereby protecting DA neurons from the toxic insult. Increasing evidence points to aberrant protein folding/protesomal processing as central to the molecular pathogenesis of neural degeneration in PD (Betarbet, 2005). Animal models represent an important facet of PD research and our understanding of the pathophysiology of this movement disorder. The TAT-α-syn A30P rat model of PD, with its characteristic stable damage of nigrostrital dopaminergic function and impairment in motor activity, reproduces the initial stages and slow development of human PD. Several studies show parkin to be neuroprotective against various insults, i.e. in rats lentiviral parkin over-expression protects the SNpc from pathology associated with lentiviral over-expression of α-syn (Lo Bianco, 2004). Our results are in agreement with previous findings that intranigral injection of TAT-α-syn A30P is toxic, and we also found that neither TAT-parkin is devoid of toxic effects. Only a low TAT-parkin dose (0.0375 μg) seems to be devoid of a toxic effect, while stimulating a massive DA release under nicotine stimulation,

TAT-parkin at a dose of 0.15 µg has a better neuroprotective effect when used in concert with a 50% higher dose of TAT-α-syn A30P, while co-administration of TAT-parkin at 0.0375 µg (instead of 0.15 µg) results in a higher DA release. Collectively, the data obtained here favor the view that a correlation between TAT-parkin activity and the extent of dopaminergic lesion exists. However, it remains to be clarified how parkin may help in protecting against TAT-α-syn A30P toxicity in this animal model. It will be for future experimentation to answer this question.

References

Abbas, N., Lucking, C.B., Ricard, S., Durr, A., Bonifatti, V., De Michele, G., Bouley, S., Vaughan, J.R., Gasser, T., Marconi, R., Broussolle, E., Brefel-Courbon, C., Harhangi, B.S., Oostra, B.A., Fabrizio, E., Boheme, G.A., Pradier, L., Wood, N.W., Filla, A., Meco, G., Denefle, P., Agid, Y., Brice, A., 1999. A wide variety of mutations in the pakin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. *Hum. Mol. Genet*. 8: 567-74.

Abeliovich, A., Schmitz, Y., Farinas, I., Choi-Lundberg, D., Ho, W.H., Castillo, P.E., Shinsky, N., Verdugo, J.M., Armanini, M., Ryan, A., 2000. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* 25: 239-52.

Ahn, B.H., Rhim, H., Kim, S.Y., Sung, Y.M., Lee, M.Y., Choi, J.Y., Wolozin, B., Chang, J.S., Lee, Y.H., Kwon, T.K, 2002. Alpha-synuclein interacts with phospolipase D isozimes and inhibits pervanadate induced phospolipase D activation in human embryonic kidney 293 cells. *J. Biol. Chem.* 277: 12334-42.

Alam, M., Schmidt, W.J., 2002. Rotenone destroys dopaminergic neurons and induces parkinsonian symptoms in rats. *Behav. Brain Res*. 136: 317-24.

Alam, M., Mayerhofer, A., Schmidt, W.J., 2004. The neurobehavioral changes induced by bilateral rotenone lesion in medial forebrain bundle of rats are reversed by L-DOPA. *Behav. Brain Res*. 151: 117-24.

Albani, D., Peverelli, E., Rametta, R., Batelli, S., Veschini, L., Negro, A., Forloni, G., 2004. Protective effect of TAT-delivered α-synuclein: relevance of the C-terminal domain and involvement of HSP70. *FASEB J*. 18, 1713–1715.

Ashkan, K., Walace, B., Bell, B.A., Benabid, A.L., 2004. Deep brain stimulation of the subthalamic nucleus in Parkinson's disease 1993-2003: where are we 10 years on? *Br. J. Neurosurg*. 18: 19-34.

Baba, Y., Wharen, R.E., Uitti, R.J., 2005. Parkinson's disease: surgical treatment – stereotactic procedures. CRC press. 1, Parkinso's disease. I Ebadi, Manuchair S. II Pfeiffer, Ronald., 745-767.

Backlund, E.O., Granberg, P.O., Hamberger, B., Knuttson, E., Martensson, A., Sedvall, G., et al., 1985. Transplantation of adrenal medullary tissue to striatum in parkinsonism first clinical trials. *J. Neurosurg*. 62: 169-173.

Bae, Y.J., Park, K.S., Kang, S.J., 2003. Genomic organization and expression of parkin in Drosphila melanogaster. *Exp. Mol. Med*. 35: 393-402.

Beck, K.D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vanden, R.A., Rosenthal, A., Hefti, F., 1995. Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* 373:339-41.

Becker-Hapak, M., McAllister, S.S., Dowdy, S.F., 2001. TAT-mediated protein transduction into mammalian cells. *Methods* 24, 247–256.

Beites, C.L., Xie, H., Bowser, R., trimble, W.S., 1999. The septin CDCrel-1 binds syntaxin and inhibits exocytosis. *Nat. Neurosci*. 2: 434-9.

Benes, F.M., 2001. Carlsson and the discovery of dopamine. *Trends Pharmac. Sci*. 22: 46-7.

Berretta, N., Freestone, P.S., Guatteo, E., De Castro, D., Geracitano, R., Bernardi, G., et al., 2005. Acute effects of 6-hydroxydopamine an dopaminergic neurons of the rat sub stantia nigra pars compacta in vitro. *Neurotoxicology* 26: 869-81.

Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V., Greenamyre, J.T., 2000. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosc*. 3: 1301-1306.

Betarbet, R., Sherer, T.B., Greenamyre, J.T., 2005. Ubiquitin-proteasome system and

Parkinson's disease. *Exp. Neurol*. 191: 17-27.

Biere, A.L., Wood, S.J., Wypych, J., Steavenson, S., Jiang, Y., Anafi, D., Jacobsen, F.W., Jaronski, M.A., Wu, G.M., Louis, J.C., Martin, F., Narhi, L.O., Citron, M., 2000. Parkinson's disease-associated α-synuclein is more fibrillogenic than β- and γ-synuclein and cannot cross-seed its homologs. *J. Biol. Chem*. 275: 34574-79.

Bjorklund, A., Dunnet, S.B., Brundin, P., Stoessl, A.J., Freed, C.R., Breeze, R.E., et al., 2003. Neuronal transplantion for the treatment of Parkinson's disease. *Lancet Neurol*. 2: 437-445.

Bonifati, V., Rizzu, P., Squitieri, F., Krieger, E., Vanacore, N., Van Swieten, J.C., Brice, A., Van Duijn, C.M., Oostra, B., Meco, G., Heutink, P., 2003. DJ-1 (PARK7), a novel gene for autosomal recessive, early onset parkinsonism. *Neurol. Sci*. 24: 159-160.

Bonifati, V., 2005. Genetics of Parkinson's disease. *Minerva Med*. 96: 175-86.

Boston, P.F., Jackson, P., Thompson, R.J., 1982. Human 14-3-3 protein, radioimmunoassay, tissue distribution, and cerebrospinal fluid levels in patients with neurological disorders. *J. Neurochem* 38: 1475-82.

Bovè, J., Prou, D., Perier, C., Przedborski, S., 2005. Toxin-induced models of Parkinson's disease. *NeuroRx*. 2: 484-94.

Bower, J.H., Maraganore, D.M., Peterson, B.J., McDonnell, S.K., Ahlskog, J.E., Rocca, W.A., 2003. Head trauma preceding PD: a case-control study. *Neurology* 60: 1610-15.

Brooks, A.I., Chadwick, C.A., Gelbard, H.A., Cory-Slechta, D.A., Federoff, H.J., 1999. Paraquat elicited neurobehavioral syndrome caused by dopaminergic neurol loss. *Brain Res*. 823: 1-10.

Carlsson, A., Lindquist, M., Magnusson, T., 1957. 3,4-dihydroxyphenilalaine and 5-hydroxytryptophan as reserpineanttagonist. *Nature* 180.

Cenci, M.A., Whishaw, I.Q., Shallert, T., 2002. Animal models of neurological deficits: how relevant is the rat? *Nat. Rev. Neurosci*. 3: 574-579.

Cenci, M.A., 2007. Dopamine dysregulation of movement control in L-DOPA-induced dyskinesia. *Trends in Neurosc*. 30: 236-43.

Chandra, S., Chen, X., Rizo. J., Jahn, R., Sudhof, T.C., 2003. A broken alpha-helix in folded alpha-synuclein. *J. Biol. Chem*. 278: 15313-8.

Choi, P., Snyder, H., Petrucelli, L., Theisler, C., Chong, M., Zhang, Y., Lim. K., Chung, K.K., Kehoe, K., D'Adamio, L., Lee, J.M., Cochran, E., Bowser, R., Dawson, T.M., Wolozin, B., 2003. SEPT_v2 is a parkin-binding protein. *Brain Res. Mol. Brain Res.* 117: 179- 89.

Chung, K.K., Zhang, Y., Lim, K.L., Tanaka, Y., Huang, H., Gao, J., Ross, C.A., Dawson, V.L., Dawson, T.M., 2001. Parkin ubiquitinates the alpha-synuclein-interacting protein synphilin-1: implications for Lewy-body formation in Parkinson's disease. *Nat. Med*. 7: 114-50.

Chung, K.K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J.C., Marsh, L., Dawson, V.L., Dawson, T.M., 2004. S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science* 304: 1328-31.

Ciechanover, A., Brundin, P., 2003. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. *Neuron* 40: 427-46.

Clayton, D.F., George, J.M., 1999. Synucleins in synaptic plasticity and neurodegenerative disorders. *J. Neurosci. Res*. 58: 120-129.

Cohen, G., 2000. Oxidative stress, mitochondrial respiration, and parkinson's disease. *Ann. NY Acad. Sci*. 899: 112-20.

Conway, K.A., Harper, J.D., Lansbury, P.T., 1998. Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. *Nat. Med*. 4: 1318-20.

Conway, K.A., Harper, J.D., Lansburry, Jr, P.T., 2000. Fibils formed in vitro from α-

synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. *Biochemistry* 39: 2552-63.

Conway, K. A., Rochet, J. C., Bieganski, R.M., Lansbury, P.T.Jr., 2001.kinetic stabilization of the alpha-synuclein adduct. *Science* 294: 6-9.

Conway, K.A., Harpr, J.D., Lansbury, P.T., 1998. Accelerated *in vitro* fibril formation by a mutant α-synuclein linked toearly-onset parkinson's disease. *Nat. Med*. 11: 1318-20.

Cookson, M.R., 2005. The biochemistry of Parkinson's disease. *Annu. Rev. Biochem*. 74: 29-52.

Creese, I., Burt, D.R., Snyder, S.H., 1988. Dopamine receptor binding anhancement accompanies lesion-induced behavioural supersensitivity. *Science* 197:596-8.

Currier, R.D., 1996. Did John Hunter give James Parkinson an idea? *Arch. Neurol*. 53(4): 377-8.

Day, B.J., Patel, M., Calavetta, L., Chang, L.Y., Stamler, J.S., 1999. A mechanism of paraquat toxicity involving nitric oxide synthase. *Proc. Natl. Acad. Sci*. USA 96: 12760-65.

Dauer, W., przedborski, S., 2003. Parkinson's disease: mechanisms and models. *Neuron* 39: 889-909.

Davidson, W.S., Jonas, A., Clayton, D.F., George, J.M., 1998. Stabilization of alpha-synuclein secondary structure upn binding to synthetic membranes. *J. Biol. Chem*. 273: 9443-9.

Dawson, T.M., Dawson, V.L., 2010. The role of parkin in familial and sporadic Parkinson's disease. *Movement Disorders* 25: S32-9.

Dietz, G.P.H. and Bähr, M. 2004. Delivery of bioactive molecules into the cell: Trojan horse approach. *Mol.Cell. Neurosci*. 27, 29-37.

Dunnett, S.B., Bjorklund, A., Shmidt, R.H., Stenevi, U., Iversen, S.D., 1983a. Intracerebral grafting of neuronal cell suspensions. V. Behavioural recovery in rats with bilateral 6-OHDA lesion following implantations of nigral cell suspension. *Acta. Physiol. Scand. Suppl*. 522: 39-47.

Elbaz, A., Tranchant, C., 2007. Epidemiologic studies of environmental exposure in Parkinson's disease. *J. Neurol. Sci*. 262: 37-44.

Eliezer, D., Kutluat, E., Bussell, R. Jr., Browne, G., 2001. Conformational properties of alpha-synuclein in iys free and lipid-associated states. *J. Mol. Biol*. 307: 1061-73.

Elkon, H., Melamed, E., Offen, D., 2004. Oxidative stress, induced by 6-hydroxydopamine, reduces proteasome activities in PC12 cells: implications for the pathogenesis of Parkinson's disease. *J. Mol. Neurosci*. 24: 387-400.

Engelender, S., Kaminsky, Z., Guo, X., Sharp, A.H., Amaravi, R.K., Kleiderlein, J.J., Margolis, R.L., Troncoso, J.C., Lanahan, A.A., Worley, P.F., Dawson, V.L., Dawson, T.M., Ross, C.A., 1999. Synpilin-1 associates with alpha-synuclein and promotes the formation of cytosolic inclusions. *Nat. Genet*. 22:110-4.

Faernley, J.M., Less, A.J., 1991. Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain* 114:2283-301.

Farrer, M.J., 2006. Genetics of Parkinson's disease: paradigm shifts and future prospects. *Nat. Rev. Genet*. 7: 306-318.

Ferger, B., Rose, S., Jenner, A., Halliwell, B., Jenner, P., 2001. 6-hydroxydopamine increases hydroxyl free radical production and DNA damage in rat striatum. *Neuroreport* 12: 1155-1159.

Foley, P., Riederer, P., 1999. Pathogenesis and preclinical course of Parkinson's disease. *J. Neural Transm. Suppl*. 56: 31-74.

Forno, L.S., Langston, J.W., DeLanney, L.E., Irwin, I., Ricaurte, G.A., 1986. Locus ceruleus lesions

and the eosinophilic inclusions in MPTP-treated monkeys. *Ann. Neurol*. 20:449-55.

Fulceri, F., Biagioni, F., Lenzi, P., Falleni, A., Gesi, M., Ruggieri, S., Fornai, F., 2006. Nigrostriatal damage with 6-OHDA: alidation of routinely applied procedures. *Ann.NY Acad. Sci*. 1074: 344-8.

Fuxe, K., Stromberg, I., Popoli, P., Rimondini-Giorgini, R., Torvinen, M., Ogren, S.O., Franco, R., Agnati, L.F., Ferre, S., 2001. Adenosine receptors and Parkinson's disease. Relevance of adenosine and dopamine receptor interactions in the striatum. *Adv. Neurol*. 86: 345-53.

Garcià Ruiz, P.J., 2004. Prehistoria de la enfermedad de Parkinson. *Neurologia* 19 (10): 735-7.

George, J.M., 2002. The synucleins. *Genom. Biol*. 3: 3002-6.

Gerfen, C.R., 1992b. The beostriatal mosaic: multiple levels of comportamental organization in the basal ganglia. *Annu. Rev. Neurosci*. 15: 285-320.

Ghee, M., Fournier, A., Mallet, J., 2000. Rat alpha-synuclein interacts with Tat binding protein 1, a component of the 26S proteasomal complex. *J. Neurochem*. 75: 2221-4.

Giasson, B.I., Uryu, K., Trojanowski, J.Q., Lee, V.M., 1999. Mutant and wild type human alpha-synucleins assemble into elongated filaments with distinct morphologies in vitro. *J. Biol. Chem*. 274: 7619-22.

Giasson, B.I., Lee, V. M.Y., 2001. Parkin and the molecular pathways of Parkinson's disease. *Neuron* 31: 885-8.

Giasson, B.I., Lee, V.M., 2003. Are ubiquitination pathways central to Parkinson's disease? *Neuron* 31: 885-8.

Goedert, M., 2001. Alpha-synuclein and neurodegenerstive diseases. *Nat. Rev. Neurosci*. 2: 492-501.

Goldberg, M.S., Fleming, S.M., Palacino, J.J., Cepeda, C., Lam, H,A., Bhatnagar, A., Meloni, E.G., Wu, N., Ackerson, L.C., Klapstein, G.J., Gajendrian, M., Roth, B.L., Chesselet, M.F., Maidment, N.T., Levine, M.S., Shen, J., 2003. Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. *J. Biol. Chem*. 278: 43628-35.

Greenamyre, J.T., Sherer, T.B., Betarbet, R., Panov, A.V., 2001. Complex I and Parkinson's disease. *IUB MB Life* 52: 135-41.

Greene, J.C., Whitwort, A.J., Kuo, I., Andrews, L.A., Feany, M.B., Pallanck, L.J., 2003. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. *Proc. Natl. Acad. Sci. USA* 73: 2424-8.

Haywood, A.F., Staveley, B.E., 2004. Parkin counteracts symptoms in a Drosophila model of Parkinson's disease. *BMC Neuroscience* 5, 14.

Hamm, R.J., Pike, B.R., O'Dell, D.M., Lyeth, B.G., Jenkins L.W., 1994. The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. *J. Neurotrauma* 11, 187-196.

Heikkila, R.E., Nicklas, W.J., Vyas, I., Duvoisin, R.C., 1985. Dopaminergic toxicity of rotenone and the 1-methyl-4-phenylpyridinium ion after their stereotaxic administration to rats: implication for the mechanism of1-methyl1-4-phenyl-1,2,3,6-tetrahydropyridine toxicity. *Neurosci. Let*. 62:389-94.

Henn, I.H., Bouman, L., Schelehe, J.S., 2007. Parkin mediates neuroprotection through activation of lkappaB kinase/nuclear factor-kappaB signalling. *J. Neurosci* 27: 1868-78.

Huyhn, D.P., Scoles, D.R., Nguyen, D., Pulst, S.M., 2003. The autosomal recessive juvenile Parkinson disease gene product, parkin, interacts with and ubiquitinates synaptotagmin XI. *Hum. Mol. Genet.* 12: 2587- 97.Imai, Y., Soda, M., Takahashi, R., 2000. Parkin

suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J. Biol. Chem*. 275: 35661-4.

Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., Takahashi, R., 2001. An unfolded putative trans membrane polipeptide, which can lead to endoplasmic reticulum stress, is a substrate for parkin. *Cell* 105: 891.902.

Ishikawa, A., Tsuji, S., 1996. Clinical analysis of 17 patients in 12 Japanese families with autosomal-recessive type juvenile parkinsonism. *Neurology*, 47:160-6.

Itier, J.M., Ibanez, P., Mena, M, A., Abbas, N., Cohen-Salmon, C., Boheme, G.A., Laville, M., Pratt, J., Corti, O., Pradier, L., Ret, G., Joubert, C., periquet, M., Araujo, F., Negroni, J., Caserejos, M.J., Canals, S., Solano, R., Serrano, A., Gallego, E., Sanchez, M., Denefle, P., Benavides, J., Tremp, G., Rooney, T.A., Brice, A., Garcia de Yebenes, J., 2003. Parkin gene inactivation alters behaviour and dopamine neurotransmission in the mouse. *Hum. Mol. Genet*. 12: 2277-91.

Iwai, A., Yoshimoto, M., Masliah, E., Saitoh, T., 1995. The precursor protein of non-beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* 14: 467-75.

Jellinger, K.A., 1991. Pathology of Parkinson's disease. Changes other than the nigrostriatal pathway. *Mol. Chem. Neuropathol*. 14: 153-197.

Jenner, P., 2008. Functional models of Parkinson's disease: a valuable tool in the development of novel therapies. *Ann. Neurol*. 64 (Suppl 2), S16-S29.

Jensen, P.H., Islam, K., Kenney, J., Nielsen, M.S., Power, J., Gai, W.P., 2000. Microtubule-associated protein 1B is a component of cortical Lewy bodies and binds alpha-synuclein filaments. *J. Biol. Chem*. 275: 2155-7.

Kahle, P.J., Haass, C., Kretzschmar, H.A., Neumann, M.M., 2002. Structure/function of alpha-synuclein in health and disease: rational development of animals models for Prkinson's and related diseases. *J. Neurochem*. 82: 449-57.

Kandel, E.R., Schwatrz, J.M., Jessell, T.M., 2000. Principles of Neural Science, 4th ed. McGraw-Hill, New York.

Kim, S.J., Sung, J.Y., Um, J.W., Hattori, N., Mizuno, Y., Tanaka, K., Paik, S.R., Kim, J., Chung, K.C., 2003. Parkin cleaves intracellular alpa-synuclein inclusions via the activation of calpain. *J. Biol. Chem*. 278: 41890-9.

Kitada, T., Asakawa, S., Hattori, N., et al., 1998. Mutations in the parkin gene cause autosomal parkinsonism. *Nature* 392: 605-8.

Klein, C., Pramstaller, P.P., Kis, B., Page, C.C., Kann, M., Leung, J., Woodward, H., Castellan, C.C., Scherer, M., Vieregge, P., Breakefield, X.O., Kramer, P.L., Ozelius, L.J., 2000. Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype. *Ann. Neurol*. 48: 65-71.

Koehler, P.J., Keyser, A., 1997. Tremor in latin texts of Dutch physicians: 16th-18th centuries. *Mov. Disord*. 12(5): 798-806.

Korell, M., Tanner, C.M., 2005. Epidemiology of Parkinson's disease: An overview. CRC press. 1, Parkinson's disease. I Ebadi, Manuchair S. II Pfeiffer, Ronald., 39-50.

Kowall, N.W., Hantraye, P., Brouillet, E., Beal, M.F., McKee, A.C., Ferrante R.J., 2000. Mptp induces alpha-synuclein aggregation in the substantia nigra of baboons. *Neuroreport* 11:211-3.

Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Scols, L., Riess, O., 1998. Ala30Pro mutation in the gene encoding α-synuclein in Prkinson's disease. *Nat. Genet*. 18: 106-8.

Lang, A.E., Lozano, A.M., 1998. Parkinson's disease. Second of two parts. *N. Engl. J. Med*. 339:1130-43.

Langston, J.W., 1998. Epidemiology versus genetics in Parkinson's disease: progress in

resolving an age-old debate. *Ann. Neurol*. 44 (Suppl 1); 45-52.

Langston, J.W., Ballard, P.A., Jr., 1983. Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *N. Engl. J. Med*. 309, 310.

Lanska, D.J., 2010. Chapter 33: the history of movement disorders. *Handb. Clin. Neurol*. 95: 501-46.

Lashuel, H.A., Petre, B.M., Wall, J., Simon, M., Nowak, R.J., Walz.T., Lansbury, P.T., Jr. 2002. Alpha-synuclein, especially the Parkinson's disease associated mutants, forms pore-like annular and tubular protofibrils. *J. Mol. Biol*. 322: 1089-102.

Lau, C.G., Zukin, R.S., 2007. NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. *Nat. Rev. Neurosci*. 8: 413-26.

Lavedan, C., 1998. The synuclein family. *Genome Res*. 8: 871-80.

Lee, F.J., Liu, F., Pristupa, Z.B., Niznik, H.B., 2001. Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. *FASEB J*. 15: 916-26.

Lees, A.J., 2007. Unresolved issues relating to the shaking palsy on the celebration of James Parkinson's 250th birthday. *Mov. Disord*. 22 Suppl 17: S327-34.

Lindgren, M., Hallbrink, M., Prochiantz, A., Langel, U., 2000. Cell-penetrating peptides. *TiPS* 21: 99-103.

Lindvall, O., Sawle, G., Widner, H., Rothwell, J.C., Bjorklund, A., Brooks, D., Brundin, P., Frackowiak, R., Marsden, C.D., Odin, P., et al., 1994. Evidence for long-term survival and function of dopaminergic grafts in progressive Parkinson's disease. *Ann. Neurol*. 35: 172-80.

Lindvall, O., Hagell, P., 2000. Clinical observations after neuronal transplantations in Parkinson's disease. *Prog. Brain Res*. 127: 299-320.

Lo Bianco, C., Schneider, B.L., Bauer, M., Sajadi, A., Brice, A., Iwatsubo, T., Aebischer, P., 2004. Lentiviral vector delivery of parkin prevents dopaminergic de generation in alpha-synuclein rat model of Parkinson's disease. *Proc. Natl. Acad. Sci. USA* 101: 17510-15

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Shimizu, N.,1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nautre* 392(6676): 605-608.

Klapdor, K., Dulfer, B.G., Hammann, A. and Van der Staay, F.J., 1997. A low cost method to analyse footprint patterns. *J. Neurosci. Methods* 75, 49–54.

Kruger, R.,Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H, Hepplen, J.T., Schols, L., Riess, O., 1998. Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. *Nat. Genet*. 18: 106-8.

Mahlon, R. Delong, 2000. The basal ganglia. In principles of neural science. Kandel, E.R., Schwartz, J.H., Jessell, T.M., eds McGraw-Hill Publ, United States, pp 853-867.

Mandel, S., Grunblatt, E., Riederer, P., Gerlach, M., Levites, Y., Youdim M.B., 2003. Neuroprotective strategies in Parkinson's disease: an update on progress. *CNS Drugs* 17: 729.62.

Manning-Bog, A.b., McCormack, A.L., Li, J., Uversky, V.N., fink, A.L., Di Monte, D.A., 2002. The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and α-synuclein. *J. Biol. Chem*. 277: 1641-44.

Marking, L., 1988. Oral toxicity of rotenone to mammals. *Investing. Fish Control*. 94.

Maroteaux, L., Campanelli. J.T., Scheller, R.H., 1988. Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. *J. Neurosci*. 8: 2804-15.

Marsden, C.D., 1990. Parkinson's disease. *Lancet* 335: 948-952.

Marsden, C.D., 1994. Parkinson's disease. *J. Neurol. Neurosurg. Psychiatry* 57:672-681.

Marshall, J.F., Ungerstesdt, U., 1977. Supersensitivity to apomorphine following destruction

to the descending dopamine neurons: quantification using the rotational model. *Eur. Pharmacol*. 41:361-7.

Martignoni, E., Pacchetti, C., Godi, L., Micieli, G., Nappi, G., 1995. Autonomic disorders in Parkinson's disease. *J. Neural Transm*. 45: 11-19.

Matsumine, H., Saito, M., Shimoda-Matsubayashi, S., Tanaka, H., Ishikawa, A., Nagakawa-Hattori, Y., Yokochi, M., Kobayshi, T., Igarashi, S., Takano, H., Sanpei, K., Koike, R., Mori, H., Kondo, T., Mizutani, Y., Schaffer, A.A., Yamamura, Y., Nakamura, S., Kuzuhara, S., Tsuji, S.,Mizuno, Y., 1997. Localization of a gene for an autosomal recessive form of juvenile parkinsonism to chromosome 6q25.2-27. *Am. J. Hum. Genet*. 60: 588-96.

McCormack, A.L., Thiruchelvam, M., Manning-Bog, A.B., Thiffault, C., Langston, J.W., Cory-Slechta, D.A., Di Monte, D.A., 2002. Environmental risk factors in Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiol. Dis*. 10: 119-27.

McNaught, K.S., Olanow, C.W., 2003. Proteolytic stress: a unifying concept for the etiopathogenesis of Prkinson's disease. *Ann. Neurol*. 53: 73-86.

Mendez, I., Dagher, A., Hong, M., Gaudet, P., Weerasinge, S., McAlitser, V., et al., 2002. Simultaneous intrastriatal and intranigral fetal dopaminergic grafts in patients with Parkinson's disease: a pilot study. Report of three cases. *J. Neurosurg*. 96: 589-96.

Miele, M., Mura, M. A., Enrico, P., Esposito, G., Serra, P. A., Migheli, R., Zangani, D., Miele, E. and Desole M. S., 2000. On the mechanism of d-amphetamine-induced changes in glutamate, ascorbic acid and uric acid release in the striatum of freely moving rats. *Br. J. Pharmacol*. 129, 582–588.

Mink, J.W., Thach, W.T., 1993. Basal ganglia intrinsic circuits and their role in behaviour. *Curr. Opin. Neurobiol*. 3: 950-7.

Moore, D.J., Dawson, V.L., Dawson, T.M., 2003. Role of the ubiquitin-proteasome system in Parkinson's disease and other neurodegenerative brain amyloidoses. *Neuromol. Med*. 4: 95-108.

Moore, D.J., 2006. Parkin: a multifaceted ubiquitin ligase. *Biochem. Soc. Transactions* 34: 749-53.

Murphy, D.D., Rueter, S.M., Trojanowski, J.Q., Lee, V. M., 2000. Synucleins are developmentialy expressed, and alpha-synuclein regulates the size of the prsynaptic vesicular pool in primary hippocampal neurons. *J. Neurosci*. 20: 3214-20.

Negro, A., Grassato, L., Polverino De Laureto, P., Skaper, S.D., 1997. Genetic construction, properties and application of a green fluorescent protein-tagged ciliary neurotrophic factor.*Protein Eng*. 10, 1077–1083.

Nicklas, W.J., Yougster, S.K., Kindt, M.V., Heikkila, R.E., 1987. MPTP, MPP^+ and mitochondrial function. *Life Sci*. 40: 721-29.

Nutt, J.G., 1990. Levodopa-induced dyskinesia: review, observations and speculations. *Neurology* 40:340-45.

Nutt, J.G., 2001. Motor fluctuation and dyskinesia in Parkinson's disease. *Parkinsonism Relat. Disord*. 8:101-108.

Nutt, J.G., Obeso, J.A., Stocchi, F., 2000. Continuous dopamine-receptor stimulation in advanced Parkinson's disease. *Trends Neurosci*. 23, S109-15.

Obeso, J.A., Olanow, C.W., Nutt, J.G., 2000a. levodopa motor complications in Parkinson's disease. *Trends Neuroscience* 23: S2-7.

Obeso, J.A., Rodriguez-Oroz, M.C., Rodriguez, M., Lanciego, J.L., Artieda, J., Gonzalo, N., Olanow, C.W., 2000c. pathophysiology of the basal ganglia in Parkinson's disease. *Trends Neuroscience* 23:S8-19.

Olanow, C.W., 1993. A radical hypothesis for neurodegeneration. *Trends Neurosci*. 16: 439-

44.

Olanow, C.W., 2004. The scientific basis for the courrent treatment of Parkinson's disease. *Annu. Rev. Med.* 55: 41-60.

Oluwatosin-Chibu, Y., Robbins, A., Scott, C.W., Arizza, J.L., Reid, J.D., Zysk, J.R., 2003. Parkin suppresses wild-type α-synuclein-induced toxicity in SHSY-5Y cells. *Biochem. Biophys. Res. Commun*. 309: 679-84.

O'Neill, R.D., 2005. Long-term monitoring of brain dopamine metabolism in vivo with carbon paste electrodes. *Sensors* 5: 317-42.

Ostrerova, N., Petrucelli, L., Farrer, M., Metha, N., Choi, P., Hardy, J., Wolozin, B., 1999. Alpha-synuclein shares physical and functional homology with 14-3-3- proteins. *J. Neurosci*. 19: 5782-91.

Owen, A.M., 2004. Cognitive dysfunction in Parkinson's disease: the role of fronto-striatal circuitry. *Neuroscientist* 10:199-202.

Owen, A.M., James, M., Leigh, P.N., Summers, B.A., Marsden, C.D., Quinn, N.P., 1992. Fronto-striatal cognitive deficits at different stages of Parkinson's disease. *Brain* 115 (Pt 6): 1727-1751.

Pakkenberg, B., Moller, A., Gundersen, H.J., Mouritzen Dam, A., Pakkenberg, H., 1991. The absolute number of nerve cells in substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method. J. Neurol. Neurosurg. Psychiatry 54: 30-3.

Palacino, J.J., Sagi, D., Goldberg, M.S., Krauss, S., Motz, C., Wacker, M., Klose, J., Shen, J., 2004. Mithocondrial dysfunction and oxidative damage in parkin-deficient mice. *J. Biol. Chem*. 279: 18614-22.

Parkinson, J., (2002). An essay on the shaking palsy. 1817. *J. Neuropsychiatry Clin. Neurosci.* 14, 223-36; discussion 222.

Paxinos, G. and Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates (2nd Ed.), *San Diego Academic Press*.

Paxinou, E., Chen, Q., Wiesse, M., Giasson, B.I., Norris, E.H., Rueter, S.M., Trojanowski, J.Q., Lee, V.M., Ischiropoulos, H., 2001. Induction of alpha-synuclein aggregation by intracellular nitrative insult. *J. Neurosci*. 21: 8053-61.

Periquet, M., Fulga, T., Myllykangas, L., Schlossmacher, M.G., Feany, M.B., 2007. Aggregated α-synuclein mediates dopaminergic neurotoxicity *in vivo*. *J. Neurosci*. 27: 3338-46.

Petrucelli, L., O'Farrell, C., Lockhart, P.J., Baptista, M., Kehoe, K., Vink, L., Choi, P., Wolozin, B., Farrer, M., Hardy, J., Cookson, M.R., 2002. Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. *Neuron* 36: 1007-19.

Prasad, K.N., Clarkson, E.D., La Rosa, F.G., Edwards-Prasad, J., Freed J., 1998. Efficacy of grafted immortalized dopamine neurons in an animal model of parkinsonism: a review. *Mol. Genet. Metab.* 65: 1-9.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., et al., 1997. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276: 2045-47.

Quinn, N.P., 1998. Classification of fluctuations in patients with parkinson's disease. *Neurology* 51:S25-9.

Rajagoplan, S., Andersen, J.K., 2001. Alpha-synuclein aggregation: is it the toxic gain of function responsible for neurodegeneration in Parkinson's disease? *Mech. Ageing. Dev*. 122: 1499-510.

Recchia, A., Debetto, P., Negro, A., Guidolin, D., Skaper, S.D., Giusti, P., 2004. Alpha-synuclein and Parkinson's disease. *FASEB J*. 18-617-626.

Recchia, A., Rota, D., Debetto, P., Peroni, D., Guidolin, D., Negro, A., Skaper, S.D., Giusti,

P., 2008. Generation of alpha-synuclein-based rat model of Parkinson's disease. *Neurobiol. Dis*. 30: 8-18.

Ren, Y., Zhao, J., Feng, J., 2003. Parkin bids to alpha/beta tubulin and increases their ubiquitination and degradation. *J. Neurosci*. 23: 3316-24.

Riederer, P., Wuketich, S., 1976. Time course of nigrostriatal degeneration in parkinson's disease. A detailed study of influential factors in human brain amine analisys. *J. Neural Transm*. 38: 277-301.

Rioux, L., Gaudin, D.P., Gagnon, C., Di Paolo, T., Bedard, P.J., 1991b. decrease of behavioral and biochemical denervation supersensitivity of rat striatum by nigral transplants. *Neuroscience* 40: 123-31.

Rosen K.M., Veereshawarayya, V., Moussa, C.E., Fu, Q., Goldberg, M.S., Schlossmacher, M.G., Shen, J., Querfurth, H.W., 2006. Parkin protects against mitochondrial toxins and beta-amyloid accumulation in skeletal muscle cells. *J. Biol.Chem*. 281: 12809-16.

Rouse, S.T., Michael, J.M., Bradley, M.S., Aad, H., Wittmann, M., Conn, P.J., 2000. Distribution and roles of metabotropic glutamate receptor in the basal ganglia motor circuit: implications for treatment of Parkinson's disease and related disorders. *Pharmacology & Therapeutics* 88: 427-35.

Schrang, A., 2004. Psychiatric aspects of Parkinson's disease: an update. *J. Neurol*. 251: 795-804.

Schubert, U., Anton, L.C., Gibbs, J., Norbury, C.C., Yewdell, J.W., Bennik, J.R., 2001. Rapid degradation of a large fraction of newly synthetized proteins by proteasomes. *Nature* 404: 770-4.

Schwarzschild, M.A., Agnati, L., Fuxe, K., Chen, J.F., Morelli, M., 2006. Targeting adenosine A2a receptors in Parkinson's disease. *Trends Neurosci*. 29:647-54.

Serpell, L.C., Berriman, J., Jakes, R., Goedert, M., Crowther, R.A., 2000. Fiber diffraction of synthetic α-synuclein filaments shows amyloid-like cross-β conformation. *Proc. Natl. Acad. Sci*. USA 97:4897-02.

Serra, P.A., Migheli, R., Rocchitta, G., Taras, M.G., Mura, M.P., Delogu, M.R., Esposito. G., Desole, M.S., Miele, E., Miele, M., 2003°. Role of the nitric oxide/cyclic GMP pathway and ascorbic acid in 3-morpholonosydnonimine (SIN-1)-induced increases in dopamine secretion from PC12 cells. A microdialysis in vitro study. *Neurosci. Let*. 353:5-8.

Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., Suzuki, T., 2000. Familial Parkinson disease gene product, parkin, is a ubiqiutin protein ligase. *Nat. Genet*. 25: 302-5.

Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosic, K.S., Selkoe, D.J., 2001. Ubiquitination of a new form of apla-synuclein by parkin from human brain: implications for Parkinson's disease. *Science* 293: 263- 9.

Schwarze, S.R., Hruska, K.A., Dowdy, S.F., 2000. Protein transduction: unrestricted delivery into all cells? *Cell Biology* 10: 290-6.

Sian, J.,Gerlach, M., Youdim, M.B., Riederer, P., 1999. Parkinson's disease: a major hypochinetic basal ganglia disorder. *J. Neural Transm*. 106:443-476.

Singleton, A.B., Farrer, M., Johnson, J., Singleton, J., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M.R., Muenter, M., Baptista, M., Miller, D., Blancato, J., Hardy, J., Gwinn-Hardy, K., 2003. A-synuclein locus triplication causes Parkinson's disease. *Science* 302: 841.

Smith, J.G., 1988. Paraquat poisoning by skin absorption: a review. *Hum. Toxicol*. 7: 15-19.

Snyder, H., Mensah, K., Theisler, C., Lee, J., Matouschek, A., Wolozin, B., 2003. Aggregated

and monomeric alpha-synuclein bind to the S6' proteasomal protein and inhibit proteasomal function. *J. Biol. Chem*. 278: 11753-9.

Spillantini, M.G., Divane, A., Goedert, M., 1995. Assignment of human alpha-synuclein (SNCA) and beta-synuclein (SNCB) gene sto chromosomes 4q21 and 5q35. *Genomics* 27: 379-81.

Staropoli, J.F., McDermott, C., Martinat, C., Schulman, B., Demireva, E., Abeliovich, A., 2003. Parkin is a component of SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainite excitotoxcicity. *Neuron* 37: 735- 49.

Stefanis, L., Larsen, K.E., Rideout, H.J., Sulzer, D., Greene. L.A., 2001. Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. *J. Neurosci*. 21: 9549- 60.

Takeuchi, Y., Sawada, T., Blunt, S., Jenner, P., Marsden, C.D., 2003. Effects of 6-hydroxydopamine lesions of the nigrostriatal pathway on striatl serotonin innervations in adult rats. *Brain Res*. 562: 301-305.

Talpade, D.J., Greene, J.G., Higgins, Jr., D.S., Greenamyre, J.T., 2000. In vivo labelling of mitochondrial complex I (NADH: ubiquinone oxidoreductase) in rat brain using [(3)]dihydrorotenone. *J. Neurochem*. 75: 2611-21.

Tanaka, K. Suzuki, T., Chiba, T., Shimura, H., Hattori, N., Mizuno, Y., 2001. Parkin is linked to the ubiquitin pathway. *J. Mol. Med*. 79: 482-94.

Tanaka, Y., Engelender, S., Igarashi, S., Rao, R.K., Wanner, T., Tanzi, R.E., Sawa, A., Dawson, L., Dawson, T.M., Ross, C.A., 2001. Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. *Hum. Mol. Genet*. 10: 919-26.

Tanner, C.M., Goldman, S.M., 1996. Epidemiology of Parkinson's disease. *Neuropepidemiology* 14: 317-35.

Tanner, C.M., 2003. Is the cause of Parkinson's disease environmental or hereditary? Evidence from twin studies. *Adv. Neurol*. 91: 133-42.

Tanner, C.M., Freya, K., Webster Ross, G., Hoppin, J.A., Goldman, S.M., Korell, M., et al., 2011. Rotenone, Paraquat, and parkinson's disease. *Environ. Health Perspect*. 119:866-72.

Thacker, E.L., Chen, H., PPatel, A.V., McCullough, M.L., Calle, E.E., Thun, M.J., Schwarzschild, M.A., Ascherio, A., 2008. Recreational physical activity and risk of Parkinson's disease. *Mov. Disord*. 23: 69-74.

Thomas, B., Beal, M.F., 2007. Parkinson's disease. *Hum. Mol. Genet*. 16: 183-94.

Tofaris, G.K., Layfield, R., Spillantini, M.G., 2001. Alpha-synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome. *FEBS Lett*. 509: 22-6.

Tomac, A., Lindqvist, E., Lin, L.F., Ogren, S.O., Young, D., Hoffer, B.J., Olson, L., 1995. Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature* 373: 335-9.

Ueda, K., Fukushima, H., Masliah, E., Xia, i., Iwai, A., Yoshimoto, M., et al., 1993. Molecular cloning of c DNA encoding in unrecognized component of amyloid in Alzheimer disease. *Proc. Natl. Acad. Sci*. USA 98: 11282-6.

Ungerstedt, U., 1968. 6-hydroxydopamine induced degeneration of central monoamine neurons. *Eur. J. Pharmacol*. 5: 107-110.

Ungerstedt, U., 1971a. Adipsia and aphagia after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system. *Acta Physiol. Scand. Suppl*. 367: 95-122.

Ungerstedt, U., 1971b. Postsynaptic supersensitivity after 6-hydroxydopamine induced

degeneration of the nigro-striatal dopamine system. *Acta Physiol. Scand. Suppl*. 367:69-73.

Volles, M.J., Lee, S.J., Rochet, J.C., Shtilerman, M.D., Ding, T.T., Kessler, J.C., Landsbury, P.T., Jr. 2001. Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinsons disease. *Biochemistry* 40: 7812-9.

Von Coeln, R., Dawson, V.L., Dawson, T.M., 2004. Parkin-associated Parkinson's disease. *Cell. Tissue Res*. 318: 175-84.

West, A., Farrer, M., Petrucelli, L., Cookson, M., Lockhart, P., Hardy, J., 2001. Identification and characterization of the human parking ne promoter. *J. Neurochem*. 78: 1146-52.

West, A., Periquet, M., Lincoln, S., Lucking, C.B., Nicholl, D., Bonifatti, V., Rawal, N., Gasser, T., Lohmann, E., Deleuze, J.F., Maraganore, D., Levey, A., Wood, N., Durr, A., Hardy, J., Brice, A., Farrer, M., 2002. Complex relationship between parkin mutations and Parkinson's disease. *Am. J. Med. Genet*. 114: 584-91.

West, A.B., Lockhart, P.J., O'Farell, C., Farrer, M.J., 2003. Identification of a novel gene linked to parkn via a bi-directional promoter. *J. Mol. Biol*. 326: 11-9.

Wightman, R.M., Robinson, D.L., 2002. Transient change in mesolimbic dopamine and their association with 'reward'. *J. Neurochem*. 82: 721-35.

Winkler, C., Bentlage, C., Nikkaha, G., Samii, M., Bjorklund, A., 1999. Intranigral transplants of GABA-rich striatal tissue induce behavioural recovery in the rat Parkinson model and promote the effects obtained by intrastriatal dopaminergic transplants. *Exp. Neurol*. 155: 165-86.

Winklhofer, K.F., Henn, I.H., Kay-Jackson, P.C., Heller, U., Tatzelt, J., 2003. Inactivation of parkin by oxidative stress and C-terminal truncations: a protective role of molecular chaperones. *J. Biol. Chem.* 278: 47199- 208.

Winklhofer, K.F., 2007. The parkin protein as a therapeutic target in Parkinson's disease. *Expert. Opin. Ther. Targets* 11: 1543- 52.

Wolters, E.C., Berendese, H.W., 2001. Management of psychosis in Parkinson'a disease. *Curr. Opin. Neurol*.14: 499-504.

Wood, S.J., Wypych, J., Steavenson, S., Louis, J.C., Citron, M., Biere, A.L., 1999. α-synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease. *J. Biol. Chem*. 274: 19509-12.

Xu, J., Kao, S.Y., Lee, F.J., Song, W., Jin, L.W., Yankner, B.A., 2002. Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson's disease. *Nat. Med*. 8: 600-6.

Yang, Y., Nishimura, I., Imai, Y., Takahashi, R., Lu, B., 2003. Parkin suppreses dopaminergic neuron-selective neurotoxicity induced by Pael-R in *Drosophila*. *Neuron* 37: 911-24.

Yamamura, Y, Sobue, I., Ando, K., Iida, M., Yanagi, T., 1973. Paralysis agitans of esrly onset with marked diurnal fluctuation of symptoms. *Neurology* 23:239-44.

Zhang, Y., Gao, J., Chung, K.K., Huang, H., Dawson, V.L., Dawson, T.M., 2000. Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc. Natl. Acad. USA* 97: 13354-59.

Zigmond, M.J., Stricker, E.M., 1972. Deficits in feeding bahavior after intraventricular injection of 6-hydroxydopamine in rats. *Science* 177: 1211-14.

Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., Kachergus, J., Hulihan, M., Uitti, R.J., Calne, D.B., et al., 2004. Mutations in LRRK2 cause autosomal-domonant parkinsonism with pleomorphic pathology. *Neuron* 44:601-7.