UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Regulation of DJ-1 Expression by Neuron-Astrocyte Interaction

Mónica Calado Sardinha

Dissertação para obtenção do Grau de Mestre em
Bioquímica
(2º ciclo de estudos)

Orientador: Prof.ª Doutora Graça Baltazar
Coorientador: Prof. Doutor Bruno Manadas

Covilhã, outubro de 2014

Principles for the Development of a Complete Mind: Study the science of art. Study the art of science. Develop your senses- especially learn how to see. Realize that everything connects to everything else."
— Leonardo da Vinci-

Acknowledgments

Gostaria de começar por agradecer às pessoas mais importantes da minha vida, os meus pais. Obrigado pelo amor e o carinho incondicional, por sempre me encorajarem a lutar por tudo o que desejo e por todos os bons valores com que sempre me educaram. Obrigado por sempre me incentivarem a querer estudar e a perceber que a educação é uma das nossas maiores riquezas. Sobretudo obrigado por todos os sacrifícios realizados todos os dias para que estes 5 anos se tornassem realidade.

Obrigado ao Luís por todo o amor, compreensão, pelo seu apoio incondicional, por estar sempre presente e sobretudo por todo a calma que me transmite. Agradeço-te, ainda, toda a ajuda preciosa na elaboração desta dissertação.

Obrigado a todos os meus amigos pelo apoio. Obrigado ao Sérgio, Rúben, Carolina, Ana e Andreia por se revelarem bons amigos e por todas as partilhas ao longo destes 5 anos de curso. Obrigado às minhas meninas Sónia, Vera e Nélia por estarem sempre presentes e por nunca me terem deixado de apoiar. Um agradecimento muito especial à Sónia por ser uma irmã de coração em todos os aspetos.

Um agradecimento especial à Rita por me ter recebido tão bem no CICS, por me ter ensinado tudo o que sabia e que tão útil foi para a realização deste projeto e por estar sempre disponível a ajudar. Obrigado ainda aos restantes elementos do meu grupo de trabalho: Filipa, Julieta, Daniela e Diogo pelo apoio sempre prestado porque mais do que colegas foram sempre bons amigos ao longo deste ano. Foi um prazer trabalhar/conviver com vocês.

Obrigado à minha "Família Musical" e um pedido de desculpas por não poder ter estado presente tanto quanto gostaria.

Por último mas não menos importante, gostaria de agradecer à Prof.ª Doutora Graça Baltazar pela oportunidade de desenvolver este projeto na área que eu tanto queria, pela disponibilidade, pelo apoio e por todos os conhecimentos transmitidos. Agradeço ainda ao Prof. Doutor Bruno Manadas pela disponibilidade demonstrada em ser meu co-orientador.

Resumo

A doença de Parkinson (DP) é a segunda desordem neurodegenerativa mais comum, caracterizada por uma progressiva disfunção motora decorrente da perda seletiva de neurónios dopaminérgicos da *substantia nigra pars compacta* (SNpc), resultando numa diminuição dos níveis de dopamina no estriado. Apesar de se encontrar estabelecido que fatores genéticos e ambientais contribuem para o desenvolvimento da doença, os mecanismos precisos a nível molecular ainda não se encontram totalmente esclarecidos. Contudo, a disfunção mitocondrial e o *stress* oxidativo têm sido apontados como os principais responsáveis pela neurodegeneração, sendo que neste estudo o nosso foco recaiu especificamente sobre o stress oxidativo.

Os radicais livres são moléculas altamente reativas caraterizadas por possuírem eletrões desemparelhados, altamente reativos que quando produzidas em pequenas quantidades nas células, como é o caso das espécies reativas de oxigénio, desempenham funções essenciais ao organismo. Contudo, estas espécies moleculares devem ser balanceadas com a produção de antioxidantes de forma a manter a homeostase celular. Assim, o aumento da concentração de radicais livres relativamente à concentração de antioxidantes conduz ao desencadear de uma condição de *stress* oxidativo.

Os astrócitos são as células gliais com maior abundância no cérebro funcionando como células de suporte ou metabólicas. Estas células são conhecidas por possuírem uma poderosa maquinaria neuroprotetora que lhes confere a capacidade de secretar fatores neurotróficos bem como de ativar a maquinaria necessária às funções antioxidantes, contribuindo para o suporte e sobrevivência neuronal. Dadas estas características, estudos têm sugerido que os astrócitos possuem um papel importante no controlo de doenças neurodegenerativas incluindo a DP e a sua disfunção tem sido demonstrada em amostras de cérebro humano ou em modelos experimentais da DP.

Mutações no gene (PARK7) que codifica para a DJ-1 resultam na perda funcional da proteína causando a forma familiar ou esporádica da DP, assim, a elucidação da função fisiológica da proteína contribuirá para importantes avanços no conhecimento da DP. A DJ-1 é uma proteína multifuncional que se encontra expressa tanto em neurónios como em astrócitos, sendo que, de todas as funções que lhe são atribuídas, a mais relevante para a DP é o seu papel neuroprotetor, provavelmente devido ao seu envolvimento na proteção contra o *stress* oxidativo funcionando como um sensor, uma vez que, a sua atividade é regulada pelo seu estado oxidativo e por esse motivo, a sobre-oxidação corresponde à forma inativa da proteína.

Estudos *postmortem* realizados em cérebros de doentes com DP esporádica demonstraram que a DJ-1 é abundantemente expressa em astrócitos reativos. Dadas as propriedades neuroprotetoras anti-oxidantes e anti-apoptóticas da DJ-1, este aumento de expressão pode representar um mecanismo protetor compensatório, visto inúmeros estudos demonstrarem que a DJ-1 tem a capacidade de proteger os neurónios contra insultos tóxicos ou oxidativos. A vulnerabilidade neuronal presente nos processos neurodegenerativos associados à DP pode não resultar apenas da disfunção dos mecanismos neuronais mas, por outro lado, resultar da alteração das funções astrocitárias ou mesmo da interação neurónio-astrócito.

Tendo em conta estes fatos, o objetivo deste estudo foi determinar como a interação neurónio-astrócito influencia a expressão da DJ-1 quando o *stress* oxidativo está presente e de que forma essa expressão contribui para a neuroprotecção.

Assim, de forma a atingir o objetivo proposto, determinámos a expressão da DJ-1 em culturas primárias de astrócitos ou de neurónios do mesencéfalo ventral e em coculturas neurónio-astrócito expostas a concentrações crescentes de H_2O_2. Da observação dos resultados, concluímos que, tal como anteriormente descrito, a DJ-1 é expressa em todos os tipos de culturas embora não se tenham verificado aumentos significativos nos níveis intracelulares da proteína com o aumento da concentração de H_2O_2 utilizada. Além disso, também o contacto entre neurónios e astrócitos apenas através do meio de cultura pareceu não afetar os níveis intracelulares da DJ-1 quando condições controlo foram comparadas com condições oxidativas. Contudo esta abordagem não nos permitiu confirmar qual o perfil de secreção da DJ-1 para o meio de cultura perante um insulto oxidativo, como previamente descrito, ou a influência dessa secreção na proteção neuronal.

Dada a necessidade de clarificar a influência da secreção da DJ-1 na sobrevivência neuronal, realizámos as culturas anteriormente descritas, mas adicionámos em simultâneo com o estímulo de H_2O_2 um anti-corpo anti-DJ-1, para promover a imunodepleção da DJ-1. Os resultados mostraram que o *stress* oxidativo provocado pela exposição a H_2O_2 levou ao aumento da morte dopaminérgica sendo a extensão da morte provocada por este estímulo significativamente maior quando se promoveu o bloqueio da DJ-1 presente no meio, o que sugere que a DJ-1 contribui significativamente para a proteção mediada pelos astrócitos.

Uma vez provada a importância da DJ-1 secretada para o meio de cultura na neuroproteção mediada pelos astrócitos, o próximo passo foi verificar se os neurónios teriam a capacidade de endocitar a DJ-1 aplicada exogenamente, e marcada com um fluoróforo de modo a que pudesse ser seguida a sua posição. A análise de fluorescência em conjunto com a marcação por imunocitoquímica para a DJ-1 permitiu concluir que, de facto, os neurónios têm a capacidade

de endocitar a DJ-1 exógena, podendo ser através deste mecanismo que a proteína exerce as suas funções intracelulares.

A análise destes resultados permitiu-nos sugerir que possivelmente a DJ-1 possa ser secretada pelos astrócitos para o meio de cultura quando estes são estimulados por um insulto oxidativo, neste caso H_2O_2, sendo consequentemente endocitada pelos neurónios, onde exercem a sua ação, direta ou indireta na proteção neuronal mediada pelos astrócitos.

Palavras-chave

DJ-1; Astrócitos; Neuroproteção; *Stress* oxidativo; H_2O_2; Doença de Parkinson

Abstract

Parkinson's disease (PD) is the second common neurodegenerative disorder, characterized by massive dopaminergic (DAergic) neuronal death in the substantia nigra and resulting in a reduced level of dopamine (DA) in the striatum. Although both genetic and environmental factors are thought to affect the onset of PD, the precise mechanisms at the molecular level have not been fully elucidated. However, mitochondrial dysfunction and oxidative stress are suggested as major causes of neurodegeneration in PD.

Astrocytes are the most abundant glial cell type in the brain. These cells are known to harbor powerful neuroprotective machinery allowing them to secrete neurotrophic factors and activate anti-oxidative stress pathways, thereby supporting neurons and ensuring their survival. Therefore, astrocyte dysfunction may compromise neuronal survival and, in this way, facilitate neurodegeneration. This dysfunction was already demonstrated in human samples and in experimental models of PD.

Mutations in DJ-1 gene (PARK7) leading to a loss of functional protein cause familial PD and enhances the sensitivity to oxidative insults, so by elucidating DJ-1 physiological function important insights into PD will be achieved. DJ-1 is a ubiquitous redox-responsive cytoprotective protein with diverse functions but, of all the functions that have been attributed, perhaps the most important to PD is its neuroprotective role, most likely by its involvement in protection against oxidative stress. In recent studies, an increase in DJ-1's expression was found in reactive astrocytes of PD brains and extensive research demonstrated that DJ-1 has the ability to protect neurons against oxidative and toxic insults. However, the function of astrocytic DJ-1 is still unknown.

Taking these facts together, the aim of this study was to determine how neuron-astrocyte interaction influences the expression of DJ-1 when oxidative stress is present and how this expression contributes to neuroprotection.

The present study allowed us to conclude that DJ-1 is expressed in primary midbrain cell cultures, and this expression is not altered when an oxidative stress insult is present. We also showed that the survival of DAergic neurons was affected when they were exposed to oxidative stress, even when astrocyes were present. Moreover, that lack of DJ-1 in culture media combined with oxidative insult impairs the astrocyte-mediated neuroprotection leading to a drastic reduction of neuronal survival. Furthermore, incubation of neurons with a fluorofore-coupled DJ-1 applied exogenously allowed us to demonstrate that in fact neurons have the ability to endocytose extracellular DJ-1.

These results suggest that DJ-1 may be released into the culture medium probably by astrocytes when challenged with an oxidative stress insult and consequently be taken up by neurons, exerting a direct or indirect neuroprotective role.

Keywords

DJ-1; Astrocytes; Neuroprotection, Oxidative stress; H_2O_2; Parkinson's disease

Index

Figures List

Tables List

Acronyms Lists

6-OHDA *6-hydroxidopamine*

AD..... *Autosomal dominant*

Ank .....*Ankyrin-like repeat*

AR.....*Autosomal recessive*

ASK1 ..... *Apoptosis Signal-Regulating kinase 1*

CAPS.....*Ciclohexilamin Acid Propanosulphonic*

COR..... *c-terminal of ROC*

DA.....*Dopamine*

DP..... *Doença de Parkinson*

EOPD ..... *Rare early-onset PD*

FBS.....*Fetal Bovine Serum*

GWAS.....*Genome-wide association studies*

HI .....*Heat-inactivated*

JNK ..... *c-Jun N-terminal kinase*

JOPD.....*juvenile-onset PD*

LB .....*Lewy bodies*

LRR .....*Leucine rich repeats*

LRRK2 ..... *Leucine-rich repeat kinase 2*

MAP3 K5 ..... *Mitogen-activated protein kinase-kinase-kinase 5*

MNB..... *Neurobasal Medium*

MTS .....*Mitochondrial targeting sequence*

NAC .....Nonamyloid component

NO ..... *Nitric oxide*

p38^{MAPK} ..... *p38 mitogen-activated protein kinase*

PBS .....Phosphate buffer saline

PBS-T .....*PBS with 0.1% Tween 20*

PD..... *Parkinson's Disease*

PFA ..... *Paraformaldehyde Fixate Solution*

PI3 K..... *Phosphoinositide-3 kinase*

PINK1 .....*PTEN-induced putative kinase 1 gene*

PSA ..... *Ammonium persulphate*

PVDF.....*Polyvinylidene difluoride*

ROC.....*Ras of complex proteins*

ROS ..... *Reactive oxygen species*

SDS.....*Sodium Dodecyl Sulfate*

SNpc .....*Substantia nigra pars compacta*

TBS-T..... *Tris-buffered saline with 0.1% Tween 20*

TEMED..... *Tetramethylethylenediamine*

TH..... *Tyrosine Hydroxylase*

TLR .....*Toll-like receptors*

TM .....*Putative transmembrane domain*

UBL .....*Ubiquitin-like*

WT .....*Wild-type*

YOPD .....*Young-onset PD*

Chapter 1

Introduction

1. Parkinson's Disease

Parkinson's disease (PD) was first described in 1817 by James Parkinson, who established the clinical symptoms, in his monograph "Essay on the Shaking Palsy" (Dauer and Przedborski, 2003).

PD is a complex progressive disease that mainly inhibits fine motor ability while also has a damaging effect on cognitive function (Foltynie et al., 2004). PD is the most frequent age-related movement disorder and, after Alzheimer's disease, is the second most prevalent neurodegenerative disorder (Dauer and Przedborski, 2003, Wirdefeldt et al., 2011).

PD is a multifactorial disease, likely arising from a combination of genetic susceptibility, ageing and gender (men are slightly more prone to develop PD, environmental exposures, and gene-environment interactions (Hindle, 2010, Elbaz and Moisan, 2008).

The incidence of this disease is similar worldwide and the percentage of affected individuals is about 1-2% of people above 65 years, increasing to 4% in people over 85 years (Dauer and Przedborski, 2003b). The sporadic PD or idiophatic PD has a mean age of onset around 60 years but when rare early-onset PD (EOPD), or familial PD, is present this symptoms occurs after the age of 50 (de Lau and Breteler, 2006).

EOPD frequently has a monogenic origin and can be divided in two different groups: juvenile-onset PD (JOPD), where the symptoms begin before the age of 20 years, and young-onset PD (YOPD) with an age of onset between 21 and 50 years (de Lau and Breteler, 2006). Although EOPD cases account for 10% of total cases of PD, investigations of the functions of EOPD gene products have provided great insights into the molecular mechanisms of the onset of PD and these gene products are thought to also play specific roles in the pathogenesis of sporadic PD (Hauser and Hastings, 2013).

The progression of PD is related to the substantial decrease of DAergic neurons located in the SNpc leading to loss of DA in the striatum (Figure 1) (Mori et al., 2006) and is accompanied by the presence of Lewy bodies (LB), cytoplasmic protein inclusions, and Lewy neurites in the surviving DAergic neurons (Dauer and Przedborski, 2003b, Wirdefeldt et al., 2011). These LB are composed by fibrils of insoluble polymers of α-synuclein that deposit in the cell body of

neurons (Mandel et al., 2010). Although some studies suggest that, in some monogenic forms of PD, this typical LB pathology is absent, this feature remains fundamental for confirm the diagnosis of the disease (Crosiers et al., 2011). Additionally, a 5-10% loss of dopamine neurons befalls each decade of aging (Fearnley and Lees, 1991). However, the process by which it occurs has not been established.

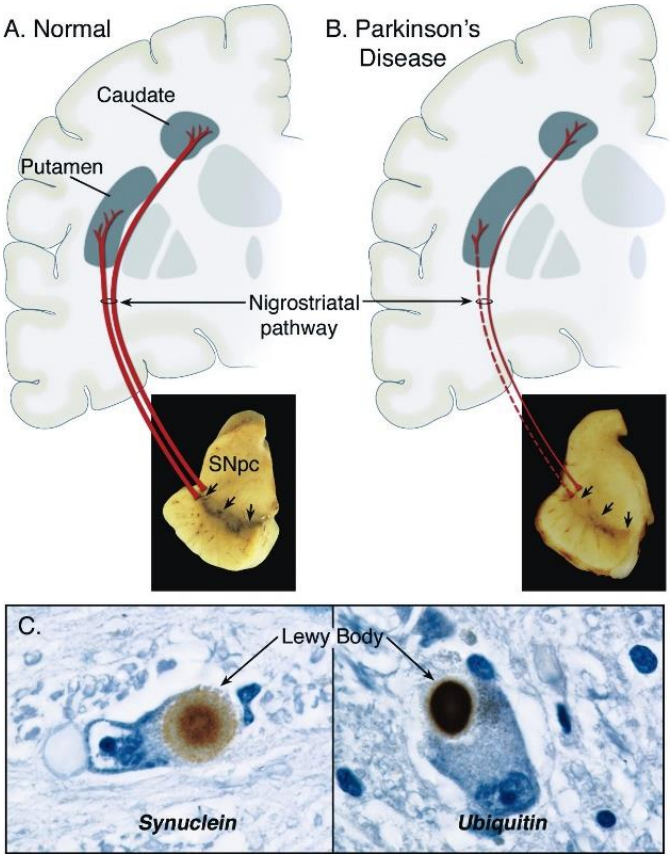


Figure 1 – **Neuropathology of PD.** (A) Schematic representation of the normal nigrostriatal pathway (in red). It is composed of DAergic neurons whose cell bodies are located in SNpc (see arrows). These neurons project their axons (thick solid red lines) to the basal ganglia and synapse in the striatum (i.e., putamen and caudate nucleus). The image shows the normal pigmentation of the SNpc. (B) Schematic representation of the diseased nigrostriatal pathway (in red). In PD, the nigrostriatal pathway degenerates. There is a marked loss of DAergic neurons that project to the putamen (dashed line) and a much more modest loss of those that project to the caudate (thin red solid line). The image shows depigmentation (i.e., loss of dark-brown pigment; arrows) of the SNpc due to the marked loss of DAergic neurons. (C) Immunohistochemical labelling of intraneuronal inclusions, termed LB, in a SNpc DAergic neuron. Immunostaining with an antibody against α-synuclein reveals a LB (black arrow) with an intensely immunoreactive central zone surrounded by a faintly immunoreactive peripheral zone (left image). Conversely, immunostaining with an antibody against ubiquitin yields more diffuse immunoreactivity within the Lewy body (right image) (Dauer and Przedborski, 2003a).

The diagnosis of PD is usually focused on the presence of cardinal motor symptoms since the definitive diagnosis of PD can only be established when post-mortem tissues are observed and for this reason is frequently used the term Parkinsonism instead of PD.

The cardinal motor symptoms are evident when approximately 80% of striatal DA and 50% of nigral neurons are lost (Bekris et al., 2010) and consist in a progressive lack of movement control encompassing clinical features such as resting tremor, bradykinesia, gait difficulties, postural instability and rigidity (Martin et al., 2011). However, some patients could also suffer several non-motor symptoms of PD, such as autonomic insufficiency, cognitive impairment, depression, olfactory deficits, psychosis, sleep disturbance and dementia during the course of the disease (Bekris et al., 2010).

Nowadays, there are a lot of treatments focused on the replacement of striatal DA in order to attenuate the symptoms (Table 1) (Bekris et al., 2010, Stayte and Vissel, 2014). The therapy most used and most responsiveness for symptomatic treatment of PD is the oral levodopa therapy and, for this reason, it has become a key diagnostic criterion (Bekris et al., 2010) (Zappia et al., 2010). Despite these therapies, there is a newly emerging non- DAergic therapeutic strategy for PD, including drugs targeting adenosine, glutamate, adrenergic, and serotonin receptors, as well as GLP-1 agonists, calcium channel blockers, iron chelators, anti-inflammatories, neurotrophic factors, and gene therapies (more information (Stayte and Vissel, 2014)). However, more studies are required to determine the specific molecular mechanisms that cause neurodegeneration in PD in order to provide an effective treatment that can delays or arrest the natural progression of the disease.

Table 1 - **Current treatment strategies in clinical use (Stayte and Vissel, 2014).**

Drug class	Drug name/route	Clinical use	Advantages	Disadvantages
Levodopa (with Dopa Decarboxylase inhibitors)	Sinemet, Parcopa, Atamet	Monotherapy	Increase levels of endogenous DA	Motor fluctuations Dyskinesias
COMT Inhibitors	Entacapone, Tolcapone	Adjunct therapy	Decrease metabolism of L-Dopa Decrease in daily dose of L-Dopa required Increase daily "on" time and UPDRS scores	Dyskinesias Diarrhea Hepatic toxicity (tolcapone) Dizziness Insomnia Nausea
Dopamine agonists	Piribedil, Pramipexole, Pramipexole extended release, Ropinirole, Rotigotine, Cabergoline, Pergolide Bromocriptine	Monotherapy (younger patients) Adjunct therapy	Increase levels of endogenous DA Decrease motor symptoms in early stages of disease	Sedation Impulse control disorder Somnolence Edema
Levodopa, Carbidopa, Entacapone combination	Stalevo	Monotherapy	Increase levels of endogenous DA Decrease metabolism of L-Dopa	Dyskinesias may appear sooner Side effects are the same as for individual drug classes
MAO-B Inhibitors	Rasagiline, Selegiline, Safinamide	Initial monotherapy (mild PD patients) Adjunct therapy	Decrease catabolism of DA Decrease rate of progression on UPDRS	Generally well tolerated Mild nausea Constipation Confusion
Continuous L-Dopa	Intravenous bolus, Intravenous infusion, Intestinal carbidopa/L-Dopa gel	Monotherapy	Decrease pulsatile DA levels Increase control of on/off periods Decrease dyskinesia severity and duration Decrease non motor symptoms (e.g., mood shifts, dribbling and urinary function changes)	Large volumes required (intravenous) Requires surgery and prosthetic device Mechanical problems Gastronomy complications
Anticholinergics	Trihexyphenidyl, Benztropine, Orphenadrine, Procyclidine, Biperiden	Monotherapy Adjunct Therapy	Decrease acetylcholine levels Decrease tremor	Dry mouth Blurred vision Constipation Nausea Impaired sweating

Abbreviations: COMT, catechol-O-methyl transferase; DA, dopamine; L-Dopa, levodopa; MAO-B, monoamine oxidase B; UPDRS, Unified Parkinson's Disease Rating Scale.

1.1 Hereditary or monogenic Parkinsonism

Until the discovery in 1997 of the mutation in the α-synuclein gene, that causes an inherited form of PD (Dauer and Przedborski, 2003b), PD was considered a non-genetic disease. Since then, several other gene mutations have been identified and, nowadays, 5-10% of patients with this disease are known to have the monogenic form (de Lau and Breteler, 2006).

Since these genetic studies increased, the knowledge of the disease has increased largely. These studies enabled the development of animal models and allowed a direct insight into gene and protein function as well (Farrer, 2006, Hardy and Orr, 2006). Genome-wide association

studies (GWAS) have been helpful to identify loci at which common genetic variants increase risk of developing apparently sporadic disease (Martin et al., 2011).

Through the GWAS (Table 2) or linkage analysis (Table 3) a total of 18 PD loci have been identified, the so-called PARK locus, associated both autosomal dominant (AD) [SNCA/PARK1/4 and Leucine- rich repeat kinase 2 (LRRK2)/PARK8] and autosomal recessive (AR) PD [parkin/PARK2, PTEN- induced kinase 1 (PINK1)/PARK6, DJ-1/PARK7, ATP13A2/PARK9] (Table 3). Besides these, variations in two other genes not assigned to a PARK locus (MAPT and GBA) are also well-validated risk factors for PD (Table 2) (Martin et al., 2011) (Bekris et al., 2010) (Spatola and Wider, 2014).

Mutations in six genes (SNCA, LRRK2, PRKN, DJ-1, PINK1 and ATP12A2) are associated with hereditary Parkinsonism, and all except the ATP12A2 results in disease that closely resembles the clinical features of idiopathic PD (Figure 2) (Bekris et al., 2010).

Here we discuss only the most important genes relevant to PD.

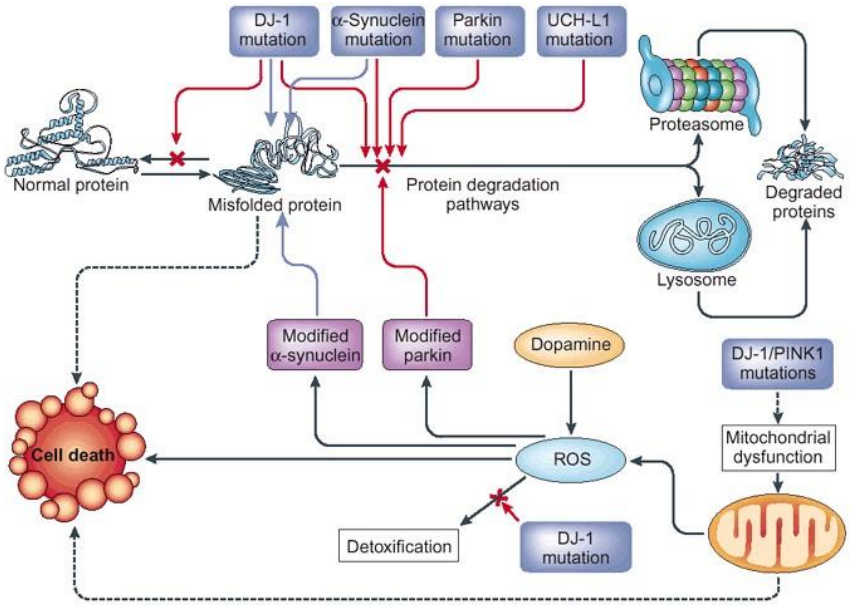


Figure 2 – **Genetic mutations and the pathogenesis of PD.** Misfolded proteins may contribute to PD neurodegeneration. Mutant α-synuclein and DJ-1 may be misfolded (blue arrows), thus overloading the ubiquitin (proteasomal) and lysosomal degradation pathways. Other mutant proteins, such as parkin and UCH-L1, may lack their wild-type function. Both of these proteins, which belong to the ubiquitin-proteasome system, upon mutation may no longer exert their ubiquitin ligase activity, thus compromising the ability of the cellular machinery to detect and degrade misfolded proteins (red arrows). Mutations in DJ-1 may also alter its supposed chaperone activity, disrupting the refolding of damaged proteins or the targeting and delivery of damaged proteins for degradation (red arrows). These different alterations may lead to the accumulation of unwanted proteins, which, by unknown mechanisms (dashed arrows), may lead to neurodegeneration. Oxidative stress generated by mitochondrial dysfunction and DA metabolism may also promote protein misfolding as a result of post-translational modifications, especially of α-synuclein and parkin. Oxidative stress in PD may also result from a defect in the reduced capacity of DJ-1 to detoxify reactive oxygen species (ROS), whereas the mitochondrial dysfunction may, at least in part, derive from defective activity and mislocation of DJ-1 and PINK1. Mitochondrial dysfunction, oxidative stress and protein mishandling are thus tightly interconnected in this hypothesized pathogenic cascade. Additional possible interactions have been omitted for clarity (Vila and Przedborski, 2004).

Table 2 - **Susceptibility Genes/Loci for PD (Bekris et al., 2010).**

PARK Locus	Gene	Map Position	Risk variants	OR[2]
Well-validated loci/genes				
PARK1/PARK4	*SNCA*	4q21	Repeat polymorphism, multiple SNPs	1.2-1.4
PARK8	*LRRK2*	12q12	G2385R, R1628P	2.0-2.2
Not assigned	*MAPT*	17q21.1	H1 haplotype	1.4
Not assigned	*GBA*	1q21	>300 mutations including: N370S and L444P	5.4
Putative loci/genes				
PARK16	Unknown	1q32	Multiple SNPs from GWASs	1.3-1.4
PARK17	*GAK*	4p16	Multiple SNPs from GWASs	1.5
PARK18	*HLA-DRA*	6p21.3	Multiple SNPs from GWASs	1.3

Abbreviations: GWASs, genome wide association studies; OR, odds ratio; PD, Parkinson disease; SNP, single nucleotide polymorphism.

Table 3 - **AD and AR forms of PD (Spatola and Wider, 2014).**

Gene	Mutations	Inheritance (penetrance)	Gene product	Phenotype[a]
Autosomal dominant PD				
LRRK2, *Leucine-rich repeat kinase 2* (PARK 8)	G2019S, N1437H, R1441C/G/H, Y1699C, I2020T	AD (incomplete, age dependent)	Lrrk2 (dardarin)	PD
SNCA (PARK 1/4)	Triplication Duplication A53T, A30P, H50Q, G51D, E46K	AD (high)	α-synuclein	PD, MDD
VPS35	D620N	AD (incomplete)	Vacuolar protein sorting 35 homolog	PD
EIF4G1	R1205H	AD	Eukaryotic translation initiation factor 4-gamma 1	PD
Autosomal recessive PD				
PRKN (PARK2)	>100 different mutations	AR	Parkin, E3 protein ligase	EO PD
PINK1 (PARK6)	>40 different mutations	AR	PTEN-induced kinase 1	EO PD
DJ-1 (PARK7)	>10 different mutations	AR	Daisuke-Junko-1	EO PD
ATP13A2 (PARK9)	Duplications G877R, L1059, F182L, G504R	AR	Lysosomal P-type ATPase	EO PD, P-P
PLA2G6 (PARK14)	R741Q, R747W, Q452X, R635Q, R632W, D331Y	AR	Calcium-independent. phospholipase A2	EO PD, P-P
FBXO7 (PARK 15)	R378G, R498X, T22M	AR	F-box only protein 7	PD, P-P
DNAJC6		AR	Neuronal-specific clathrin-uncoating co-chaperone auxilin	CO P-P

[a] CO, childhood onset; EO, early onset; LO, late onset; MDD, myoclonus, dementia, dysautonomia (atypical forms); P-P, parkinsonism-pyramidal syndrome; PD, classical PD (levodopa-responsive parkinsonism).

1.1.1. PARK1/4 / SNCA

SNCA gene was the first to be linked to Parkinsonism with mutations often associated with an EOPD that is usually more aggressive, progresses more rapidly and in some cases presents atypical clinical features (Bekris et al., 2010).

The SNCA gene encodes for α-synuclein, a small protein with 140 KDa that is the major component of LB (Lesage and Brice, 2009). α-synuclein has a high propensity to aggregate in vitro and the presence of α-synuclein aggregates in LB has led to the hypothesis that aggregation may be important to α-synuclein toxicity (Martin et al., 2011).

Three missense mutations were identified in this gene: A53T, A30P and E46K (Figure 3). A53T and A30P mutations preferentially promote the formation of α-synuclein oligomers. PD patients sometimes lack LB, suggesting that oligomeric α-synuclein may be the primary toxic species in PD pathogenesis (Martin et al., 2011). Studies showed that when A53T mutation is present, there is an early onset age (<50 years), rapid disease progression, atypical symptoms such as prominent cognitive deterioration, central hypoventilation, myoclonus and severe postural instability (Crosiers et al., 2011). E46K mutation leads to parkinsonism with LB formation that progressed to dementia accompanied by visual hallucinations and fluctuations in consciousness (Crosiers et al., 2011).

Numerous studies have also established a link between EOPD and duplications or triplications in the SNCA gene (Nuytemans et al., 2010). In fact, gene triplication leads to earlier onset and faster progression of disease than duplication indicating that disease severity is dependent on α-synuclein expression levels.

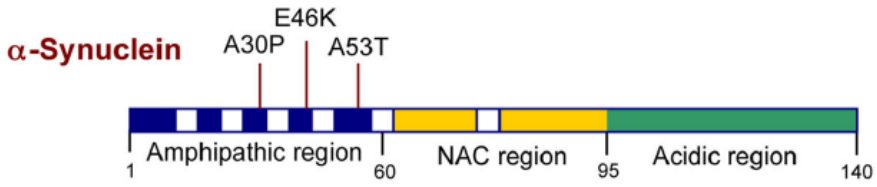


Figure 3 **- α-synuclein domains and missense mutations: A53T, A30P AND E46K.** A number of imperfect KTKEGV repeat sequences (white stripes) are shown in the N-terminal region and central NAC (nonamyloid component) region. Mutations that segregate with PD are annotated at their approximate position along the protein's length. Adapted from (Martin et al., 2011)

1.1.2. PARK8 / LRRK2

Mutations on LRRK2 gene (Figure 4) was the second causal gene linked to AD inherited PD. This transcript contains 51 exons coding for a leucine-rich repeat kinase 2 (LRRK2), also called as Dardarin (Paisan-Ruiz et al., 2004). LRRK2 protein is expressed in striatum and comprises several functional domains that were suggested to be involved in protein-protein interactions, maintenance of neurites, and regulation of neuronal survival (Belin and Westerlund, 2008). Until now, 80 missense mutations have been identified over the entire LRRK2 protein and affect all predicted functional domains. Nevertheless, some mutations have much higher frequencies than others, for example, p.Gly2019Ser and mutations altering codon Arg1441 (Nuytemans et al., 2010) and only five mutations have been associated with an increased risk for sporadic PD (Wider et al., 2010, Martin et al., 2011). Four of these five mutations, Y1699C, I2020T, R1441C, and R1441G (Figure 4) have been associated with AD PD (Zimprich et al., 2004) and the G2019S (Figure 4) mutation, the most common in this gene, have been related with both familial and sporadic PD (Dachsel and Farrer, 2010).

Clinically, patients with LRRK2 mutations typically have a late onset form of the disease and exhibit a wide range of pathologies that can differ between or within families and for this reason cannot be distinguished from sporadic PD cases even when LB pathology is absent (Wider et al., 2010, Martin et al., 2011).

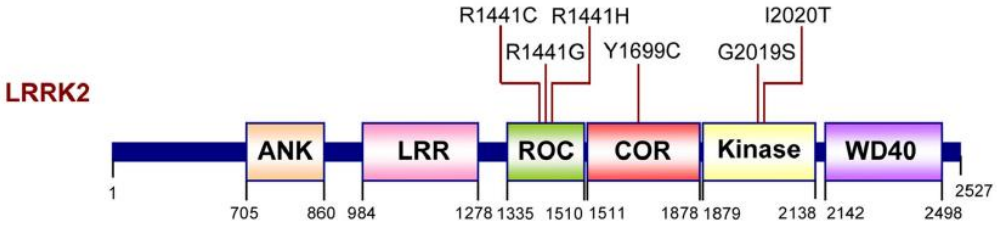


Figure 4 - **LRRK2 domains and pathogenic mutations: Y1699C, I2020T, R1441C, R1441G, and G2019S.** Ank (ankyrin-like repeats), LRR (leucine rich repeats), ROC (Ras of complex proteins) GTPase domain, COR (C-terminal of ROC), kinase, and WD40. Mutations that segregate with PD are annotated at their approximate position along the protein's length. Adapted from (Martin et al., 2011).

1.1.3. PARK2 / Parkin

Parkin is expressed in neurons and functions as an ubiquitin E3 ligase targeting specific substrates for degradation by the proteasome or lysosome (Dawson and Dawson, 2010).

Mutations in parkin (Figure 5) are the most common cause of AR PD (Kitada et al., 1998) and may play a role in sporadic PD through common and frequent mutations (Pilcher, 2005). In

addition, this protein is inactivated when key pathogenic processes, such as nitrosative stress, DAergic stress and oxidative stress, in sporadic PD are present which indicates that loss of parkin activity may not only play a role in AD PD but also in AR PD (Dawson and Dawson, 2010).

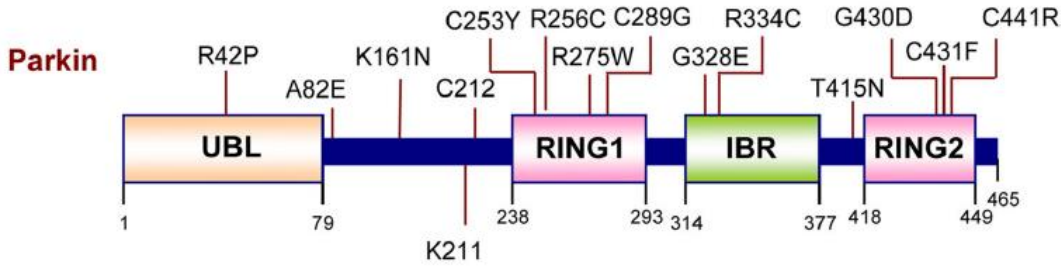


Figure 5 - **Parkin domains and pathogenic mutations.** UBL (ubiquitin-like) and two RING domains separated by an IBR (in-between RING) domain. Mutations that segregate with PD are annotated at their approximate position along the protein's length. Adapted from (Martin et al., 2011).

1.1.4. PARK6 / PINK1

PTEN-induced putative kinase 1 gene (PINK1) is a rare causal gene of recessively inherited, EOPD with a prevalence below 4% (Crosiers et al., 2011). This gene encodes for PINK1 protein that is a putative/threonine kinase localized at mitochondria and widely expressed in the human brain (Cookson, 2010). PINK1 plays an important role in mitochondrial response to cellular and oxidative stress (Valente et al., 2004).

Most of the known PINK1 mutations (Figure 6) occur within its kinase domain, supporting a loss-of-function mechanism, although other pathogenic mutations exist outside this domain.

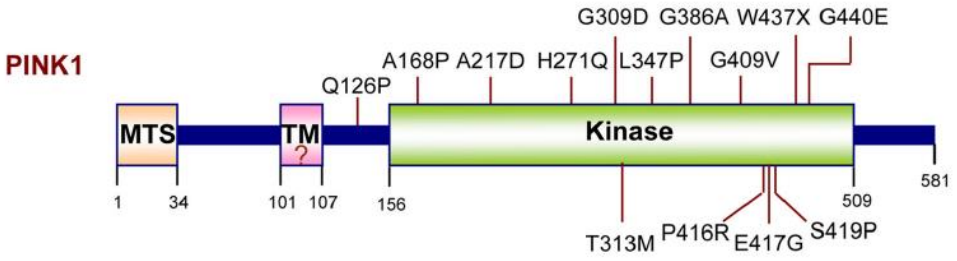


Figure 6 **- PINK1 domains and pathogenic mutations.** MTS (mitochondrial targeting sequence), TM (putative transmembrane domain), and serine/threonine kinase. Mutations that segregate with PD are annotated at their approximate position along the protein's length. Adapted from (Martin et al., 2011).

1.1.5. PARK9 / ATP13A2

ATP13A2 is a lysosomal membrane protein with an ATPase domain. Loss- of-function mutations in ATP13A2 gene underlying an AR form of EOPD with pyramidal degeneration and dementia. Whereas the wild-type protein was located in the lysosome of transiently transfected cells, the unstable truncated mutants were retained in the endoplasmic reticulum and degraded by the proteasome (Ramirez et al., 2006).

1.1.6. PARK7 / DJ-1

DJ-1, also known as PARK7, is a homodimer that belongs to the peptidase C56 family of proteins. It is located on chromosome 1p36.23, with a transcript length of 949 bp with 7 exons and encodes a protein consisting of 189 amino acids (Bekris et al., 2010).

In 2003, mutations in the DJ-1 gene were found to be associated with AR EOPD (Bonifati et al., 2003). Missense mutations, a frameshift mutation, a splice site mutation and whole exon deletions have been found and it is estimated that these mutations affects less than 1% of all patients with EOPD, being the age of onset before 40 years (Wider et al., 2010). The mutations L166P, E64D, M26I, A104T, and D149A (Figure 7) have been shown to create structural perturbations of DJ1 protein resulting in global destabilization, unfolding of the protein structure, heterodimer formation, or reduced antioxidant activity (Bekris et al., 2010).

Patients with these mutations have a slow progression of the disease, good response to DAergic therapy, psychiatric disturbances, dystonic features, and clinical heterogeneity in disease severity (Dekker et al., 2003).

DJ-1, protein encoded by this gene, has been connected to different cellular functions, however the most important with regard to PD is its putative role in response to oxidative stress, which is not yet well understood. A more in-depth description of all the different biological and biochemical features of this protein will be discussed in the next section of this chapter.

Figure 7 - **DJ-1 domain and pathogenic mutations.** Mutations that segregate with PD are annotated at their approximate position along the protein's length. Adapted from (Martin et al., 2011).

1.2. DJ-1 and its role in Neuroprotection

DJ-1 was discovered in 1997 as an oncogene product that transforms mouse NIH3T3 cells in cooperation with activated ras (Nagakubo et al., 1997). DJ-1 comprises 189 amino acids with seven ß-strands and nine α-helices and is present as a homodimer (Figure 8) (Tao and Tong, 2003, Wilson et al., 2003).

DJ-1 is a multifunctional protein with diverse biological implications such as mitochondrial regulation (Ren et al., 2011, Thomas et al., 2011, Wang et al., 2012, Heo et al., 2012b), chaperone activity (Shendelman et al., 2004, Zhou et al., 2006), protease activity (Koide-Yoshida et al., 2007, Chen et al., 2010), antioxidant (Yanagida et al., 2009, Canet-Aviles et al., 2004, Kinumi et al., 2004b) and regulation of transcription (Shinbo et al., 2005, Xu et al., 2005, Clements et al., 2006, Fan et al., 2008a).

As described above, the involvement of DJ-1 in neurodegenerative diseases became apparent when the PARK7 gene was associated with AR EOPD. 23 pathogenic deletion and point mutations of PARK7 gene were found in PD patients, with the most dramatic point mutation being the L166P (Bonifati et al., 2003), that causes severe destabilization of the protein (Gorner et al., 2004, Macedo et al., 2003).

DJ-1 is almost ubiquitously expressed (Nagakubo et al., 1997), it is localized mainly in the cytoplasm but also in the nucleus, in combination with mitochondria or even in extracellular location. Its secretion has been described in various cultured cells or tissues, such as astrocytes or cancer cells (Yanagida et al., 2009, Koide-Yoshida et al., 2007). This subcellular distribution is dynamic and nuclear or mitochondrial translocations can occur (Canet-Aviles et al., 2004, Junn et al., 2009).

DJ-1 as a redox-reactive protein in neurodegenerative diseases. DJ-1 contains three cysteine residues -C46, C56, C106- and the most susceptible to oxidative stress is the C106 that can be oxidized as SOH, SO_2H and then SO_3H (Canet-Aviles et al., 2004, Kinumi et al., 2004a). For this reason, mutation of C106 results in a loss of all DJ-1's functions (Figure 9) (Taira et al., 2004, Canet-Aviles et al., 2004, Ariga et al., 2013). Moreover, it was also described that this cysteine oxidation is required for the nuclear translocation of DJ-1 (Kim et al., 2012). Therefore, the inactive forms of the protein occur when the cysteine is in the form SO_3H because this excessive oxidized DJ-1 has been observed in brains of patients with PD and Alzheimer's disease (Bandopadhyay et al., 2004, Choi et al., 2006).

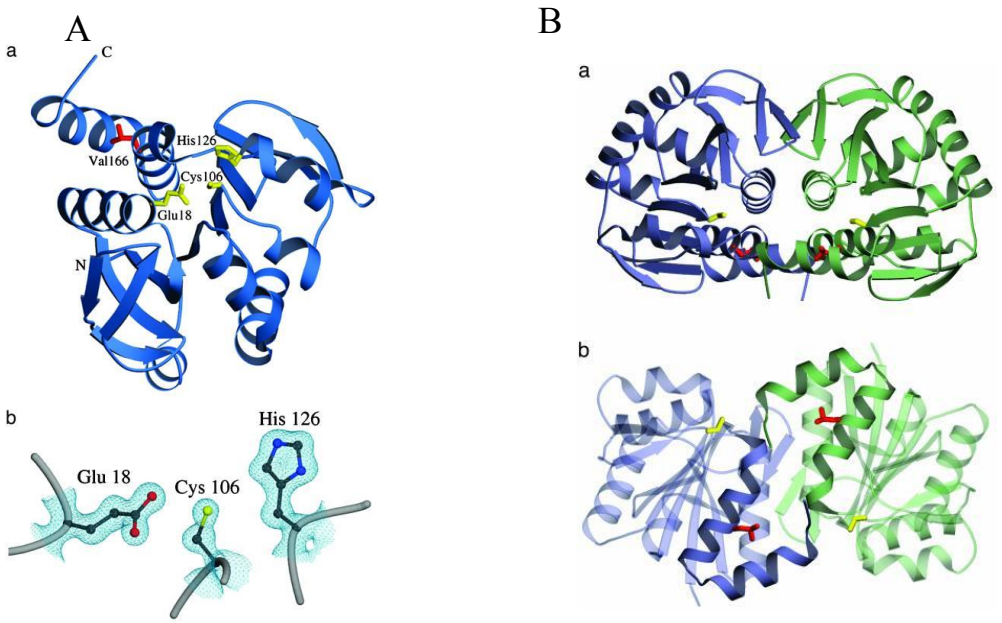


Figure 8 - **DJ-1 monomer structure.** (A) Representation of DJ-1 monomer, with residues Glu-18, Cys-106, and His-126 in yellow (a). The Leu-166 residue in red is mutated to proline in PARK7 familial PD. A closer view of the "nucleophile elbow" region (b). (B) (a and b) Two views of the DJ-1 crystallographic dimer. Monomer A is purple and monomer B is green. The view in b is rotated by 90° with respect to the view in a. In both views, Cys-106 is yellow and Leu-166, which is mutated to proline in PARK7 familial PD, is red. Adapted from (Wilson et al., 2003).

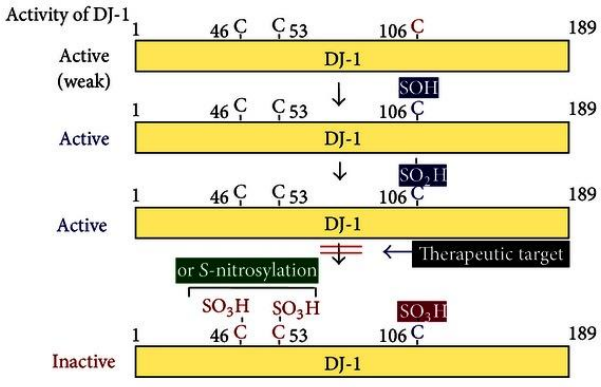


Figure 9 - **Cysteine oxidation and activation of DJ-1.** DJ-1 contains three cysteine residues at amino acids numbers 46, 54 and 106 (C46, C54 and C106, resp.). C106 is sequentially oxidized with SOH, SO_2H and SO_3H, and then C46 and C54 are oxidized or S-nitrosylated (Ariga et al., 2013).

1.2.1. Cytoprotective Functions of DJ-1

1.2.1.1. Role of DJ-1 in Mitochondrial Homeostasis

Mitochondria are ubiquitous organelles, critical for cell survival. Their main function is to support aerobic respiration and to provide energy substrates for intracellular metabolic pathways, but they are also involved in cell signaling particularly in signalling for apoptotic cell death (Schapira, 2006).

Mitochondrial dysfunction is closely related to increase of ROS formation in PD and the deterioration of DAergic neurons is triggered by the pathogenic mitochondrial mechanism enabling ROS production (Yan et al., 2013).

The engagement of mitochondrial dysfunction and ROS production, in addition to the contribution of DA and its oxidation products, plays a vital role in the pathogenesis of PD (Hastings, 2009, Belluzzi et al., 2012).

Under normal conditions, part of the DJ-1 protein is present in mitochondria where it binds specifically to subunits of mitochondrial complex I to regulate its activity (Hayashi et al., 2009). PD patients showed a reduction of mitochondrial complex I activity as well as a reduction of mitochondrial membrane potential (Orth and Schapira, 2002). Mice and cells that do not express DJ-1 also exhibit these dysfunctional characteristics (Hao et al., 2010, Giaime et al., 2012) together with mitochondria fragmentation (Irrcher et al., 2010, Krebiehl et al., 2010, Wang et al., 2012).

On the other hand, the synergistic transcriptional activities of DJ-1 and PGC-1α, that is an important mediator of mitochondrial biogenesis, are suggestive of a role of DJ-1 in general mitochondrial maintenance (Zhong and Xu, 2008). Under oxidative stress conditions, translocation of DJ-1 to the outer mitochondrial membrane is enhanced. The central C106 is essential for this process and, contrariwise, C106A-mutant DJ-1 abolishes the cytoprotective effect of DJ-1 against the mitochondrial toxin MPP+ (Canet-Aviles et al., 2004). It is thought that localization of DJ-1 as a dimer in mitochondria is required for DJ-1 to exert its antioxidant role, and that the presence of the monomeric form of the protein (such as M26I and L166P DJ-1 mutants) in mitochondria is in contrast harmful to cells (Canet-Aviles et al., 2004, Maita et al., 2013).

DJ-1 has no mitochondrial-targeting sequence and, for this reason, binds to several chaperones, including mitochondrial Hsp70/mortalin/Grp75, suggesting that translocation of DJ-1 into mitochondria depends on other proteins, including mortalin (Li et al., 2005). Another hypothesis suggests that a small fraction of the cytosolic wild-type DJ-1 is recruited to the vicinity of

mitochondria in cells under oxidative stress. Mitochondrial DJ-1 translocation seems to be transient within a 3h time frame in H_2O_2-treated cells, followed by nuclear import after 12h.

Besides the antioxidant role, it was suggested that DJ-1 induces mitophagy, mitochondrial-specific autophagy, after translocation into the same when the membrane potential is decreased (Krebiehl et al., 2010, Thomas et al., 2011, Heo et al., 2012a).

1.2.1.2. Regulation of Transcription by DJ-1

Transcription factors whose activity is regulated by DJ-1 involve the sterol regulatory element binding protein (SREBP) (Yamaguchi et al., 2012), p53 (Shinbo et al., 2005, Fan et al., 2008a, Kato et al., 2013), nuclear factor erythroid-2-related factor 2 (Nrf2) (Ren et al., 2011), the androgen receptor (Tillman et al., 2007, Niki et al., 2003) and the polypyramidine tract-binding protein-associated splicing factor (PSF) (Zhong et al., 2006).

The regulation of p53 and Nrf2 transcription by DJ-1 is determinant for the oxidative stress response.

p53 is a tumor suppressor protein and plays roles in induction of senescence and apoptosis in cells and in regulation of mitochondrial homeostasis against oxidative stress. DJ-1 may regulates p53 activity in various ways; DJ-1 binds to the DNA-binding region of p53 to inhibit p53 transcriptional activity when affinity of p53 or its mutants to DNA is low, leading to cell cycle progression (Kato et al., 2013). It has also been reported that DJ-1 decreased the expression of Bcl-2 associated X protein (Bax) by repressing p53 transcriptional activity, leading to inhibition of p53-Bax-caspase apoptotic pathway (Fan et al., 2008b). This information was complemented with a later study where showing the ability of DJ-1 to repress p53 is dependent on post-translational modification of DJ-1, more specifically it is dependent on proper sumoylation of DJ-1 which is responsible for its translocation to the nucleus (Figure 10) (Fan et al., 2008a).

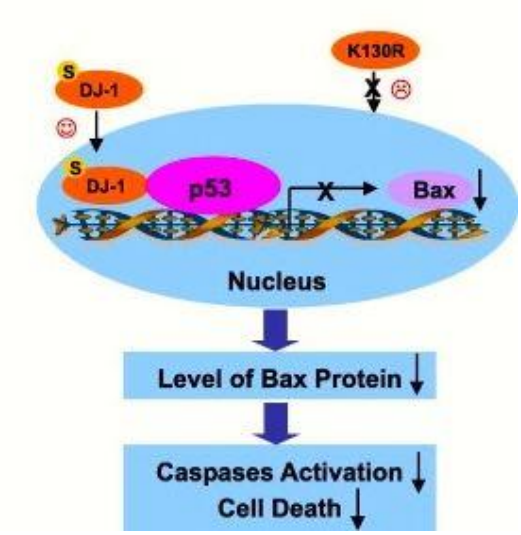


Figure 10 - **DJ-1, but not its K130R mutant, inhibits p53 transcriptional activity on Bax promoter.** A hypothetical model illustrating the role of DJ-1 in rescuing cell death through p53-Bax-caspase pathway. DJ-1 may bind to p53 physically in the nucleus, decrease Bax expression by attenuating p53 transcriptional activities, inhibit subsequent caspase activation and finally rescue cell death. Whereas, the non-sumoylatable K130R mutant of DJ-1, loses its cytoprotective effect due to the sequestration in cytoplasm. Adapted from (Fan et al., 2008a).

Nrf2 is a master transcriptional factor for antioxidant molecules, such as glutathione, and the expression of several detoxication enzymes, such as NAD(P)H quinone oxireductase 1 (NQO1). This factor is localized in cytoplasm in a complex with Keap1. When oxidative stress conditions are present, DJ-1 sequesters Keap1, resulting in translocation of Nrf2 into the nucleus to activate various antioxidative stress genes, thereby decreasing ROS level (Figure 11) (Clements et al., 2006).

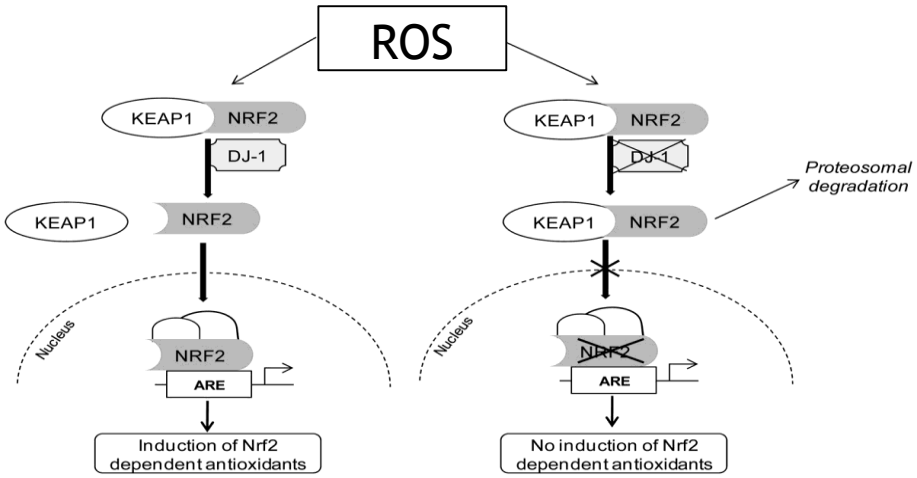


Figure 11 **- Schematic drawing showing the working model for DJ-1-mediated regulation of the Nrf2 pathway**. ARE=antioxidant response element. Adapted from reference (Malhotra et al., 2008).

Studies also report that DJ-1 is important for the increased expression of SOD3 (superoxide dismutase) and glutathione ligase, enzymes important in reducing ROS (Nishinaga et al., 2005, Zhou and Freed, 2005). Knockdown of DJ-1 in NIH3T3 cells reduced expression of the extracellular SOD3, but not SOD1 or SOD2/MnSOD (Nishinaga et al., 2005). However, a possibly compensatory up-regulation of mitochondrial SOD2 was observed in aged DJ-1 knockout mouse brain (Andres-Mateos et al., 2007).

Finally, there is also evidence that DJ-1 positively regulates human Tyrosine Hydroxylase (TH) gene expression by sequestering transcriptional repressor PSF, and supressing its sumoylation, from human TH gene promoter. Based on that, there is evidence that DJ-1 could act as a regulator of protein sumoylation directly linking loss of DJ-1 expression and/or transcriptional dysfunction to impaired DA synthesis (Zhong et al., 2006, Ishikawa et al., 2010).

1.2.1.3. DJ-1 Mediated Signaling Pathways against Oxidative Stress

Free radicals are highly reactive molecules with unpaired electrons that may be detrimental to the body. These molecular species are produced in cells as a result of normal physiological processes, and to maintain homeostasis and avoid cellular damage this must be balanced with antioxidant production (Jezek and Hlavata, 2005).

In pioneering studies, the cytoprotective functions of DJ-1 against (mitochondrial) ROS were associated to a direct quenching effect of DJ-1 (Taira et al., 2004, Takahashi-Niki et al., 2004). However, when subsequent studies compared this effect of DJ-1 with the effect exerted by specialized antioxidant enzymes such as peroxiredoxins, it was found that the antioxidative enzyme activity of the DJ-1 could be 1-2 orders of magnitude slower or even be negative (Martinat et al., 2004, Andres-Mateos et al., 2007). This difference may be due to the fact that the DJ-1 in its over-oxidized state is not reduced again, as would be expected for other antioxidant enzymes, resulting in their cellular accumulation (Duan et al., 2008) and subsequent accumulation in brains of patients with neurodegenerative diseases (Choi et al., 2006). There are suggestions that DJ-1 does not act directly as an antioxidant enzyme in vivo but rather as an inducer of protein expression of other proteins with higher antioxidant power, by gene regulation (Clements et al., 2006).

DJ-1 has the ability to activate signaling pathways against oxidative stress, preventing cell death and promoting cell growth. DJ-1 can facilitate the prosurvival Akt pathway that is activated when a growth signal is received. This stimulation promotes the phosphorylation of Akt by phosphoinosite-3 kinase (PI3 K), which in turn activates a continuous phosphorylation cascades, resulting in stimulation of cell growth (Takeuchi and Ito, 2010). PTEN is a lipid phosphatase that inhibits PI3 K and acts as a negative regulator of Akt/PI3 K pathway (Yotsumoto et al., 2009). When an oxidative insult occurs DJ-1 has the ability to directly bind

PTEN inhibiting its repressor activity and thereby promoting continuous activation of the signalling pathway which is in accordance with the reduced levels of phosphorylated Akt in DJ-1-knockout mice and the increased neuronal cell death (Kim et al., 2005, Kim et al., 2009).

DJ-1 was associated with other cell growth pathway, ERK pathway, which starts with Ras, followed by Raf, MEK and ERK. DJ-1 plays a protective role when DA concentrations are toxic to the cell, suffering a mutually activation with ERK (Lev et al., 2009). DJ-1 may also protect DAergic neurons against rotenone-induced apoptosis by enhancing ERK-dependent mitophagy (Gao et al., 2012).

DJ-1 seems to have also a critical role in keeping the Apoptosis Signal-Regulating Kinase 1 (ASK1) under control, a function that is lost by the PD-causing L166P mutant isoform of DJ-1 (Junn et al., 2005). ASK1 is a mitogen-activated protein kinase-kinase-kinase 5 (MAP3 K5) that activates c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases ($p38^{MAPK}$) in response to a stimulus such as oxidative stress in the presence of Daxx, a death domain-associated protein (Hattori et al., 2009, Shiizaki et al., 2013). DJ-1 may bind to both ASK1 and Daxx inhibiting oxidative stress-induced apoptosis in H_2O_2-treated culture cells and MPTP-administered-PD model mice (Junn et al., 2005, Karunakaran et al., 2007). Mutations associated with PD may inhibit the suppressive activity that DJ-1 exerts on ASK1 pathway, leading to their increased activity and consequently increase the neurodegeneration (Junn et al., 2005, Waak et al., 2009a).

ROS not always play a cytotoxic role. They are produced in cells in tiny amounts to facilitate signal transduction pathways via reversible oxidation of catalytic cysteine residues (Janssen-Heininger et al., 2008). For example, ASK1 signaling pathway activated by tumor necrosis factor and Toll-like receptor (TLR) ligands is ROS-dependent (Matsuzawa and Ichijo, 2008). Furthermore, it is described that DJ-1 itself may directly regulate signaling through TLR4 because DJ-1 knockout mouse primary astrocytes cultures produce excessive amounts of nitric oxide (NO) in response to lipopolysaccharide due to the selective induction of type II NO synthase via ROS- and $p38^{MAPK}$-dependent pathway. In the same study, this same effect was demonstrated using co-cultures of primary neurons (Waak et al., 2009b).

1.2.2. Astrocyte-mediated Neuroprotection

In normal human brain, DJ-1 is moderately expressed in neurons and astrocytes throughout the CNS (Bandopadhyay et al., 2004, Rizzu et al., 2004). In patients with PD or other neurodegenerative diseases, the expression of this protein is strongly increased, especially in reactive astrocytes, as a resulting of increased oxidative stress (Yanagida et al., 2009).

Biochemically, it is known that in the brain of patients with PD, DJ-1 undergoes a shift to a more acidic form (oxidized) (Bandopadhyay et al., 2004), and this modification can provide the role of DJ-1 as a redox-reactive protein in neurodegenerative diseases.

First studies on this subject showed that an insult with H_2O_2 induces the release of astrocytic DJ-1, which may contribute to astrocyte-mediated neuroprotection. In the same study it was demonstrated that recombinant GST-DJ-1 protein directly trapped •OH and inhibited H_2O_2-induced cell death (Yanagida et al., 2009). In fact, DJ-1 knockdown in astrocytes impaired the astrocyte-mediated neuroprotection against rotenone, and its overexpression enhances their capacity to protect neurons (Mullett and Hinkle, 2009, Mullett et al., 2013). A study using the 6-hydroxidopamine (6-OHDA) PD model showed that the lack of DJ-1 leads to astrocytic dysfunction, altering their ability to protect neuronal cells by direct contact and altering the secretion of soluble protective factors. The authors also found reduced activation of the Nrf2 system in DJ-1 knockout astrocytes (Lev et al., 2013). Larsen and colleagues concluded that, in astrocytes culture, DJ-1 deficiency significantly reduced mitochondrial motility in cellular processes. However, this behavior was quite different in neuron-astrocyte contact co-culture (Larsen et al., 2011).

On the other hand, a recombinant GST-DJ-1 injected in the SNpc of rats exposed to 6-OHDA exerted a protective effect, even when the administration was post-lesion (Inden et al., 2006). The uptake of recombinant DJ-1 was demonstrated both in the presence and absence of an oxidative stress insult. However this incorporation was not seen in L166P mutant of DJ-1 (Inden et al., 2006). This result may be explained with the instability and/or insoluble characteristic of this mutant DJ-1 (Macedo et al., 2003).

All these results suggest that DJ-1 shuttles between the inside and outside of cells (Tsuboi et al., 2008). Moreover, DJ-1 besides exerting an antioxidant or antiapoptotic role in neurons could also play a role in the control of transcellular damage mediated by reactive astrocytes (Figure 12).

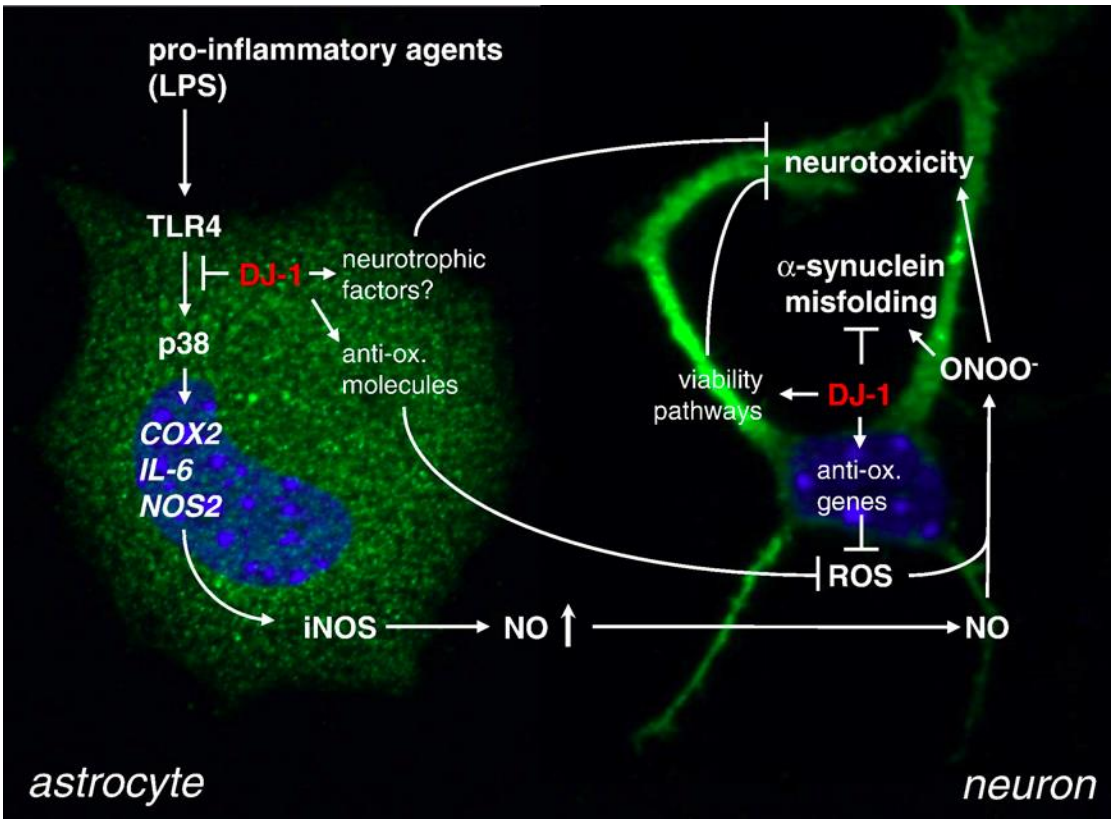


Figure 12 - **Cellular signaling pathways influenced by DJ-1** (Kahle et al., 2009).

Chapter 2

Objectives

DJ-1 plays an important role in neuroprotection mechanisms, acting as an antioxidant stress sensor in oxidative stress conditions, such as when a H_2O_2 stimulus is present. In addition it has been already described that the protein is expressed both in neurons and astrocytes (Baulac et al., 2004, Bader et al., 2005) and that this expression is increased in reactive astrocytes in neurodegenerative diseases (Bandopadhyay et al., 2004, Rizzu et al., 2004, Neumann et al., 2004, Mullett et al., 2009).

Taking these facts together, the main goal of this study was to determine how DJ-1 expression is affected in primary midbrain cell cultures when an oxidative environment is present and, on the other hand, give some insights about the neuroprotective mechanisms mediated by astrocytes in primary midbrain neuron cultures.

To clarify this objective, we propose:

- Evaluate DJ-1 expression in control situations or under oxidative conditions in:
    - Neuron, astrocyte cultures or neuron-astrocyte cocultures;
    - Neuron-astrocyte Banker Culture;
- Determine the effect of DJ-1 in astrocyte-mediated neuroprotection.
- Evaluate if exogenous DJ-1 has the capability to be endocytosed by neurons and its influence in neuroprotection.

Chapter 3

3. Materials and Methods

3.1. Animals

All experiments requiring the use of animals were conducted in compliance with protocols approved by the national ethical requirements for animal research, and with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Directive number 2010-63-EU).

All animals were kept under controlled conditions of temperature and light and with food and water available ad libitum.

3.2 Cell Cultures

3.2.1. Neuron cultures and Neuron-astrocyte co-cultures

Embryonic day 15 to 16 Wistar rat embryos were collected previously from Wistar pregnant females anesthetized with a solution containing Ketamine (87.5 mg/kg) and Xylazine (12.5 mg/kg) followed by euthanasia by cervical dislocation. In a laminar flow hood, the collected uterine horns were placed in ice-cold sterile phosphate buffer saline (PBS) and dissected to obtain the uterine sacs. Under the same conditions, the uterine sacs were then dissected and amniotic membranes removed to collect the embryos, which were placed in cleaned ice-cold sterile phosphate buffer saline (PBS: NaCl 140 mM, KCl 2.7 mM, KH2PO4 1.5 mM and Na_2HPO_4 8.1 mM, pH 7.4). Under a dissection microscope, the ventral midbrain region of each embryo was dissected and putted in ice-cold PBS.

After dissection of the ventral midbrain, the meninges were carefully stripped of, and the dissected tissue was put in PBS. The tissue was then dissociated by enzymatic digestion (Trypsin 4.5mg/ml (cat. T7409; Sigma-Aldrich) and DNAse 2.5 mg/ml (cat. DN25; Sigma-Aldrich) diluted in PBS) at 37° C for 4 minutes. To stop the enzymatic digestion, the tissue chunk was resuspended in a solution containing PBS with 10% heat-inactivated (HI) Fetal Bovine Serum (FBS) (Biochrom, Holliston, USA). Tissue chunks was washed in sterile PBS followed by mechanical digestion in Neurobasal Medium (MNB) (cat. 21103-049; Invitrogen) through micropipette-pipetting until a homogeneous suspension was observed.

The resulting cell suspension was then centrifuged at 1500 rpm for 3 min (3K18C Bioblock Scientific; Sigma Laboratory Centrifuges) and the pellet was resuspended in 10 mL of MNB containing 2% of B27 supplement (17504-044; Invitrogen), 0.5 mM of L-glutamine, 25 µM of glutamic acid and 120 µg/mL of gentamicin. For cell counting it was performed a 1:1 dilution of the final cell suspension with Trypan blue (0.4% in NaCl 0.9%) and viable cells were counted using a Neubauer chamber.

Finally, the cells were plated at a density of 0.4 x 10^6 cells/well into 24-well plates or in coverslips (BD Biosciences, San Jose, California, USA previously coated with poly-D-lysine (Sigma-Aldrich, St. Louis, USA). Cultures were kept at 37° C under a 5% CO_2 and 95% air atmosphere for 4-5 days until the desirable confluence was reached.

3.2.2. Astrocyte midbrain cultures

Postnatal Wistar rat pups with 2-4 years old were placed on ice until hypothermia. After decapitation, the brain was removed and placed in ice PBS. The correspondent midbrain region was dissected, carefully stripped of the meninges, and put in iced PBS. The resultant tissue was dissociated in a enzymatic solution containing cysteine water (1,9 mM $CaCl_2$, 1,3 mM cysteine), H&B solution (116 mM NaCl, 5,4mM KCl, 26 mM $NaHCO_3$, 12 mM NaH_2PO_4. H_2O, 1mM $MgSO_4.7H_2O$, 0,5 mM EDTA, 25 mM glicose, pH 7,3), 0,5% phenol red and 4 mg/mL papain, 4 min at 37° C. To stop the digestion, the tissue chunk was washed three times with M10 C-G Medium containing MEM (Sigma, M0268) supplemented with 2,2 g/L of sodium bicarbonate ($NaHCO_3$), 0,75% of glucose 45%, 0,12% of antibiotic (penicillin and streptomycin, Sigma), 0,02% of insulin and 10% FBS (Biochrom, Sigma). The mechanic digestion was performed as previous described.

The resulting cell suspension was then centrifuged at 1500 rpm for 3 min (3K18C Bioblock Scientific; Sigma Laboratory Centrifuges) and the pellet was resuspended in 10 mL of M10 C-G Medium. Cell counting was performed in a 1:1 dilution of the final cell suspension with Trypan blue (0.4% in NaCl 0.9%) and viable cells were counted using a Neubauer chamber.

Finally, the cells were plated at a density of 0.264 x 10^6 cells/3,85cm^2 into 12 or 24-wells plates or coverslips (BD Biosciences, San Jose, California, USA) previously coated with poly-D-lysine (Sigma-Aldrich, St. Louis, USA). Cultures were kept at 37° C under a 5% CO_2 and 95% air atmosphere during 4-5 days until they reach the desirable confluence. Twelve hours before starting the experiments the culture medium was replaced for serum free M10 C-G.

3.3. Cell Stimulation

3.3.1. H_2O_2 stimulus

When desirable confluence of the cultures was reached, after approximately 5 days, the culture medium was replaced with 1.2 mL or 0.6 mL of fresh medium for 12-well or 24 well-plate, respectively.

After 12h, different H_2O_2 concentrations (25 - 200 μM) were applied, as specified in the results section. The culture plates were then incubated at 37°C in 5% CO_2, 95% air atmosphere and the stimulus was allowed to exert its effect for an additional period of 24h. At the end of experiment the cells were washed twice with cold PBS and cells were prepared for Western Blot analysis or Immunocytochemistry assay. The experimental procedure used is represented in Figure 13.

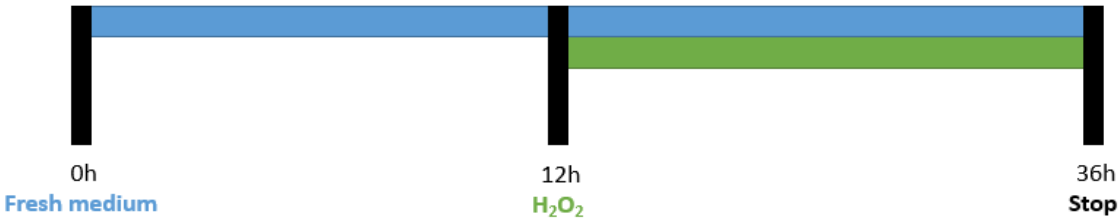


Figure 13 - **Schematic representation of H_2O_2 stimulation in primary midbrain cultures.**

3.3.2. Experiments in Banker Cultures

After 5 days, the medium of the neuronal and astrocytic primary cultures was replaced with 0.6 mL of fresh MNB medium. After 8h, the coverslips carrying astrocytes were placed above the coverslips with the neuronal culture. After the cultures were challenged with H_2O_2 25 μM (Fig 14). After 24h in the presence of the stimulus the coverslips were washed twice with cold PBS followed by fixation with Paraformaldehyde Fixate Solution 4% (PFA) during 10 minutes. The coverslips were kept in PBS until Immunocytochemical processing.

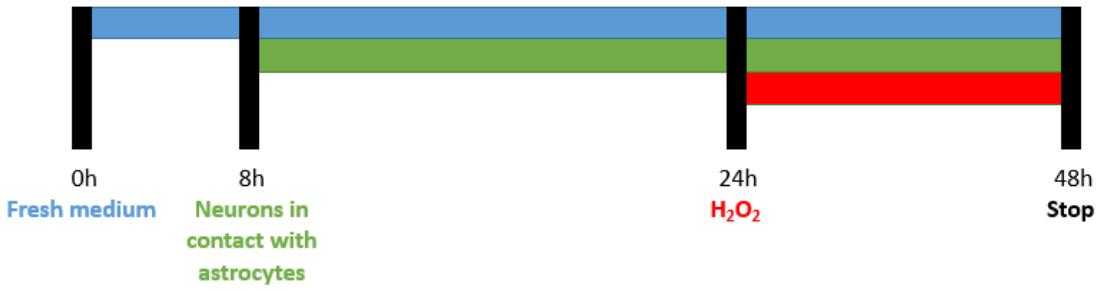


Figure 14 - **Schematic representation of H_2O_2 stimulation in Banker Cultures.**

3.3.3. DJ-1 Immunodepletion in Banker Cultures

On culture day 5 the medium of neurons and astrocytes cultures was replaced with 0.6 mL of fresh MNB medium. After 8h, the coverslips with the astrocyte culture were placed above the neuronal culture and 24h later the anti-DJ1 antibody in a concentration of 0.5 µg/mL (sc-55572, Santa Cruz Biotechnology) was added to the culture followed by stimulation with 25 µM of H_2O_2, 6h later. After 24h of stimulation, the cultures were washed twice with cold PBS followed by fixation with 4% PFA for 10 minutes. The coverslips were kept in PBS until Immunocytochemical processing.

The scheme of the experimental procedure followed is represented in figure 15.

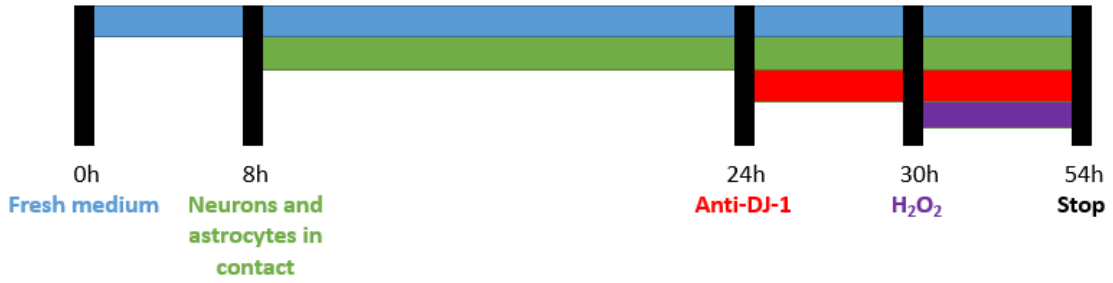


Figure 15 - **Schematic representation of H_2O_2 stimulation with DJ-1 immunodepletion in Banker Cultures.**

3.3.4. Labeling of DJ-1 peptide

DJ-1 peptide labeling was performed using the Alexa Fluor® 488 TFP Microscale Protein Labeling Kit (A30006, Invitrogen) essentially according to manufacturer protocol. Briefly, 100 µl of 100 µM DJ-1 peptide solution , 6-His-DJ-1 in an initial concentration of 2.012 mg/mL resuspended in PBS with 10% of glycerol, was pH adjusted to pH 9 with 10 µl of 1 M $NaHCO_3$, followed by addition of 5.53 µl of the H_2O solubilized reactive dye. Incubation at RT (~22°C) was performed

for 15 min followed by immediate addition of the labeling reaction mixture onto a spin column packed with a Biogel P-6 resin for removal of unbind dye. The resulting eluant (pH 7.4) was stored at 4°C and used in cellular uptake experiments.

3.3.4.1. Cellular uptake experiment of the DJ-1 conjugated to Alexa 488

After 5 days in culture and medium replacement, DJ-1 conjugated to Alexa 488 was added to neuron cultures at a concentration of 3 µM. The cultures were then incubated at 37°C in 5% CO_2, 95% air atmosphere and the stimulus was allowed to exert its effect for 4h. In the end of the stimulus, cells were washed twice followed by fixation with 4% PFA during 10 minutes. The coverslips were kept in PBS until Immunocytochemical processing, sheltered from light.

3.4. Western Blot

For Western Blot analysis, cells were lysed, on ice, with 60 µL of lysis buffer (Triton X-100 1%, Tris-HCl 50mM, pH 7.5, EGTA 10 mM, NaCl 150 mM, protease inhibitors (phenylmethylsulphonyl fluoride 2 mM, aprotinin 10 µg/µl, and leupeptin 1 µg/µl).

After quantification of total protein levels present in the supernatants, the proteins were denatured in 0.5M Tris-HCl, pH 6.8, 10% (w/v) Sodium Dodecyl Sulfate (SDS), 10% glycerol, 140 mM 2-mercaptoethanol, and 0.1% (w/v) bromophenol blue and heated at 100°C for 5 minutes.

The volumes of protein extracts correspondent to 20 µg were concentrated in the stacking gel containing 4% acrylamide, 0.5 mM Tris-HCl (pH 6.8), 10% SDS, 0.05% ammonium persulphate (PSA) and 0.05% tetramethylethylenediamine (TEMED). The proteins were then separated in 1.5 mm thick acrylamide resolving gel. The resolving gel was prepared with acrylamide at 12% in 1.5 M Tris-HCl (pH 8.8), containing 10% SDS, 0.05% PSA and 0.05% TEMED.

We carried out electrophoresis of the samples and protein standard (colored molecular weight marker) under 160 V for approximately 1 hour, held at room temperature, and using a buffer containing 960 mM Glycine, 125mM Tris and 0.5% SDS.

After the electrophoresis, the stacking gel was removed and the resolving gel was dipped in CAPS Buffer (10% Ciclohexilamin Acid propanosulphonic, 10% methanol, pH 11). In order to make the proteins accessible to antibody detection, they were transfered from the gel onto a polyvinylidene difluoride (PVDF - GE Healthcare, Amersham, UK) membrane. Before the transference, the PVDF membranes were activated by dipping the membranes for 5 seconds in methanol 100%, followed by 5 minutes in water and 10 minutes in CAPS buffer under agitation. Each membrane was placed on top of the respective gel. The electrotransference was carried out at 300 mA for about 1 hour, at 4° C.

After electrotransference of proteins, the membranes were blocked by incubation in 5% non-fat milk (Regilait, France) in 0.1% Tween 20 in Tris-buffered saline (TBS-T) for 2 h at room temperature. After blocking, the membranes were incubated with the primary antibodies (diluted in TBS-T according to Table 4), overnight at 4º C followed 1h at room temperature for anti-DJ-1 or only 1h at room temperature for housekeeping proteins.

After incubation with primary antibodies, the membranes were washed with TBS-T and then incubated for 1 hour at room temperature with the secondary antibodies diluted in TBS-T (according to Table 4).

After washing, bands were visualized by reaction with the alkaline phosphatase substrate (Enhanced ChemiFluorescent substrate, GE Healthcare, Amersham,

The specific OD was then normalized with respect to the amount of α -tubulin or GFAP loaded in the corresponding lane of the gel. Data are expressed as a percentage of values obtained under control conditions.

Table 4 - **Primary and secondary antibodies used in Western Blot**

Protein	Primary Antibody	Dilution	Company	Secondary Antibody	Dilution	Company
DJ-1	Monoclonal Anti-DJ1	1:500	Sc-55572, Santa Cruz Biotechnology, Germany	Goat Anti-Mouse	1:10000	RPN 5781, GE, Amersham, UK
Glial Fibrillary Acid Protein	Polyclonal Anti-GFAP	1:5000	Z0334, DAKO, Glostrup, Denmark	Goat Anti-Mouse	1:10000	RPN5781, GE Amersham,
Tubulin	Monoclonal Anti-α-tubulin	1:5000	T-9026, Sigma-Aldrich, USA	Goat Anti-Mouse	1:10000	RPN 5781, GE, Amersham, UK

3.5. Immunocytochemistry

To initiate the immunocytochemistry, cells were washed with PBS, permeabilized with 0.25% Triton X-100 in PBS for 10 minutes and then washed with PBS. Once washed, non-specific binding was blocked by incubation of cells with a solution of 20% FBS in PBS and 0.1% - Tween (PBS-T) for 1 hour at room temperature.

After blocking, the coverslips were washed with PBS-T and incubated with the primary antibodies (diluted in PBS-T 1% FBS, according to Table 5) overnight at 4º C. After overnight incubation, the coverslips were subjected to six washes of 15 min each with PBS-T and were then incubated with the corresponding secondary antibodies conjugated to Alexa® 488 or Alexa® 546 fluorophores and diluted in the same solution used previously to prepare the primary antibodies (according to Table 5).

After incubation with the secondary antibodies the cells were washed three times with PBS-T, counterstained with Hoescht 2μM (Invitrogen, CA, USA) during 10 minutes and mounted in fluorescent mounting medium (DAKO, Glostrup, Denmark). The images were acquired in an Axiobserver Z1 microscope (Zeiss).

3.5.1. Cell counting and Quantitative analysis

All immunocytochemical analyses were performed at least in three different experiments using two or three coverslips per experimental condition and at least 6 snaps per coverslip at the same exposure for fluorescent intensity experiment. TH positive cells were determined by counting for each staining, 20 fields (x63 magnification) of two or three coverslips for the different conditions. The results are represented as the mean value of TH positive cells in relation to total number of cells ± SEM. The quantification of fluorescence intensity of DJ-1 was

performed using ImageJ 1.48u4 software (National Institutes of Health, USA). In all images was applied threshold so to highlight only the area of interest to determine the intensity. The results are represented as the mean value of fluorescence intensity expressed in percentage of control ± SEM.

Table 5 - **Primary and secondary antibodies used in Immunocytochemistry.**

Protein	Primary Antibody	Dilution	Company	Secondary Antibody	Dilution	Company
DJ-1	Monoclonal Anti-DJ1	1:200	Sc-55572, Santa Cruz Biotechnology, Germany	Anti-Mouse conjugated to Alexa®488 or 546	1:1000	Invitrogen, UK
TH	Polyclonal Anti-TH	1:1000	Ab112, abcam, Cambridge, USA	Anti-Mouse conjugated to Alexa®488	1:1000	Invitrogen, UK

3.6. Data Analysis and Statistics

Data are expressed as percentages of values obtained in control conditions or as relative fluorescence intensity, and are presented as mean ± S.E.M. of at least three independent experiments, performed in triplicate. Statistical analysis was performed using One-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test. Values of $P<0.05$ were considered significant. All statistical procedures were performed using GraphPad Prism 6 Demo (GraphPad Sotware Inc., San Diego, CA).

Chapter 4

4. Results

4.1. Characterization of DJ-1 expression

4.1.1. Assessment of DJ-1 levels in primary ventral midbrain cell cultures under oxidative stress condition

The first part of this chapter is focused on the characterization of DJ-1 expression in primary ventral midbrain cell cultures under oxidative stress conditions exerted by H_2O_2 exposure. With this purpose, primary astrocytes cultures, obtained from newborn mice, were exposed to different concentrations of H_2O_2 (0 μM, 25 μM, 100 μM, 200 μM) and, by using these cultures, we analyzed the cellular levels of DJ-1 by Western Blot.

A 24h exposure to 25 μM and 100 μM of H_2O_2 induced a small, not statistically significant, increase of DJ-1 levels (Figure 16). At the higher concentration of H_2O_2 a tendency to a decrease below the control levels was observed, which can be indicative of H_2O_2 concentrations already affecting cellular viability.

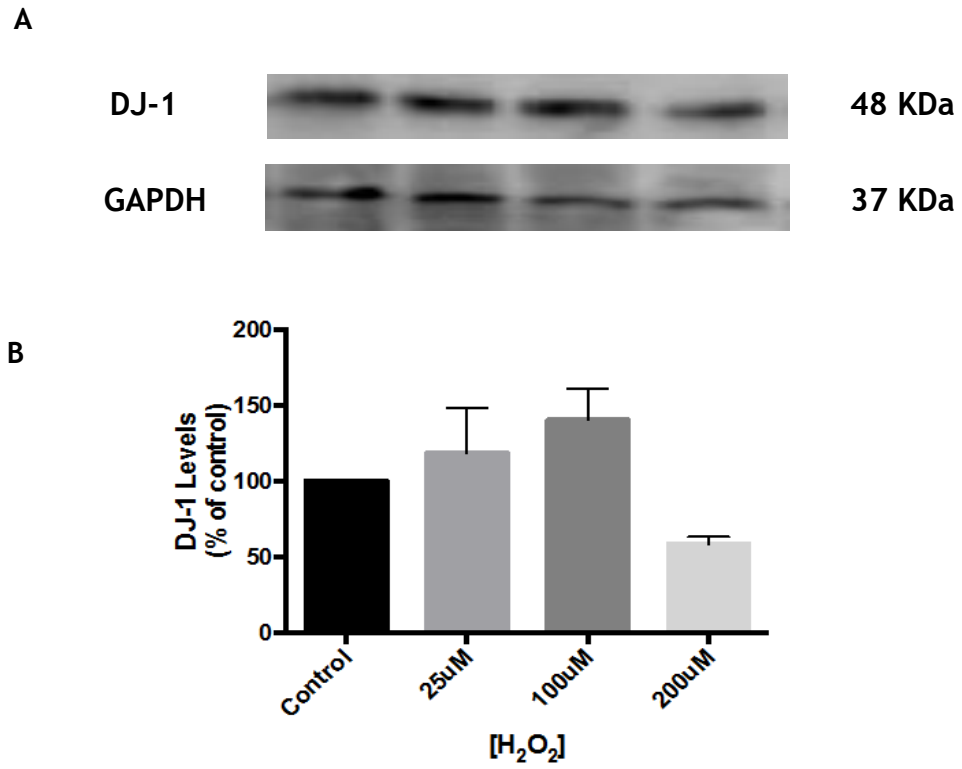


Figure 16 - **DJ-1 levels in midbrain astrocyte cultures exposed to H_2O_2.** DJ-1 expression in midbrain astrocyte cultures stimulated with H_2O_2 (25-200 μM) for 24 h. Representative Western Blot probed for mouse DJ-1 (MW: 48KDa), α-tubulin (MW: 55KDa) or GAPDH (MW: 37KDa) proteins. DJ-1 levels were determined by optical densitometry analysis in that each intensity of the DJ-1 band was normalized for the intensity of the α-tubulin or GAPDH band. Results were expressed as DJ-1/ α-tubulin or GAPDH ratio. Data shown are expressed as a percentage of control and represent the mean ± SEM of three separate experiments carried out in triplicate.

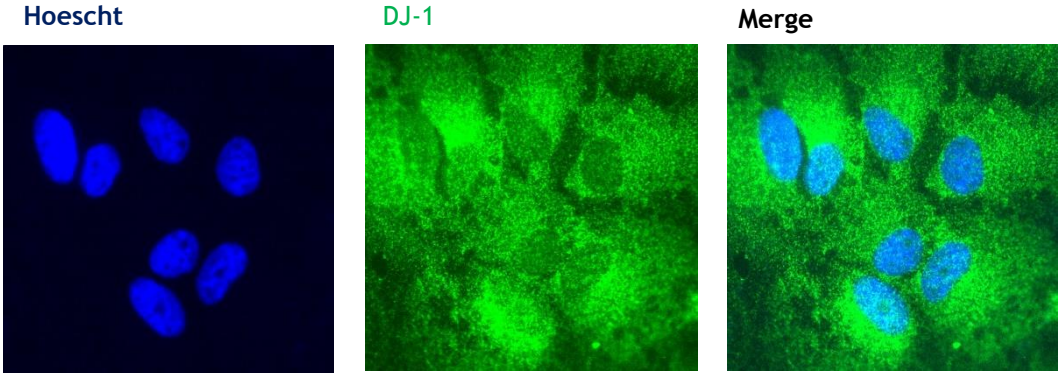
To further confirm these results we did analyse the levels of DJ-1 using a different approach, in this case DJ-1 was determined by Immunocytochemistry followed by quantification of fluorescence intensity. The results obtained show a pattern similar to the observed in the Western Blotting analysis, i.e., a slight increase for the two lower concentrations when compared to control (Figure 17B).

To compare the expression of DJ-1 in neurons and astrocytes exposed to the H_2O_2 oxidative stimulus and also how the contact between the two types of cells influences the levels of DJ-1, we exposed primary neurons or neurons-astrocytes midbrain cultures to H_2O_2 and, by Immunocytochemistry, assessed the DJ-1 levels (Figure 17C e D).

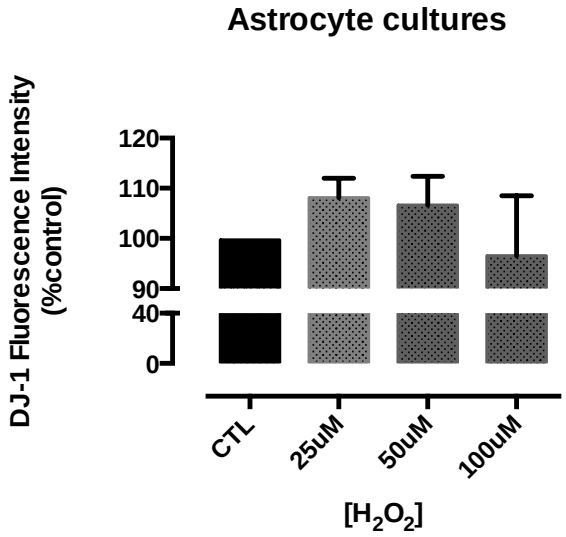
Once again, for the two types of cell cultures used the changes in DJ-1 expression were not significant. Nevertheless, the pattern of the changes is different from both the one observed in astrocytes cultures, in which the tendency for an increase was observed for the lower

concentration of the stimulus, whereas neurons culture required higher levels of H_2O_2 to promote changes in DJ-1 levels. Interestingly, the profile observed in the neuron-astrocyte cultures was more similar to the profile observed in the astrocyte culture, with the exception of the reduction of DJ-1 levels for the higher concentrations of H_2O_2.

(A)



(B)



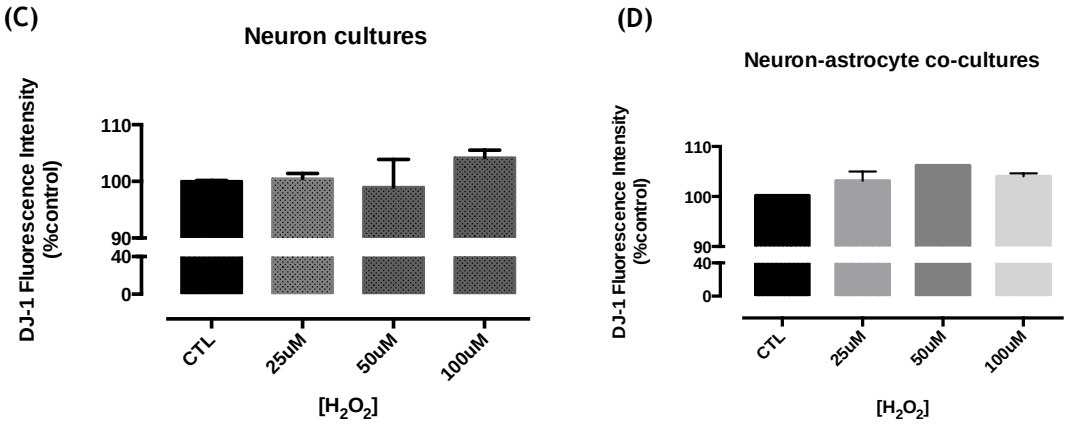


Figure 17 - **Expression of DJ-1 by midbrain cell cultures exposed to H_2O_2.** (A) Representative images of immunocytochemistry for DJ-1 expression. DJ-1 levels in midbrain astrocyte cultures (B), in midbrain neuron cultures (C) and in midbrain neuron-astrocyte cocultures (D) challenged with H_2O_2 (25-100 μM) for 24 h. DJ-1 was evaluated by immunocytochemistry followed by measurement of fluorescence intensity. Data shown are expressed as a percentage of control and represent the mean ± SEM of three separate experiments carried out in triplicate.

Moreover, we observed that the basal expression of DJ-1 in the independent cultures of neurons (65.052 u.a) and astrocytes (67.723 u.a) was lower than when the same cultures are in direct contact (72.340 u.a).

4.1.2. Effect of neuron-astrocyte crosstalk on DJ-1 levels

To further explore how the crosstalk between neurons and astrocytes mediated by secreted factors affects the expression of DJ-1, we introduced a different culture method in which neurons and astrocytes were cultured in separate coverslips and then putted in contact through the culture medium, allowing us to analyze how molecules secreted by the cells to the culture medium could affect DJ-1 expression. Since we did not observe a concentration-dependent effect, in this assays we used the lower concentration of H_2O_2 tested (25 μM).

The results obtained showed that astrocytes maintained separated seem to present higher basal levels of DJ-1, and to respond to H_2O_2 with higher increases, when compared with astrocytes that were allowed to contact with neurons through the culture medium (Figure 18). This suggest that the contact with neurons reduced the levels of DJ-1 present in astrocytes.

Furthermore, the basal levels of neuronal DJ-1 appear to be bigger than those presented by astrocytic DJ-1. Moreover, when astrocytes and neurons were placed in contact through culture medium, both showed a tendency to reduce the cellular content of DJ-1 in both cultures.

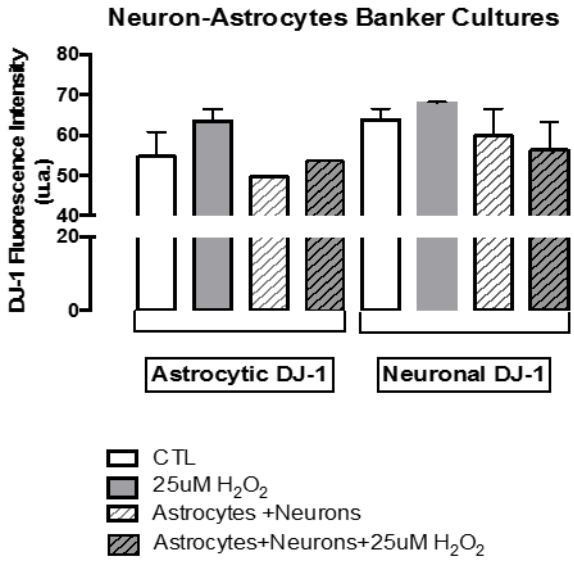


Figure 18 - **Effect of neuron-astrocyte crosstalk on DJ-1 levels both in control cultures and in cultures challenged with H_2O_2.** Neurons and astrocytes cultured separately on different coverslips were maintained in contact thought the culture medium. DJ-1 was evaluated by immunocytochemistry followed by measurement of fluorescence intensity with Image J. Data shown are expressed as a percentage of control and represent the mean ± SEM of two separate experiments carried out in triplicate.

4.2. Immunodepletion of extracellular DJ-1 enhances the DAergic lesion induced by H_2O_2

Taking into account the small effects in the cellular content of DJ-1 induced by H_2O_2 exposure and also the reports showing that H_2O_2 promotes the release of DJ-1 from astrocytes (Yanagida et al., 2009), we decided to evaluate the levels of DJ-1 in the culture medium by Western blot (after submitting the medium to a process of concentration). Unfortunately the results were not consistent and in the majority of the experiments we were unable to observe the DJ-1 bands. We then tried to use a different approach to evaluate the secretion of DJ-1 to the medium. We used the methodology described previously in section 4.1.2, but the cultures were challenged 25 μM of H_2O_2 in the presence of an antibody against DJ-1. The results showed that blocking extracellular DJ-1 with a specific antibody significantly enhances the decrease of DAergic viability induced by H_2O_2 exposure.

Immunocytochemistry against the TH protein and quantitative analysis of TH^+-neurons revealed that H_2O_2 induced a reduction of 35.68% in the number of TH^+ cells, while in the presence of the DJ-1 antibody H_2O_2 reduced the number of DAergic neurons to 68.20% of the control (Figure 19). This result indicate that extracellular DJ-1 released upon an oxidative challenge with H_2O_2 plays a critical role in the survival of DAergic neurons.

After analysing the results we conclude that it is also crucial to evaluate the effect of the DJ-1 immunodepletion in the absence of the oxidative challenge, to determine the participation of DJ-1 in neuronal survival under basal conditions.

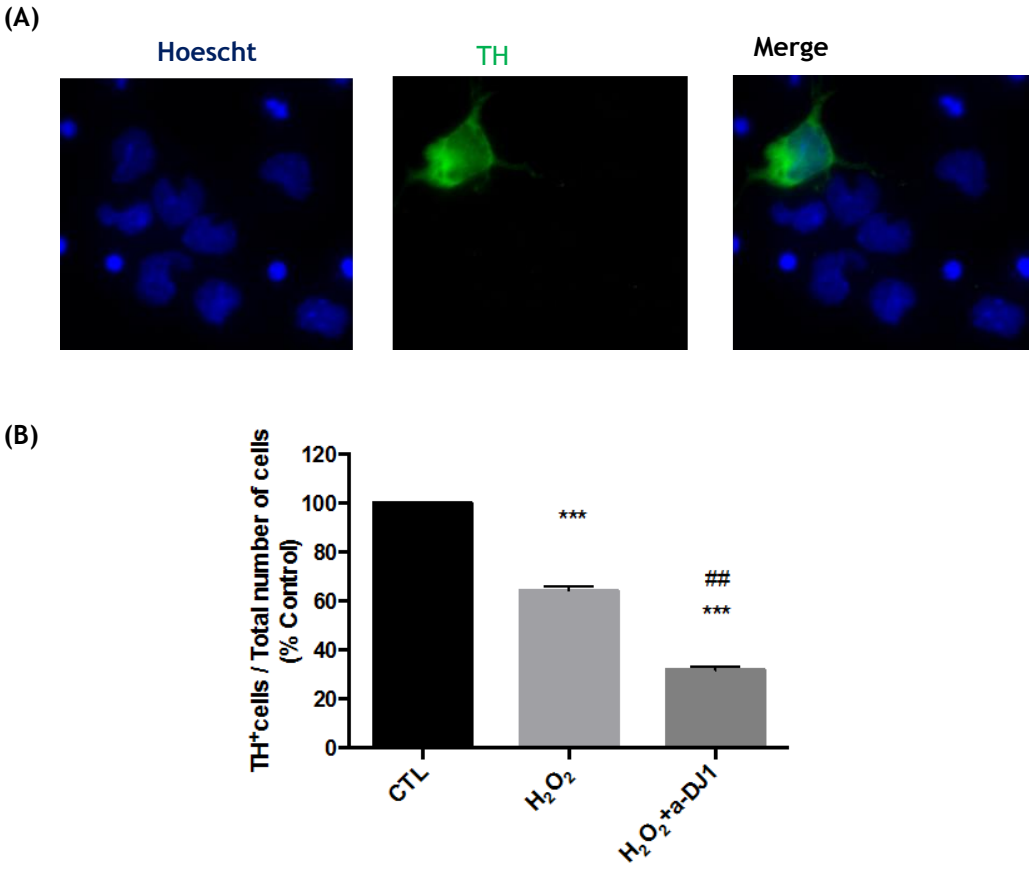


Figure 19 - **Effect of extracellular DJ-1 immunodepletion in neuronal survival mediated by astrocytes when cellular cultures are challenge with H_2O_2.** (A) Representative image of neurons and astrocytes cultured separately on different coverslips were maintained in contact thought the culture medium and were challenge with 25 uM of H_2O_2 for 24h. Data shown are expressed as a percentage of control and represent the mean ± SEM of two separate experiments carried out in triplicate. (***P<0,001;**P<0.01) One-Way ANOVA followed by Bonferroni's Multiple Comparision Test). * related to control and # related to H_2O_2 condition (B).

4.3 Neurons are capable of incorporating DJ-1 applied exogenously

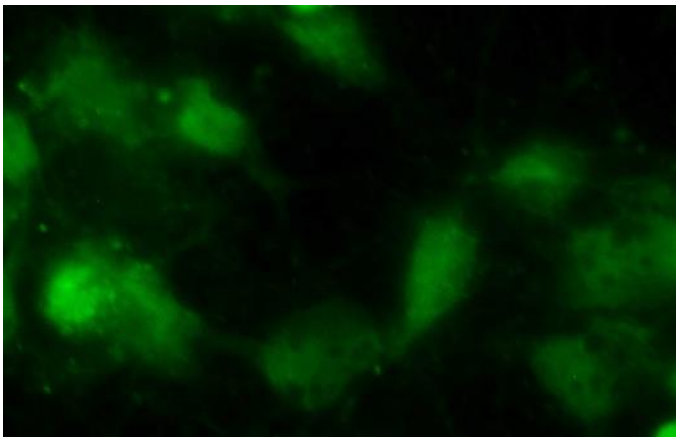
After we observed that the DJ-1 released into the medium appears to play a neuroprotective role, we hypothesized that this neuronal protection could be promoted by the neuronal capture of the DJ-1 protein secreted by astrocytes.

To clarify if neurons are indeed capable of incorporating extracellular DJ-1 we used as a tool a DJ-1 conjugated to the fluorophore Alexa 488, prepared as described in methods section. Alexa

488-coupled DJ-1 was incubated at concentration of 3 μM for 4h. After incubation, the neuronal culture was observed under a fluorescence microscope with the adequate filter. All neuronal cells were stained exhibit green fluorescence, confirming the probe internalization (Figure 20a). Although the preparation of the DJ-1:Alexa 488 conjugate includes a purification step that aims at eliminating the nonconjugated probe, we decided to determine if the staining observed was due to DJ-1 internalization and to the incorporation of the free probe by performing an immunocytochemistry against DJ-1 protein after the incubation with Alexa 488-coupled DJ-1. Even though there is a need for deeper analysis is necessary the results showed that a strong overlapping of red and green fluorescence suggesting a colocalization of Alexa 488-DJ-1 and anti-DJ-1 labeling (Figure 20b).

Since the primary neuronal cultures are frequently contaminated with a small number of other cell types it is important to repeat the experiment but using also a staining against a neuronal marker, to ensure that Alexa 488-DJ-1 staining was is present in neurons and not in other contaminant cells types. Moreover to achieve our initial goal of determining if extracellular DJ-1 exert its protective effect by being incorporated by neurons we plan to determine the protective effect of exogenous applied DJ-1. Although this was already shown by other authors using SH-SY5Y in the presence of recombinant GST-tagged human DJ-1 protein and challenged with H_2O_2 (Yanagida et al., 2009) we think that it is important to test this in our culture model.

(A)



(B)

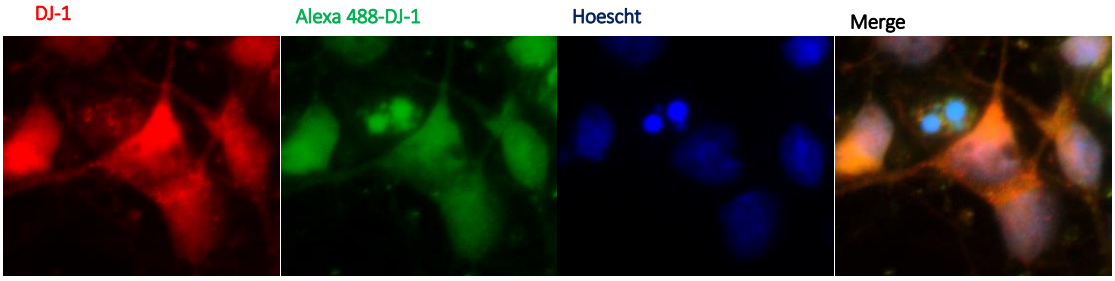


Figure 20 - **Assessment of the ability of neurons to incorporate DJ-1 applied exogenously.** Representative images of neurons cultures incubated for 4h with 3uM DJ-1 conjugated to Alexa 488. (A) Representative image of incorporation of DJ-1 applied exogenously by neurons. (B) Representative image of colocalization of Alexa 488-DJ-1 and anti-DJ-1 labeling. The images was obtained after immunocytochemistry in neuron cultures with specific antibodies for DJ-1 protein and cell nucleus.

Chapter 5

Discussion

DJ-1 was first linked to PD when deletions in its gene (PARK7) were discovered to cause familial early onset PD (Bonifati et al., 2003). DJ-1 has been described as a multifunctional protein but, of all of these functions, the most important to PD is its putative role in neuroprotection, most likely through its involvement in the protection against oxidative stress - the proposed cause of death of DAergic neurons in PD - which is defined as an imbalance between ROS generation and the anti-oxidant capacity of a cell (Giaime et al., 2010). However, it is still not fully understood by which mechanisms DJ-1 plays this neuroprotective role.

DJ-1 is expressed both in neurons and in all types of glial cells (Baulac et al., 2004, Bader et al., 2005). Although it is described that this expression is increased in reactive astrocytes in PD and other neurodegenerative diseases (Bandopadhyay et al., 2004, Rizzu et al., 2004, Neumann et al., 2004, Mullett et al., 2009). This expression may be due to the fact that astrocytes are known to harbor a powerful neuroprotective arsenal and have the ability to secrete neurotrophic factors and to activate anti-oxidative machinery, thereby contributing to neuronal survival (Vila et al., 2001, Takuma et al., 2004, Mullett and Hinkle, 2009, Yanagida et al., 2009, Lev et al., 2013a). Taking these facts together, the main goal of this study was to determine how DJ-1 expression is affected when an oxidative stress condition is present and, how the crosstalk between neurons and astrocytes modulate this process.

In the first part of this study, we characterized the expression of DJ-1 in three different midbrain cell cultures in order to understand how the expression of our protein of interest may be affected when an oxidative stimulus is present. Using Western blot (Figure 16), we found that astrocytes have the ability to express DJ-1 but the results were not consistent, so we opted to confirm these results using Immunocytochemistry (Figure 17B). Like in the first approach, we confirmed the expression of DJ-1 in astrocytes as already described in other studies (Bandopadhyay et al., 2004, Rizzu et al., 2004, Neumann et al., 2004, Mullett et al., 2009, Yanagida et al., 2009) and verified that this expression can be slightly increased when low doses of H_2O_2 are added. A decreased of DJ-1 was detected when astrocytes were stimulated with the higher dose of H_2O_2 (200 µM). Even though it is documented that astrocytes are resistance to the doses administered in this study (100 µM and 200 µM), it is possible that the higher dose of H_2O_2 used induced cellular toxicity and thus decreased the number of cells present in the culture. It is crucial to determine cell viability in order to confirm or exclude this hypothesis.

Furthermore, this decrease in intracellular DJ-1 may be associated with increased secretion of this protein to the culture medium (Yanagida et al., 2009). When we performed an immunocytochemistry on neuron cultures (Figure 17C) and neuron-astrocyte co-cultures (Figure 17D) challenged with H_2O_2, we found that, like the observed in astrocyte cultures, DJ-1 was expressed in this cultures like previously described (Bandopadhyay et al., 2004, Rizzu et al., 2004, Neumann et al., 2004, Mullett et al., 2009). However, in both cultures we did not observe any difference between the control condition and oxidative stress conditions although it is found that when neurons and astrocytes were in independent cultures the basal expression of DJ-1 was lower than when in direct contact. This suggestion raised the hypothesis that factors secreted by one type of cells were modulating the expression of DJ-1 by the other cell type.

The contact between neurons and astrocytes through de media (Figure 18) demonstrated that DJ-1 expression does not undergo significant changes when the two cultures are in contact, even when challenged with the oxidant agent. But both cultures respond to H_2O_2 challenge by increasing DJ-1 levels, although this increase is more pronounced in astrocytes. This behavior can be explained by the fact that reactive astrocytes express more protein than resting astrocytes or neurons in PD or other neurodegenerative diseases (Bandopadhyay et al., 2004, Rizzu et al., 2004, Mullett et al., 2009). On the other hand, the lower protein expression observed in the contact between the two cultures may be due to the fact that when exposed to H_2O_2 neurons stimulate the secretion of DJ-1 by astrocytes in order to give protection to the neurons and thereby exhibit fewer differences in cellular protein content. Thus, oxidative stress activates astrocytes and induces DJ-1 expression. Furthermore, the expression of neuronal DJ-1 seems to be higher in all conditions tested, in comparison with astrocytic DJ-1. The decreased expression observed when neurons and astrocytes are in contact through the culture medium led us to believe that perhaps this decrease was a consequence of a more intense secretion of DJ-1 protein from astrocytes into the medium, in order to promote neuronal protection (Yanagida et al., 2009) and not to a true decrease in the expression on cellular content.

Yanagida and his colleagues (Yanagida et al., 2009) demonstrated in a recent study that in media without H_2O_2, extracellular DJ-1 protein was undetectable whereas the amount of extracellularly released DJ-1 significantly increased, in a concentration-dependent manner when cells were exposed to H_2O_2.

With the aim of determining the DJ-1 present in medium in contact with astrocytes exposed to increasing concentrations, we tested various methodologies but we did not succeed to obtain signal corresponding DJ-1. Therefore it was necessary to use an alternative approach in order to answer this question. DJ-1 has anti-apoptotic and anti-oxidative stress properties (Junn et al., 2005, Inden et al., 2006, Meulener et al., 2006, Fan et al., 2008b, Lev et al., 2008, Lev et al., 2009) and extensive research demonstrated that DJ-1 has the capacity to protect neurons

against oxidative or toxic insults (Canet-Aviles et al., 2004, Junn et al., 2005, Choi et al., 2006, Lev et al., 2013b, Mullett et al., 2013, Lev et al., 2013a). Previous studies founded that DJ-1 knockdown in astrocytes impaired astrocyte-mediated neuroprotection against rotenone and other pesticides (Mullett and Hinkle, 2009, Mullett and Hinkle, 2011). Moreover, Waak et al. showed enhanced neurotoxicity of lipopolysaccharide when primary neuron cultures were grown on DJ-1 knockout astrocytes as compared to wild-type (WT) astrocytes, thereby demonstrating neurotoxic potential of astrocytic DJ-1 deficiency (Waak et al., 2009b). However, later studies determined that astrocytic DJ-1 deficiency did not result in toxic effects either alone or when exposure to 6-OHDA. Furthermore, cocultures experiments support the fact that even with direct contact between astrocytes and neurons, astrocytes lacking DJ-1 showed inferior protective abilities as compared to WT astrocytes (Lev et al., 2013a). In contrast, DJ-1-overexpressing astrocytes, which model sporadic PD astrocytes, exhibited an enhanced capacity to protect cocultured neurons against rotenone-induced oxidative stress (Mullett et al., 2013).

Experiments using astrocyte-conditioned media suggested that astrocyte-released soluble factors, which may be antioxidant molecules or molecules that enhance antioxidants systems, were involved in the DJ-1-dependent, astrocyte-mediated neuroprotective mechanism (Mullett et al., 2013, Lev et al., 2013a).

In order to clarify whether the DJ-1 is one of the factors released by astrocytes as well as if it has, in itself, the ability to protect neurons, we performed primary neuron-astrocyte co-cultures with indirect contact in which we combined oxidative stimulus with immunodepletion of DJ-1 protein (Figure 19). Our data support the conclusion that DJ-1 is effectively secreted into the media (Yanagida et al., 2009) and perhaps shuttle between the inside and outside of cells (Tsuboi et al., 2008). Furthermore, it was found that also in the presence of astrocytes, the survival of DAergic neurons was significantly affected when H_2O_2 is present and that the combination of the same stimulus with the removal of DJ-1 present in the media resulted, in turn, in a drastic reduction of DAergic survival. Taking these results together, we found that DJ-1 is released into the media and not only induce the expression of antioxidant molecules, being their presence vital for neuronal protection. However, although the protein is present in the culture media as previously seen, the question arises whether it can be derived from astrocytes or neurons, although the most plausible hypothesis is that of the astrocytes (Yanagida et al., 2009).

One hypothesis that remains to be clarified is how the DJ-1 secreted plays a neuroprotective role in target neurons, i.e., if it is internalized by neurons and subsequently exerts its intracellular effects. Although there is evidence for DJ-1 uptake by SH-SY5Y (Inden et al., 2006) using a recombinant DJ-1 engineered with an N-terminus GST-tag, these results could not be

confirmed in subsequent studies. Moreover, there is no evidence for a DJ-1 interaction with any membrane protein. Given these facts, the next step in this study was to evaluate the ability of DJ-1 to be captured by neurons by using a different approach. With this purpose we coupled the probe fluorescent Alexa 488 to the DJ-1 peptide (Figure 20a), mimicking extracellular DJ-1 and allowing us to track the protein in the culture. The results obtained suggested that actually neurons have the ability to capture extracellular DJ-1, since the fluorescence displayed by cells co-localize with the antibody specific for the protein under study and is therefore not due to a supposed incorporation of free probe (Figure 20b).

The results obtained allowed us to hypothesize that DJ-1 may be secreted into the culture medium probably by astrocytes when challenged with an oxidative stress insult and consequently taken up by neurons, exerting its direct or indirect neuroprotective role. However more studies are needed to validate this hypothesis.

Chapter 6

Conclusions

The main goal of this study was to determine how neuron-astrocyte crosstalk may affect DJ-1 expression and how this expression contributes to neuroprotection. To achieve this objective, we evaluated how DJ-1 expression in primary midbrain cell cultures is affected when exposed to an oxidative stress environment, such as H_2O_2 stimulus. Furthermore, we intend to give some insights about the importance of DJ-1 in the astrocyte-mediated neuroprotection and if an exogenous DJ-1, mimicking the DJ-1 secreted into the media, has the capability to be captured by neurons and exert a possible influence in neuroprotection.

This project allowed to verify that DJ-1 is expressed both in primary midbrain neurons and astrocytes. The endogenous expression in each cell type (neurons or astrocytes) was not significantly affected by the contact with the other cell type, either through direct contact or when contacting only through molecules secreted through the culture medium, either in control or in H_2O_2 exposed cells.

The release of endogenous DJ-1 significantly protected dopaminergic neuronal survival from an oxidative insult.

Ultimately, these results suggest that DJ-1 may be released into the culture medium probably by astrocytes when challenged with an oxidative stress insult and consequently taken up by neurons, exerting its direct or indirect neuroprotective role.

Future perspectives

In order to strengthen our results, it would be appropriate to continue this study with other important experiments to achieve our initial objective of determining if extracellular DJ-1 exerts its protective effect by being incorporated by neurons.

To clarify if when exposed to H_2O_2 neurons stimulate the secretion of DJ-1 by astrocytes it is necessary to quantify the DJ-1 levels secreted to the culture media by primary neurons and astrocytes cultures or neuron-astrocyte cocultures with indirect contact, in control conditions and in oxidative stress conditions.

It is also crucial to evaluate the effect of the DJ-1 immunodepletion in the absence of the oxidative challenge, to determine the participation of DJ-1 in neuronal survival under basal conditions.

Moreover, will be interesting evaluate if DJ-1 immunodepletion affects all neurons present in primary neuronal cultures or if only DAergic neurons are protected by the presence of DJ-1 in the media.

Since the primary neuronal cultures are frequently contaminated with a small number of other cell types it is important to repeat the experiment of DJ-1 applied exogenously but using also a staining against a neuronal marker, to ensure that Alexa 488-DJ-1 staining was is present in neurons and not in other contaminant cells types.

Finally, in order to achieve our initial goal of determining if extracellular DJ-1 exert its protective effect by being incorporated by neurons we plan to determine the protective effect of exogenous applied DJ-1 in neuronal survival after neurons were exposed to H_2O_2.

References

ANDRES-MATEOS, E., PERIER, C., ZHANG, L., BLANCHARD-FILLION, B., GRECO, T. M., THOMAS, B., KO, H. S., SASAKI, M., ISCHIROPOULOS, H., PRZEDBORSKI, S., DAWSON, T. M. & DAWSON, V. L. 2007. DJ-1 gene deletion reveals that DJ-1 is an atypical peroxiredoxin-like peroxidase. *Proc Natl Acad Sci U S A,* 104**,** 14807-12.

ARIGA, H., TAKAHASHI-NIKI, K., KATO, I., MAITA, H., NIKI, T. & IGUCHI-ARIGA, S. M. 2013. Neuroprotective function of DJ-1 in Parkinson's disease. *Oxid Med Cell Longev,* 2013**,** 683920.

BANDOPADHYAY, R., KINGSBURY, A. E., COOKSON, M. R., REID, A. R., EVANS, I. M., HOPE, A. D., PITTMAN, A. M., LASHLEY, T., CANET-AVILES, R., MILLER, D. W., MCLENDON, C., STRAND, C., LEONARD, A. J., ABOU-SLEIMAN, P. M., HEALY, D. G., ARIGA, H., WOOD, N. W., DE SILVA, R., REVESZ, T., HARDY, J. A. & LEES, A. J. 2004. The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. *Brain,* 127**,** 420-30.

BEKRIS, L. M., MATA IF FAU - ZABETIAN, C. P. & ZABETIAN, C. P. 2010. The genetics of Parkinson disease.

BELIN, A. C. & WESTERLUND, M. 2008. Parkinson's disease: a genetic perspective. *FEBS J,* 275**,** 1377-83.

BELLUZZI, E., BISAGLIA, M., LAZZARINI, E., TABARES, L. C., BELTRAMINI, M. & BUBACCO, L. 2012. Human SOD2 modification by dopamine quinones affects enzymatic activity by promoting its aggregation: possible implications for Parkinson's disease. *PLoS One,* 7**,** e38026.

BONIFATI, V., RIZZU, P., VAN BAREN, M. J., SCHAAP, O., BREEDVELD, G. J., KRIEGER, E., DEKKER, M. C., SQUITIERI, F., IBANEZ, P., JOOSSE, M., VAN DONGEN, J. W., VANACORE, N., VAN SWIETEN, J. C., BRICE, A., MECO, G., VAN DUIJN, C. M., OOSTRA, B. A. & HEUTINK, P. 2003. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science,* 299**,** 256-9.

BRIEGER, K., SCHIAVONE, S., MILLER, F. J., JR. & KRAUSE, K. H. 2012. Reactive oxygen species: from health to disease. *Swiss Med Wkly,* 142**,** w13659.

CANET-AVILES, R. M., WILSON, M. A., MILLER, D. W., AHMAD, R., MCLENDON, C., BANDYOPADHYAY, S., BAPTISTA, M. J., RINGE, D., PETSKO, G. A. & COOKSON, M. R. 2004. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. *Proc Natl Acad Sci U S A,* 101**,** 9103-8.

CHEN, J., LI, L. & CHIN, L. S. 2010. Parkinson disease protein DJ-1 converts from a zymogen to a protease by carboxyl-terminal cleavage. *Hum Mol Genet,* 19**,** 2395-408.

CHOI, J., SULLARDS MC FAU - OLZMANN, J. A., OLZMANN JA FAU - REES, H. D., REES HD FAU - WEINTRAUB, S. T., WEINTRAUB ST FAU - BOSTWICK, D. E., BOSTWICK DE FAU - GEARING, M., GEARING M FAU - LEVEY, A. I., LEVEY AI FAU - CHIN, L.-S., CHIN LS FAU - LI, L. & LI, L. 2006. Oxidative damage of DJ-1 is linked to sporadic Parkinson and Alzheimer diseases.

CLEMENTS, C. M., MCNALLY, R. S., CONTI, B. J., MAK, T. W. & TING, J. P. 2006. DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. *Proc Natl Acad Sci U S A,* 103**,** 15091-6.

COOKSON, M. R. 2010. Unravelling the role of defective genes. *Prog Brain Res,* 183**,** 43-57.

CROSIERS, D., THEUNS J FAU - CRAS, P., CRAS P FAU - VAN BROECKHOVEN, C. & VAN BROECKHOVEN, C. 2011. Parkinson disease: insights in clinical, genetic and pathological features of monogenic disease subtypes.

DACHSEL, J. C. & FARRER, M. J. 2010. LRRK2 and Parkinson disease. *Arch Neurol,* 67**,** 542-7.

DAUER, W. & PRZEDBORSKI, S. 2003b. Parkinson's disease: mechanisms and models. *Neuron,* 39**,** 889-909.

DAWSON, T. M. & DAWSON, V. L. 2010. The role of parkin in familial and sporadic Parkinson's disease. *Mov Disord,* 25 Suppl 1**,** S32-9.

DE LAU, L. M. L. & BRETELER, M. M. B. 2006. Epidemiology of Parkinson's disease. *The Lancet Neurology,* 5**,** 525-535.

DEKKER, M., BONIFATI, V., VAN SWIETEN, J., LEENDERS, N., GALJAARD, R. J., SNIJDERS, P., HORSTINK, M., HEUTINK, P., OOSTRA, B. & VAN DUIJN, C. 2003. Clinical features and neuroimaging of PARK7-linked parkinsonism. *Mov Disord,* 18**,** 751-7.

DUAN, X., KELSEN, S. G. & MERALI, S. 2008. Proteomic analysis of oxidative stress-responsive proteins in human pneumocytes: insight into the regulation of DJ-1 expression. *J Proteome Res,* 7**,** 4955-61.

ELBAZ, A. & MOISAN, F. Update in the epidemiology of Parkinson's disease.

FAN, J., REN, H., FEI, E., JIA, N., YING, Z., JIANG, P., WU, M. & WANG, G. 2008a. Sumoylation is critical for DJ-1 to repress p53 transcriptional activity. *FEBS Lett,* 582**,** 1151-6.

FAN, J., REN, H., JIA, N., FEI, E., ZHOU, T., JIANG, P., WU, M. & WANG, G. 2008b. DJ-1 decreases Bax expression through repressing p53 transcriptional activity. *J Biol Chem,* 283**,** 4022-30.

FARRER, M. J. 2006. Genetics of Parkinson disease: paradigm shifts and future prospects. *Nat Rev Genet,* 7**,** 306-18.

FEARNLEY, J. M. & LEES, A. J. 1991. Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain,* 114 (Pt 5)**,** 2283-301.

FOLTYNIE, T., BRAYNE CE FAU - ROBBINS, T. W., ROBBINS TW FAU - BARKER, R. A. & BARKER, R. A. The cognitive ability of an incident cohort of Parkinson's patients in the UK. The CamPaIGN study.

GAO, H., YANG W FAU - QI, Z., QI Z FAU - LU, L., LU L FAU - DUAN, C., DUAN C FAU - ZHAO, C., ZHAO C FAU - YANG, H. & YANG, H. 2012. DJ-1 protects dopaminergic neurons against rotenone-induced apoptosis by enhancing ERK-dependent mitophagy.

GIAIME, E., YAMAGUCHI, H., GAUTIER, C. A., KITADA, T. & SHEN, J. 2012. Loss of DJ-1 does not affect mitochondrial respiration but increases ROS production and mitochondrial permeability transition pore opening. *PLoS One,* 7**,** e40501.

GORNER, K., HOLTORF, E., ODOY, S., NUSCHER, B., YAMAMOTO, A., REGULA, J. T., BEYER, K., HAASS, C. & KAHLE, P. J. 2004. Differential effects of Parkinson's disease-associated mutations on stability and folding of DJ-1. *J Biol Chem,* 279**,** 6943-51.

HAO, L. Y., GIASSON, B. I. & BONINI, N. M. 2010. DJ-1 is critical for mitochondrial function and rescues PINK1 loss of function. *Proc Natl Acad Sci U S A,* 107**,** 9747-52.

HARDY, J. & ORR, H. 2006. The genetics of neurodegenerative diseases. *J Neurochem,* 97**,** 1690-9.

HASTINGS, T. G. 2009. The role of dopamine oxidation in mitochondrial dysfunction: implications for Parkinson's disease. *J Bioenerg Biomembr,* 41**,** 469-72.

HATTORI, K., NAGURO, I., RUNCHEL, C. & ICHIJO, H. 2009. The roles of ASK family proteins in stress responses and diseases. *Cell Commun Signal,* 7**,** 9.

HAUSER, D. N. & HASTINGS, T. G. 2013. Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. *Neurobiol Dis,* 51**,** 35-42.

HAYASHI, T., ISHIMORI, C., TAKAHASHI-NIKI, K., TAIRA, T., KIM, Y. C., MAITA, H., MAITA, C., ARIGA, H. & IGUCHI-ARIGA, S. M. 2009. DJ-1 binds to mitochondrial complex I and maintains its activity. *Biochem Biophys Res Commun,* 390**,** 667-72.

HEO, J. Y., PARK JH FAU - KIM, S. J., KIM SJ FAU - SEO, K. S., SEO KS FAU - HAN, J. S., HAN JS FAU - LEE, S. H., LEE SH FAU - KIM, J. M., KIM JM FAU - PARK, J. I., PARK JI FAU - PARK, S. K., PARK SK FAU - LIM, K., LIM K FAU - HWANG, B. D., HWANG BD FAU - SHONG, M., SHONG M FAU - KWEON, G. R. & KWEON, G. R. 2012a. DJ-1 null dopaminergic neuronal cells exhibit defects in mitochondrial function and structure: involvement of mitochondrial complex I assembly.

HEO, J. Y., PARK, J. H., KIM, S. J., SEO, K. S., HAN, J. S., LEE, S. H., KIM, J. M., PARK, J. I., PARK, S. K., LIM, K., HWANG, B. D., SHONG, M. & KWEON, G. R. 2012b. DJ-1 null dopaminergic neuronal cells exhibit defects in mitochondrial function and structure: involvement of mitochondrial complex I assembly. *PLoS One,* 7**,** e32629.

HILL-BURNS, E. M., FACTOR, S. A., ZABETIAN, C. P., THOMSON, G. & PAYAMI, H. 2011. Evidence for more than one Parkinson's disease-associated variant within the HLA region. *PLoS One,* 6**,** e27109.

HINDLE, J. V. 2010. Ageing, neurodegeneration and Parkinson's disease. *Age and Ageing,* 39**,** 156-161.

INDEN, M., TAIRA, T., KITAMURA, Y., YANAGIDA, T., TSUCHIYA, D., TAKATA, K., YANAGISAWA, D., NISHIMURA, K., TANIGUCHI, T., KISO, Y., YOSHIMOTO, K., AGATSUMA, T., KOIDE-YOSHIDA, S., IGUCHI-ARIGA, S. M., SHIMOHAMA, S. & ARIGA, H. 2006. PARK7 DJ-1 protects against degeneration of nigral dopaminergic neurons in Parkinson's disease rat model. *Neurobiol Dis,* 24**,** 144-58.

IRRCHER, I., ALEYASIN, H., SEIFERT, E. L., HEWITT, S. J., CHHABRA, S., PHILLIPS, M., LUTZ, A. K., ROUSSEAUX, M. W., BEVILACQUA, L., JAHANI-ASL, A., CALLAGHAN, S., MACLAURIN, J. G., WINKLHOFER, K. F., RIZZU, P., RIPPSTEIN, P., KIM, R. H., CHEN, C. X., FON, E.

A., SLACK, R. S., HARPER, M. E., MCBRIDE, H. M., MAK, T. W. & PARK, D. S. 2010. Loss of the Parkinson's disease-linked gene DJ-1 perturbs mitochondrial dynamics. *Hum Mol Genet,* 19**,** 3734-46.

ISHIKAWA, S., TAIRA, T., TAKAHASHI-NIKI, K., NIKI, T., ARIGA, H. & IGUCHI-ARIGA, S. M. 2010. Human DJ-1-specific transcriptional activation of tyrosine hydroxylase gene. *J Biol Chem,* 285**,** 39718-31.

JANSSEN-HEININGER, Y. M., MOSSMAN, B. T., HEINTZ, N. H., FORMAN, H. J., KALYANARAMAN, B., FINKEL, T., STAMLER, J. S., RHEE, S. G. & VAN DER VLIET, A. 2008. Redox-based regulation of signal transduction: principles, pitfalls, and promises. *Free Radic Biol Med,* 45**,** 1-17.

JEZEK, P. & HLAVATA, L. 2005. Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism.

JUNN, E., JANG, W. H., ZHAO, X., JEONG, B. S. & MOURADIAN, M. M. 2009. Mitochondrial localization of DJ-1 leads to enhanced neuroprotection. *J Neurosci Res,* 87**,** 123-9.

JUNN, E., TANIGUCHI, H., JEONG, B. S., ZHAO, X., ICHIJO, H. & MOURADIAN, M. M. 2005. Interaction of DJ-1 with Daxx inhibits apoptosis signal-regulating kinase 1 activity and cell death. *Proc Natl Acad Sci U S A,* 102**,** 9691-6.

KAHLE, P. J., WAAK, J. & GASSER, T. 2009. DJ-1 and prevention of oxidative stress in Parkinson's disease and other age-related disorders. *Free Radic Biol Med,* 47**,** 1354-61.

KARUNAKARAN, S., DIWAKAR, L., SAEED, U., AGARWAL, V., RAMAKRISHNAN, S., IYENGAR, S. & RAVINDRANATH, V. 2007. Activation of apoptosis signal regulating kinase 1 (ASK1) and translocation of death-associated protein, Daxx, in substantia nigra pars compacta in a mouse model of Parkinson's disease: protection by alpha-lipoic acid. *FASEB J,* 21**,** 2226-36.

KATO, I., MAITA, H., TAKAHASHI-NIKI, K., SAITO, Y., NOGUCHI, N., IGUCHI-ARIGA, S. M. & ARIGA, H. 2013. Oxidized DJ-1 inhibits p53 by sequestering p53 from promoters in a DNA-binding affinity-dependent manner. *Mol Cell Biol,* 33**,** 340-59.

KIM, R. H., PETERS, M., JANG, Y., SHI, W., PINTILIE, M., FLETCHER, G. C., DELUCA, C., LIEPA, J., ZHOU, L., SNOW, B., BINARI, R. C., MANOUKIAN, A. S., BRAY, M. R., LIU, F. F., TSAO, M. S. & MAK, T. W. 2005. DJ-1, a novel regulator of the tumor suppressor PTEN. *Cancer Cell,* 7**,** 263-73.

KIM, S. J., PARK, Y. J., HWANG, I. Y., YOUDIM, M. B., PARK, K. S. & OH, Y. J. 2012. Nuclear translocation of DJ-1 during oxidative stress-induced neuronal cell death. *Free Radic Biol Med,* 53**,** 936-50.

KIM, Y. C., KITAURA, H., TAIRA, T., IGUCHI-ARIGA, S. M. & ARIGA, H. 2009. Oxidation of DJ-1-dependent cell transformation through direct binding of DJ-1 to PTEN. *Int J Oncol,* 35**,** 1331-41.

KINUMI, T., KIMATA J FAU - TAIRA, T., TAIRA T FAU - ARIGA, H., ARIGA H FAU - NIKI, E. & NIKI, E. 2004a. Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide-mediated oxidation in vivo in human umbilical vein endothelial cells.

KINUMI, T., KIMATA, J., TAIRA, T., ARIGA, H. & NIKI, E. 2004b. Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide-mediated oxidation in vivo in human umbilical vein endothelial cells. *Biochem Biophys Res Commun,* 317**,** 722-8.

KITADA, T., ASAKAWA, S., HATTORI, N., MATSUMINE, H., YAMAMURA, Y., MINOSHIMA, S., YOKOCHI, M., MIZUNO, Y. & SHIMIZU, N. 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature,* 392**,** 605-8.

KOIDE-YOSHIDA, S., NIKI, T., UEDA, M., HIMENO, S., TAIRA, T., IGUCHI-ARIGA, S. M., ANDO, Y. & ARIGA, H. 2007. DJ-1 degrades transthyretin and an inactive form of DJ-1 is secreted in familial amyloidotic polyneuropathy. *Int J Mol Med,* 19**,** 885-93.

KRAUSE, K. H. & BEDARD, K. 2008. NOX enzymes in immuno-inflammatory pathologies. *Semin Immunopathol,* 30**,** 193-4.

KREBIEHL, G., RUCKERBAUER, S., BURBULLA, L. F., KIEPER, N., MAURER, B., WAAK, J., WOLBURG, H., GIZATULLINA, Z., GELLERICH, F. N., WOITALLA, D., RIESS, O., KAHLE, P. J., PROIKAS-CEZANNE, T. & KRUGER, R. 2010. Reduced basal autophagy and impaired mitochondrial dynamics due to loss of Parkinson's disease-associated protein DJ-1. *PLoS One,* 5**,** e9367.

LARSEN, N. J., AMBROSI, G., MULLETT, S. J., BERMAN, S. B. & HINKLE, D. A. 2011. DJ-1 knock-down impairs astrocyte mitochondrial function. *Neuroscience,* 196**,** 251-64.

LESAGE, S. & BRICE, A. 2009. Parkinson's disease: from monogenic forms to genetic susceptibility factors. *Hum Mol Genet,* 18**,** R48-59.

LEV, N., BARHUM, Y., BEN-ZUR, T., MELAMED, E., STEINER, I. & OFFEN, D. 2013. Knocking out DJ-1 attenuates astrocytes neuroprotection against 6-hydroxydopamine toxicity. *J Mol Neurosci,* 50**,** 542-50.

LEV, N., ICKOWICZ, D., BARHUM, Y., LEV, S., MELAMED, E. & OFFEN, D. 2009. DJ-1 protects against dopamine toxicity. *J Neural Transm,* 116**,** 151-60.

LI, H. M., NIKI, T., TAIRA, T., IGUCHI-ARIGA, S. M. & ARIGA, H. 2005. Association of DJ-1 with chaperones and enhanced association and colocalization with mitochondrial Hsp70 by oxidative stress. *Free Radic Res,* 39**,** 1091-9.

MACEDO, M. G., ANAR, B., BRONNER, I. F., CANNELLA, M., SQUITIERI, F., BONIFATI, V., HOOGEVEEN, A., HEUTINK, P. & RIZZU, P. 2003. The DJ-1L166P mutant protein associated with early onset Parkinson's disease is unstable and forms higher-order protein complexes. *Hum Mol Genet,* 12**,** 2807-16.

MAITA, C., MAITA H FAU - IGUCHI-ARIGA, S. M. M., IGUCHI-ARIGA SM FAU - ARIGA, H. & ARIGA, H. 2013. Monomer DJ-1 and its N-terminal sequence are necessary for mitochondrial localization of DJ-1 mutants.

MAITA, C., TSUJI, S., YABE, I., HAMADA, S., OGATA, A., MAITA, H., IGUCHI-ARIGA, S. M., SASAKI, H. & ARIGA, H. 2008. Secretion of DJ-1 into the serum of patients with Parkinson's disease. *Neurosci Lett,* 431**,** 86-9.

MALHOTRA, D., THIMMULAPPA, R., NAVAS-ACIEN, A., SANDFORD, A., ELLIOTT, M., SINGH, A., CHEN, L., ZHUANG, X., HOGG, J., PARE, P., TUDER, R. M. & BISWAL, S. 2008. Decline in NRF2-regulated antioxidants in chronic obstructive pulmonary disease lungs due to loss of its positive regulator, DJ-1. *Am J Respir Crit Care Med,* 178**,** 592-604.

MANDEL, S. A., MORELLI M FAU - HALPERIN, I., HALPERIN I FAU - KORCZYN, A. D. & KORCZYN, A. D. Biomarkers for prediction and targeted prevention of Alzheimer's and Parkinson's diseases: evaluation of drug clinical efficacy.

MARTIN, I., DAWSON VL FAU - DAWSON, T. M. & DAWSON, T. M. 2011. Recent advances in the genetics of Parkinson's disease.

MARTINAT, C., SHENDELMAN, S., JONASON, A., LEETE, T., BEAL, M. F., YANG, L., FLOSS, T. & ABELIOVICH, A. 2004. Sensitivity to oxidative stress in DJ-1-deficient dopamine neurons: an ES- derived cell model of primary Parkinsonism. *PLoS Biol,* 2**,** e327.

MATSUZAWA, A. & ICHIJO, H. 2008. Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. *Biochim Biophys Acta,* 1780**,** 1325-36.

MORI, F., NISHIE M FAU - KAKITA, A., KAKITA A FAU - YOSHIMOTO, M., YOSHIMOTO M FAU - TAKAHASHI, H., TAKAHASHI H FAU - WAKABAYASHI, K. & WAKABAYASHI, K. 2006. Relationship among alpha-synuclein accumulation, dopamine synthesis, and neurodegeneration in Parkinson disease substantia nigra.

MULLETT, S. J., DI MAIO, R., GREENAMYRE, J. T. & HINKLE, D. A. 2013. DJ-1 expression modulates astrocyte-mediated protection against neuronal oxidative stress. *J Mol Neurosci,* 49**,** 507-11.

MULLETT, S. J. & HINKLE, D. A. 2009. DJ-1 knock-down in astrocytes impairs astrocyte-mediated neuroprotection against rotenone. *Neurobiol Dis,* 33**,** 28-36.

NAGAKUBO, D., TAIRA, T., KITAURA, H., IKEDA, M., TAMAI, K., IGUCHI-ARIGA, S. M. & ARIGA, H. 1997. DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. *Biochem Biophys Res Commun,* 231**,** 509-13.

NIKI, T., TAKAHASHI-NIKI, K., TAIRA, T., IGUCHI-ARIGA, S. M. & ARIGA, H. 2003. DJBP: a novel DJ-1-binding protein, negatively regulates the androgen receptor by recruiting histone deacetylase complex, and DJ-1 antagonizes this inhibition by abrogation of this complex. *Mol Cancer Res,* 1**,** 247-61.

NISHINAGA, H., TAKAHASHI-NIKI, K., TAIRA, T., ANDREADIS, A., IGUCHI-ARIGA, S. M. & ARIGA, H. 2005. Expression profiles of genes in DJ-1-knockdown and L 166 P DJ-1 mutant cells. *Neurosci Lett,* 390**,** 54-9.

NUYTEMANS, K., THEUNS, J., CRUTS, M. & VAN BROECKHOVEN, C. 2010. Genetic etiology of Parkinson disease associated with mutations in the SNCA, PARK2, PINK1, PARK7, and LRRK2 genes: a mutation update. *Hum Mutat,* 31**,** 763-80.

ORTH, M. & SCHAPIRA, A. H. 2002. Mitochondrial involvement in Parkinson's disease. *Neurochem Int,* 40**,** 533-41.

PAISAN-RUIZ, C., JAIN, S., EVANS, E. W., GILKS, W. P., SIMON, J., VAN DER BRUG, M., LOPEZ DE MUNAIN, A., APARICIO, S., GIL, A. M., KHAN, N., JOHNSON, J., MARTINEZ, J. R., NICHOLL, D., CARRERA, I. M., PENA, A. S., DE SILVA, R., LEES, A., MARTI-MASSO, J. F., PEREZ-TUR, J., WOOD, N. W. & SINGLETON, A. B. 2004. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron,* 44**,** 595-600.

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

PILCHER, H. 2005. Parkin implicated in sporadic Parkinson's disease. *Lancet Neurol,* 4**,** 798.

RAMIREZ, A., HEIMBACH A FAU - GRUNDEMANN, J., GRUNDEMANN J FAU - STILLER, B., STILLER B FAU - HAMPSHIRE, D., HAMPSHIRE D FAU - CID, L. P., CID LP FAU - GOEBEL, I., GOEBEL I FAU - MUBAIDIN, A. F., MUBAIDIN AF FAU - WRIEKAT, A.-L., WRIEKAT AL FAU - ROEPER, J., ROEPER J FAU - AL-DIN, A., AL-DIN A FAU - HILLMER, A. M., HILLMER AM FAU - KARSAK, M., KARSAK M FAU - LISS, B., LISS B FAU - WOODS, C. G., WOODS CG FAU - BEHRENS, M. I., BEHRENS MI FAU - KUBISCH, C. & KUBISCH, C. 2006. Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase.

REN, H., FU, K., WANG, D., MU, C. & WANG, G. 2011. Oxidized DJ-1 interacts with the mitochondrial protein BCL-XL. *J Biol Chem,* 286**,** 35308-17.

RIZZU, P., HINKLE, D. A., ZHUKAREVA, V., BONIFATI, V., SEVERIJNEN, L. A., MARTINEZ, D., RAVID, R., KAMPHORST, W., EBERWINE, J. H., LEE, V. M., TROJANOWSKI, J. Q. & HEUTINK, P. 2004. DJ-1 colocalizes with tau inclusions: a link between parkinsonism and dementia. *Ann Neurol,* 55**,** 113-8.

SCHAPIRA, A. H. 2006. Mitochondrial disease. *Lancet,* 368**,** 70-82.

SHENDELMAN, S., JONASON, A., MARTINAT, C., LEETE, T. & ABELIOVICH, A. 2004. DJ-1 is a redox-dependent molecular chaperone that inhibits alpha-synuclein aggregate formation. *PLoS Biol,* 2**,** e362.

SHIIZAKI, S., NAGURO, I. & ICHIJO, H. 2013. Activation mechanisms of ASK1 in response to various stresses and its significance in intracellular signaling. *Adv Biol Regul,* 53**,** 135-44.

SHINBO, Y., TAIRA, T., NIKI, T., IGUCHI-ARIGA, S. M. & ARIGA, H. 2005. DJ-1 restores p53 transcription activity inhibited by Topors/p53BP3. *Int J Oncol,* 26**,** 641-8.

SPATOLA, M. & WIDER, C. 2014. Genetics of Parkinson's disease: the yield. *Parkinsonism Relat Disord,* 20 Suppl 1**,** S35-8.

STAYTE, S. & VISSEL, B. 2014. Advances in non-dopaminergic treatments for Parkinson's disease. *Front Neurosci,* 8**,** 113.

TAIRA, T., SAITO, Y., NIKI, T., IGUCHI-ARIGA, S. M., TAKAHASHI, K. & ARIGA, H. 2004. DJ-1 has a role in antioxidative stress to prevent cell death. *EMBO Rep,* 5**,** 213-8.

TAKAHASHI-NIKI, K., NIKI, T., TAIRA, T., IGUCHI-ARIGA, S. M. & ARIGA, H. 2004. Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson's disease patients. *Biochem Biophys Res Commun,* 320**,** 389-97.

TAKEUCHI, K. & ITO, F. 2010. EGF receptor in relation to tumor development: molecular basis of responsiveness of cancer cells to EGFR-targeting tyrosine kinase inhibitors. *FEBS J,* 277**,** 316-26.

TAO, X. & TONG, L. 2003. Crystal structure of human DJ-1, a protein associated with early onset Parkinson's disease. *J Biol Chem,* 278**,** 31372-9.

THOMAS, K. J., MCCOY, M. K., BLACKINTON, J., BEILINA, A., VAN DER BRUG, M., SANDEBRING, A., MILLER, D., MARIC, D., CEDAZO-MINGUEZ, A. & COOKSON, M. R. 2011. DJ-1 acts in

parallel to the PINK1/parkin pathway to control mitochondrial function and autophagy. *Hum Mol Genet,* 20**,** 40-50.

TILLMAN, J. E., YUAN, J., GU, G., FAZLI, L., GHOSH, R., FLYNT, A. S., GLEAVE, M., RENNIE, P. S. & KASPER, S. 2007. DJ-1 binds androgen receptor directly and mediates its activity in hormonally treated prostate cancer cells. *Cancer Res,* 67**,** 4630-7.

TSUBOI, Y., MUNEMOTO, H., ISHIKAWA, S., MATSUMOTO, K., IGUCHI-ARIGA, S. M. & ARIGA, H. 2008. DJ-1, a causative gene product of a familial form of Parkinson's disease, is secreted through microdomains. *FEBS Lett,* 582**,** 2643-9.

VALENTE, E. M., SALVI, S., IALONGO, T., MARONGIU, R., ELIA, A. E., CAPUTO, V., ROMITO, L., ALBANESE, A., DALLAPICCOLA, B. & BENTIVOGLIO, A. R. 2004. PINK1 mutations are associated with sporadic early-onset parkinsonism. *Ann Neurol,* 56**,** 336-41.

VILA, M. & PRZEDBORSKI, S. 2004. Genetic clues to the pathogenesis of Parkinson's disease. *Nat Med,* 10 Suppl**,** S58-62.

WAAK, J., WEBER, S. S., GORNER, K., SCHALL, C., ICHIJO, H., STEHLE, T. & KAHLE, P. J. 2009a. Oxidizable residues mediating protein stability and cytoprotective interaction of DJ-1 with apoptosis signal-regulating kinase 1. *J Biol Chem,* 284**,** 14245-57.

WAAK, J., WEBER, S. S., WALDENMAIER, A., GORNER, K., ALUNNI-FABBRONI, M., SCHELL, H., VOGT-WEISENHORN, D., PHAM, T. T., REUMERS, V., BAEKELANDT, V., WURST, W. & KAHLE, P. J. 2009b. Regulation of astrocyte inflammatory responses by the Parkinson's disease-associated gene DJ-1. *FASEB J,* 23**,** 2478-89.

WANG, X., PETRIE, T. G., LIU, Y., LIU, J., FUJIOKA, H. & ZHU, X. 2012. Parkinson's disease-associated DJ-1 mutations impair mitochondrial dynamics and cause mitochondrial dysfunction. *J Neurochem,* 121**,** 830-9.

WARAGAI, M., NAKAI, M., WEI, J., FUJITA, M., MIZUNO, H., HO, G., MASLIAH, E., AKATSU, H., YOKOCHI, F. & HASHIMOTO, M. 2007. Plasma levels of DJ-1 as a possible marker for progression of sporadic Parkinson's disease. *Neurosci Lett,* 425**,** 18-22.

WIDER, C., FOROUD, T. & WSZOLEK, Z. K. 2010. Clinical implications of gene discovery in Parkinson's disease and parkinsonism. *Mov Disord,* 25 Suppl 1**,** S15-20.

WILSON, M. A., COLLINS, J. L., HOD, Y., RINGE, D. & PETSKO, G. A. 2003. The 1.1-A resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease. *Proc Natl Acad Sci U S A,* 100**,** 9256-61.

WIRDEFELDT, K., ADAMI, H. O., COLE, P., TRICHOPOULOS, D. & MANDEL, J. 2011. Epidemiology and etiology of Parkinson's disease: a review of the evidence. *Eur J Epidemiol,* 26 Suppl 1**,** S1-58.

XU, J., ZHONG, N., WANG, H., ELIAS, J. E., KIM, C. Y., WOLDMAN, I., PIFL, C., GYGI, S. P., GEULA, C. & YANKNER, B. A. 2005. The Parkinson's disease-associated DJ-1 protein is a transcriptional co-activator that protects against neuronal apoptosis. *Hum Mol Genet,* 14**,** 1231-41.

YAMAGUCHI, S., YAMANE, T., TAKAHASHI-NIKI, K., KATO, I., NIKI, T., GOLDBERG, M. S., SHEN, J., ISHIMOTO, K., DOI, T., IGUCHI-ARIGA, S. M. & ARIGA, H. 2012. Transcriptional activation of low-density lipoprotein receptor gene by DJ-1 and effect of DJ-1 on cholesterol homeostasis. *PLoS One,* 7**,** e38144.

YAN, M. H., WANG, X. & ZHU, X. 2013. Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. *Free Radic Biol Med,* 62**,** 90-101.

YANAGIDA, T., TSUSHIMA, J., KITAMURA, Y., YANAGISAWA, D., TAKATA, K., SHIBAIKE, T., YAMAMOTO, A., TANIGUCHI, T., YASUI, H., TAIRA, T., MORIKAWA, S., INUBUSHI, T., TOOYAMA, I. & ARIGA, H. 2009. Oxidative stress induction of DJ-1 protein in reactive astrocytes scavenges free radicals and reduces cell injury. *Oxid Med Cell Longev,* 2**,** 36-42.

YOTSUMOTO, F., SANUI, A., FUKAMI, T., SHIROTA, K., HORIUCHI, S., TSUJIOKA, H., YOSHIZATO, T., KUROKI, M. & MIYAMOTO, S. 2009. Efficacy of ligand-based targeting for the EGF system in cancer. *Anticancer Res,* 29**,** 4879-85.

ZAPPIA, M., COLOSIMO C FAU - POEWE, W. & POEWE, W. 2010. Levodopa: back to the future.

ZHONG, N., KIM, C. Y., RIZZU, P., GEULA, C., PORTER, D. R., POTHOS, E. N., SQUITIERI, F., HEUTINK, P. & XU, J. 2006. DJ-1 transcriptionally up-regulates the human tyrosine hydroxylase by inhibiting the sumoylation of pyrimidine tract-binding protein-associated splicing factor. *J Biol Chem,* 281**,** 20940-8.

ZHONG, N. & XU, J. 2008. Synergistic activation of the human MnSOD promoter by DJ-1 and PGC-1alpha: regulation by SUMOylation and oxidation. *Hum Mol Genet,* 17**,** 3357-67.

ZHOU, W. & FREED, C. R. 2005. DJ-1 up-regulates glutathione synthesis during oxidative stress and inhibits A53T alpha-synuclein toxicity. *J Biol Chem,* 280**,** 43150-8.

ZHOU, W., ZHU, M., WILSON, M. A., PETSKO, G. A. & FINK, A. L. 2006. The oxidation state of DJ-1 regulates its chaperone activity toward alpha-synuclein. *J Mol Biol,* 356**,** 1036-48.