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CICLO XX

TOXIC EFFECTS OF THE OXIDIZED PRODUCTS OF THE NEUROTRANSMITTER DOPAMINE ON MITOCHONDRIA AND THEIR IMPLICATIONS IN PARKINSON'S DISEASE

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DATA CONSEGNA TESI
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Summary

Parkinson's disease (PD) is a chronic, progressive, neurodegenerative disorder clinically characterized by motor symptoms such as tremor at rest, rigidity, slowness of movement (bradykinesia), and postural instability.

One pathological hallmark of the disease is the progressive and striking loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). These neurons reside in the midbrain and project axons rostrally to the forebrain where they release dopamine (DA) into the striatum. DA release in the striatum is critical for the coordination and initiation of movement.

It is unclear why SNpc neurons die during PD. However, several biochemical hallmarks of the disease exist, and likely reveal clues to the underlying etiology of PD. Mitochondrial dysfunctions, at the level of complex I of the electronic transport chain (ETC), have been reported in the SNpc of PD patients. Mitochondria are the main providers of cellular energy, which is generated through the flow of electrons down the electron transport chain (ETC) coupled with production of adenosine tri-phosphate (ATP) form Adenosine di-phosphate (ADP).

Deficits in complex I observed in PD patients are indicative of a bioenergetic defect and increased reactive oxygen species (ROS) production due to electron leak from ETC. Consistent with this, lipid peroxidation and protein carbonyls are present in striatum of post-mortem PD brains, indicative of exposure to ROS. Additionally, a specific decrease in the anti-oxidant glutathione (GSH) has been reported in the SNpc of PD patients, suggesting that these neurons may not be able to buffer the ROS. All these findings suggest that dopaminergic neurons are under oxidative stress in PD, and that the ability of dopaminergic neurons to scavenge free radicals and ROS is compromised. Taken together, mitochondrial dysfunction and oxidative stress are likely key factors that are responsible for the loss of SNpc neurons in PD.

It has been hypothesized that the reason dopaminergic neurons of SNpc are vulnerable in PD is due to the toxic properties of the DA itself. DA is a highly reactive molecule, normally stored in synaptic vesicles. The acidic environment of the synaptic vesicle prevents DA oxidation. However, DA is synthesized and metabolized in the cytoplasm and in this environment cytoplasmic DA can undergo spontaneous oxidation. DA oxidation gives arise to several toxic ROS and the dopamine quinone molecules (DAQs). Increasing cytosolic dopamine in neurons and its oxidized metabolites has many deleterious effects, including increases in oxidative stress, mitochondrial dysfunction, as well as the promotion and stabilization of a potentially toxic protein called α-synuclein. Therefore, the selective loss of SNpc DA neurons in PD may result from increased oxidation of cytoplasmic DA and the oxidative stress induced by ROS-mediated oxidative damage to the neurons. Moreover, the ability of DAQs to react and covalently

modify cellular molecules and macromolecules has been reported. In particular electrophilic DAQs have high affinity for free cysteine, reduced glutathione (GSH) and sulfhydryl groups of proteins to form cysteinyl-catechol conjugates

The overall aim of the research that this dissertation comprises is to identify the mechanism that contributes to the selective degeneration of dopaminergic neurons in PD. The research described herein utilizes in vitro and cellular model approaches to test the hypothesis that the oxidized products of the neurotransmitter DA (DAQs) induce toxic effects on mitochondria. If this hypothesis is correct any pathological event that impairs DA synthesis, storage or metabolism, leading to the cytoplasmic accumulation of DA can increase the presence of oxidized products of DA and their toxic effects in the cell with mitochondria as one of the target. Mitochondrial dysfunctions derived from exposure to DA-oxidized products can account for the specific vulnerability of dopaminergic neurons and their degeneration in PD.

Results from this research demonstrate that DAQs have the ability to enter the mitochondria crossing both the outer and the inner mitochondrial membranes (OMM, IMM). DAQs react and covalently modify several proteins within mitochondria and modifications of subunits of the complex I and complex V of the ETC were identified.

DAQs induce the permeability transition (PT) in mitochondria. PT is an increase in the permeability of the mitochondrial membranes to molecules of less than 1500 Daltons in molecular weight due to the opening a proteinaceous pore that is formed in the IMM and lead to mitochondrial swelling, disruption of the IMM structure and the release in the medium of the pro-apoptotic factors normally located in the inter-membrane space. The mechanism of DAQ-induced PT was studied in vitro. Oxidation of the pyridine nucleotide (PN) pool in DAQ-treated mitochondria was identified. The redox state of the PN is a key factor of PT ore conformation and oxidation of PN trigger PT.

The ability of DAQs to depolarize mitochondria by inducing PT in a cellular model is also reported.

DAQs can exert their toxic effects on mitochondria inhibiting mitochondrial respiration. The inhibitory effect of DAQ on isolated mitochondria complex I was determined. Toxic effect of this inhibition were compared with the ones of the specific complex I inhibitor rotenone in a cellular model expressing the rotenone-insensitive single subunit NADH dehydrogenase of Saccharomices cerevisiae (Ndi1). Ndi1 subunit allows electron transfer through the ETC avoiding electrons leakage and ROS production as consequence of rotenone inhibition. Ndi1-expressing cells are protected from DAQs toxicity suggesting that DAQs, as rotenone, can exert their toxic effects increasing electron leakage from ETC, at least, at the level of complex I and increasing ROS production.

Taken together the results described in this dissertation details a unique, novel, mechanism that may render SNpc DA neurons vulnerable to oxidative stress, ROS-mediated oxidative damage, and neurodegeneration in PD. The contribution of the neurotransmitter DA to oxidative stress represents a valid explanation for the peculiar vulnerability of these neurons in PD and also represents a logical therapeutic target for protecting these neurons.

Riassunto

La malattia di Parkinson è una malattia cronica, progressiva, clinicamente caratterizzata da disfunzioni motorie come tremore a riposo, lentezza nei movimenti (bradicinesia) e instabilità posturale. L'analisi post mortem del cervello di pazienti affetti da malattia di Parkinson rivela la massiva e specifica degenerazione dei neuroni dopaminergici della Substantia nigra pars compacta (SNpc) e presenza di inclusioni proteiche nel citoplasma dei neuroni dopaminergici rimanenti. La perdita dei neuroni dopaminergici della SNpc provoca una diminuzione del rilascio del neurotrasmettitore dopamina (DA) nello striato specificamente espresso in questi neuroni. Il rilascio di DA nello striato è un evento chiave nell'inizio e nella coordinazione dei movimenti e la sua diminuzione nei pazienti affetti dalla malattia di Parkinson è la ragione dei sintomi motori.

La ragione per la quale i neuroni dopaminergici della SNpc degenerano nella malattia di Parkinson è tuttora sconosciuta. Evidenze biochimiche di disfunzioni mitocondriali nei neuroni della SNpc sono state riportate. In particolare, il complesso I della catena respiratoria è inibito in pazienti affetti dalla malattia di Parkinson. Inoltre i neuroni della SNpc di questi pazienti sono caratterizzati dalla presenza di markers per lo stress ossidativo, come aumentati perossi-lipidi, proteine carbonilate e glutatione ossidato. Tutte queste evidenze portano alla conclusione che i neuroni dopaminergici nella malattia di Parkinson sono esposti ad un elevato stress ossidativo e che la loro capacità di eliminare le molecole radicali o le specie reattive dell'ossigeno (ROS) è compromessa.

L'ipotesi che la molecola DA contribuisca allo stress ossidativo dei neuroni dopaminergici è stata formulata da molto tempo. La molecola DA è altamente reattiva e normalmente sequestrata in vescicole sinaptiche dov'è stabilizzata dall'ambiente acido.

Difetti nella sintesi o nel metabolismo della DA portano ad un suo accumulo nel citoplasma. Nel citoplasma l'anello catecolo della DA può essere ossidato e questa reazione porta alla formazione di ROS tra cui lo ione super-ossido, l'anione ossidrile e i chinoni della DA. Numerosi effetti citotossici sono stati riportati per i metaboliti ossidati della DA, tra cui aumento dello stress ossidativo, modificazione covalente e inibizione dell'attività di diverse proteine e disfunzioni mitocondriali.

Lo scopo generale della ricerca presentata in questa tesi è l'identificazione di meccanismi che contribuiscono alla morte selettiva dei neuroni dopaminergici nella malattia di Parkinson. L'ipotesi che i prodotti ossidati della DA (DAQ) inducano effetti tossici nei mitocondri è stata testata utilizzando sistemi in vitro e modelli cellulari. In questo scenario, qualsiasi evento patologico che alteri il catabolismo-metabolismo della DA – aumentandone la concentrazione nel citoplasma – all'aumento dei prodotti ossidati della DA nel citoplasma e, di

conseguenza, ad effetti citotossici. Uno dei target cellulari dei DAQ possono essere i mitocondri. Le disfunzioni mitocondriali derivanti dalla presenza dei DAQ nel citoplasma possono spiegare la peculiare vulnerabilità dei neuroni dopaminergici e la loro degenerazione nella malattia di Parkinson.

I risultati del lavoro di ricerca qui presentato mostrano che i DAQ sono in grado di penetrare entrambe le membrane mitocondriali e giungere nella matrice. I DAQ, inoltre, reagiscono e modificano covalentemente diverse proteine mitocondriali, tra queste subunità del complesso I e del complesso F1FO ATP sintasi (complesso V).

I DAQ, inoltre, sono in grado di indurre il fenomeno di transizione di permeabilità mitocondriale. Questo fenomeno è dovuto all'apertura di un poro costituito da proteine non ancora identificate nella membrana interna del mitocondrio. Quando questo poro si apre, la membrana interna diventa permeabile a soluti con massa inferiore ai 1500 Da e i mitocondri si rigonfiano per effetto osmotico. Questo rigonfiamento porta alla perdita della struttura ripiegata della membrana interna e alla rottura della membrana esterna, con rilascio dei fattori pro-apoptotici che normalmente risiedono nello spazio inter-membrana. La transizione di permeabilità è considerata uno dei fattori che scatenano la via mitocondriale di morte cellulare programmata. Il meccanismo di induzione di permeabilità da parte dei DAQ è stato studiato in vitro. I risultati di questi esperimenti indicano che i DAQ provocano l'ossidazione dei piridin nucleotidi nella matrice e, dato che lo stato redox dei piridin nucleotidi è uno dei fattori che regola la conformazione aperta/chiusa del poro di permeazione, questo effetto dei DAQ è suggerito come fattore decisivo per l'inizio della transizione di permeabilità. È stata inoltre descritta la capacità dei DAQ di depolarizzare i mitocondri e indurre la transizione di permeabilità in un sistema cellulare.

Data la capacità dei DAQ di legarsi covalentemente a proteine dei complessi della catena respiratoria, è stato studiato l'effetto dei DAQ sulla respirazione mitocondriale. I DAQ sono in grado di inibire la respirazione mitocondriale e, in particolare, è stato evidenziato un effetto inibitorio a livello del complesso I della catena respiratoria. Alcune tossine (come MPTP e rotenone), la cui azione specifica è quella di inibire il complesso I della catena respiratoria, sono usate per ottenere modelli animali della malattia di Parkinson. Un modello cellulare che esprime un complesso I resistente al rotenone è stato impiegato per comparare il meccanismo inibitorio dei DAQ e del rotenone. Queste cellule mostrano di essere protette, almeno in parte, anche dall'azione citotossica dei DAQ. Dato che la tossicità del rotenone risiede nella sua capacità di aumentare la produzione di ROS a livello del complesso I bloccando il normale flusso di elettroni nella catena respiratoria, si ipotizza che lo stesso meccanismo possa valere per i DAQ.

In conclusione, i risultati della ricerca presentati in questa tesi descrivono un meccanismo che può spiegare la ragione della specifica vulnerabilità dei neuroni dopaminerici della SNpc nella malattia di Parkinson. L'aumento delle specie reattive derivanti dall'ossidazione del neurotrasmettitore DA specificamente espresso da questi neuroni rappresenta una valida spiegazione per la vulnerabilità di questa famiglia di neuroni e rappresenta un logico target terapeutico per proteggere queste cellule.

Introduction

Parkinson's Disease

Parkinson's disease (PD) is a chronic, progressive, neurodegenerative disorder clinically characterized by motor symptoms such as tremor at rest, rigidity, slowness of movement (bradykinesia), and postural instability (*1*). The motor symptoms of the disease were first described in "An Essay on the Shaking Palsy" by James Parkinson in 1817. It wasn't until many years later that the underlying pathology responsible for the development of the motor impairments was revealed. One pathological hallmark of the disease is the progressive and striking loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). These neurons reside in the midbrain and project axons rostrally to the forebrain where they release dopamine (DA) into the striatum. DA release in the striatum is critical for the coordination and initiation of movement. The loss of this DA input is primarily responsible for the manifestation of motor symptoms in PD. A second pathological hallmark of PD is the presence of eosinophilic, intracytoplasmic, proteinaceous inclusions called Lewy bodies (LB) found in neurons at post-mortem analysis of the brain (*1*). Lewy bodies were first described by Friedrich Lewy in 1912 as characteristic hyaline inclusions with a dense eosinophilic core surrounded by a clear halo. They are found primarily in the SNpc and another midbrain nucleus, the locus coeruleus (LC) in PD patients (*2*). The presence of Lewy bodies in the midbrain is required for the post-mortem diagnosis of PD.

It is unclear why SNpc neurons die during PD. However, several biochemical hallmarks of the disease exist, and likely reveal clues to the underlying etiology of PD. Mitochondrial dysfunctions, at the level of complex I of the electronic transport chain (ETC), have been reported in the SNpc of PD patients (*3, 4*). Mitochondria are the main providers of cellular energy, which is

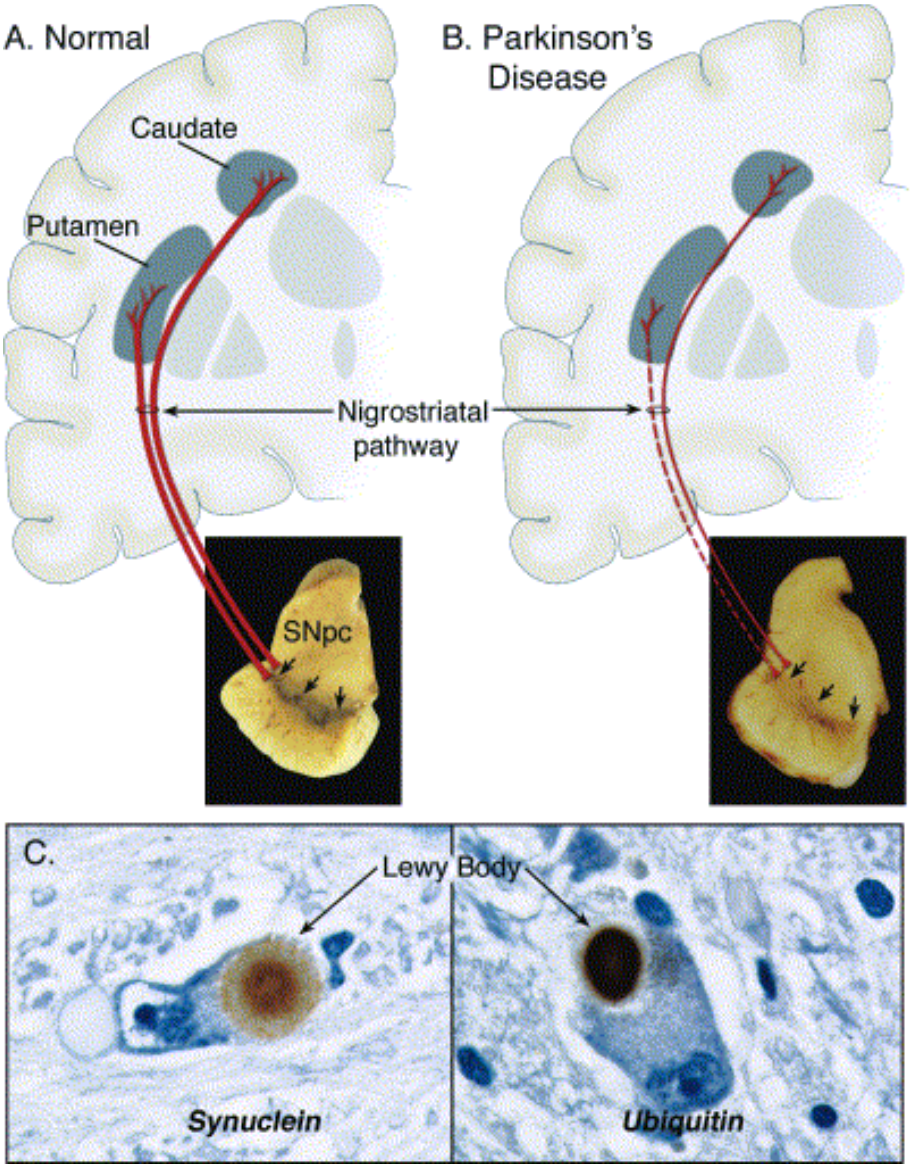

Figure 1 Clinical hallmarks of Parkinson's disease

(A) Schematic representation of the normal nigrostriatal pathway (in red). Dopaminergic neurons have the neuromelanin-containing cell bodies that reside in the substantia nigra pars compacta (SNpc) and they project (thick solid red lines) in the striatum (i.e., putamen and caudate nucleus). (B) Dopamineric neurons degenerate in PD (loss of dark-brown pigment neuromelanin; arrows). There is a marked loss of dopaminergicneurons that project to the putamen (dashed line) and much less of those that project to the caudate (thin red solid line). (C) Lewy bodies. Immunohistochemical labeling of intraneuronalinclusions, in SNpc dopaminergic neurons. Immunostaining with an antibody against α-synuclein (left photograpf) and against ubiquitin (right photograph). (from Dauer and Prezdborski 2003)

generated through the flow of electrons down the electron transport chain (ETC) coupled with production of adenosine tri-phosphate (ATP) form adenosine di-phosphate (ADP). The ETC is located on the inner mitochondrial membrane (IMM) and consists of 4 membrane spanning enzyme complexes. These comprise complex I (NADH–ubiquinone reductase), which oxidizes NADH, complex II (succinate–ubiquinone oxidoreductase) which oxidizes FADH2, complex III (ubiquinol cytochrome c oxidoreductase) and complex IV (cytochrome c oxidase). The ETC transfers electrons through a series of oxidation–reduction reactions, culminating in the generation of a proton gradient across the inner mitochondrial membrane. Depolarization of this membrane potential is used to induce the phosphorylation of ADP to ATP. Thus, the deficits in complex I observed in PD patients are indicative of a bioenergetic defect and increased reactive oxygen species (ROS) production due to electron leak from ETC. Consistent with this, lipid peroxidation and protein carbonyls are present in striatum of post-mortem PD brains (*5*), typical of exposure to ROS. Additionally, a specific decrease in the anti-oxidant glutathione (GSH) has been reported in the SNpc of PD patients,

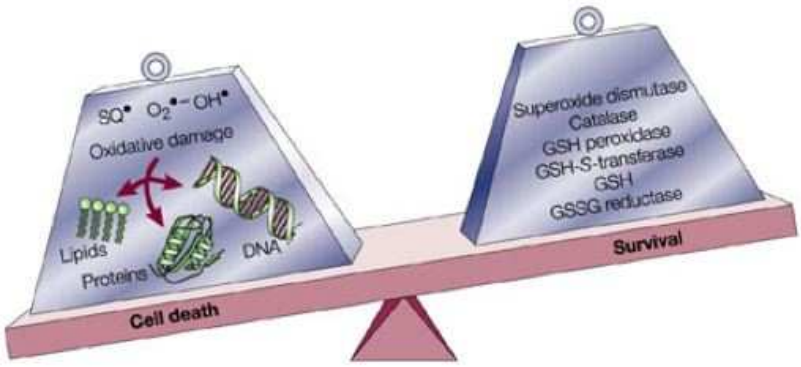

Figure 2 Oxidative damage arises from an unbalance between ROS production and ROS scavenging

Increase production of ROS or defects in ROS scavenging (i.e. supeoxide dismutase (SOD), catalase, glutathione (GSH)) lead to oxidative stress. This oxidative damage can induce cell death and its one of the main mechanism claimed for the degeneration of dopaminergic neurons in PD. DA itself, the neurotransmitter expressed by dopaminergic neurons, has been for long hypothesized as one cause of the increased oxidative stress that this neurons experience since reactive species are produced during its normal metabolism and DA can undergoes to spontaneous oxidation in the cytoplasm leading to the formation of ROS as superoxide ion and hydroxyl anion and DA quinone. (fromLotharius and Brundin 2002).

suggesting that these neurons may not be able to buffer the ROS (*6*). All these findings imply that dopaminergic neurons are under oxidative stress in PD, and that the ability of dopaminergic neurons to scavenge free radicals and ROS is compromised. Taken together, mitochondrial dysfunction and oxidative stress are likely key factors that are responsible for the loss of SNpc neurons in PD.

Mitochondria and the Selective Vulnerability of SNpc Dopaminergic Neurons

It is currently unknown why SNpc neurons selectively degenerate in PD. While many factors may ultimately contribute to the degeneration of these neurons, there appear to be two final common pathways that eventually result in neuronal demise. First, the mitochondrial-stimulated programmed cell death pathway is predominantly activated in the SN of patients with PD (*7, 8*). Secondly, aggregation of misfolded proteins in the Lewy bodies is likely a contributing factor. Nonetheless, the factors that render SNpc neurons vulnerable likely lie upstream of these two pathological outcomes, since the mitochondrial-stimulated apoptotic pathway is conserved among all the cells and cannot account for the specific loss of SNpc dopaminergic neurons. Secondly, despite many studies, it remains unclear whether Lewy bodies are toxic to or protective for dopaminergic neurons. Postmortem studies of PD brains reveal high oxidative damage and mitochondrial dysfunction with decreased complex I activity (*3*). Whether mitochondrial dysfunction is a cause or consequence of the oxidative stress in PD remains to be elucidated. However, neurotoxicity studies in animals suggest that mitochondrial dysfunction and oxidative stress may be responsible for the selective loss of SNpc neurons. Following exposure to the mitochondrial complex I inhibitors, MPTP or rotenone, which affect many different neuronal populations, there is a selective loss of SNpc DA neurons (*9, 10*). ATP depletion and increased production of ROS due to leakage of electrons at the level of complex I is the mechanism proposed for these toxins (*8, 11*). Therefore, the selective vulnerability and loss of SNpc DA neurons in PD likely involves a bioenergetic failure and increased exposure to ROS.

It has been hypothesized that the reason SNpc neurons are vulnerable in PD is due to the toxic properties of the DA itself. DA is a highly reactive molecule, normally stored in synaptic vesicles. The acidic environment of the synaptic vesicle prevents DA oxidation. However, DA is synthesized and metabolized in the cytoplasm and cytoplasmic DA can undergo to spontaneous oxidation. DA oxidation gives rise to several toxic ROS among these super-oxide anions (O_2^-), hydroxyl radicals ($OH^\bullet$), and the dopamine quinone molecules (DAQs) (*12*). Increasing cytosolic dopamine in neurons and its oxidized metabolites has many deleterious effects, including increases in oxidative stress, mitochondrial dysfunction, as well as the promotion and stabilization of a potentially toxic protein called α-synuclein (*13, 14*). Therefore, the selective loss of SNpc DA neurons in PD may result from increased oxidation of cytoplasmic DA and the oxidative stress induced by ROS-mediated oxidative damage to the neurons.

The overall aim of the research that this dissertation comprises is to identify the mechanism that contributes to the selective degeneration of dopaminergic neurons in PD. The research described herein utilizes *in vitro* and cellular model approaches to test the hypothesis that the oxidized products of the neurotransmitter DA induce toxic effects on mitochondria. If this hypothesis is correct any pathological event that impairs DA synthesis, storage or metabolism, leading to the cytoplasmic accumulation of DA can increase the presence of oxidized products of DA and their toxic effects in the cell with mitochondria as one of the target. Mitochondrial dysfunctions derived from exposure to DA-oxidized products can account for the specific vulnerability of dopaminergic neurons and their degeneration in PD.

Mitochondrial dysfunction in PD

Environmental Toxins of complex I: MPTP and rotenone

Mitochondria were first implicated in PD in the 1980's when several designer-drug abusers developed an acute and irreversible parkinsonian syndrome

as result of accidental exposure to the environmental toxin 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) (*10*). MPTP is able to cross the blood-brain barrier, and is converted to the active metabolite N-methyl-4-phenylpyridinium ion (MPP^+) by MAO-B in the glial cells. MPP^+ is released from glial cell by an unknown mechanism and, due to its affinity for the dopamine transporter (DAT), accumulates into dopaminergic neurons. Once in the neurons, MPP^+ binds to mitochondrial complex I, inhibiting its activity and increasing ROS production by mitochondria (*8*). The bioenergetic deficit and ROS formation lead to the death of the SNpc DA neurons.

Chronic administration of the highly selective complex I inhibitor rotenone in rats induce a PD-like syndrome characterized by SNpc neuronal degeneration, protein accumulation similar to Lewy bodies, and characteristic motor impairments (*9*). Rotenone is a naturally occurring mitochondrial complex I inhibitor developed for use as an insecticide. Rotenone is highly lipophilic and can easily cross the blood-brain barrier and gain access to all cells. Following systemic injection, rotenone is found evenly distributed throughout the brain (*9*). Once rotenone enters cells, it accumulates in mitochondria, where it binds to complex I and inhibits the transfer of electrons, thus inhibiting mitochondrial respiration and ATP production. Additionally, binding to complex I inhibits the transfer of electrons and promote the generation of super oxide ion and other ROS (*15*).

Cellular toxicity following rotenone is due to ROS generation and ATP depletion. Despite the ability of rotenone to enter all cell types, chronic intravenous administration of the drug produces a relatively selective degeneration of dopaminergic neurons and also produces intraneuronal inclusions similar to Lewy-bodies (*9*). These results suggest that the primary pathological hallmarks of PD, the unique vulnerability of dopaminergic neurons and Lewy-body formation, can be produced as a result of systemic inhibition of mitochondrial complex I. Also, the chronic nature of dopaminergic cell loss induced by rotenone more accurately reflects the progressive nature of PD since it causes motor dysfunctions including abnormal animal posture and decreased spontaneous movement.

Role of mitochondrial DNA in PD

Several lines of evidence point to a pathological role of the mitochondrial DNA (mtDNA) itself in PD. mtDNA encodes 13 proteins, all of which are components of the electron transport chain (ETC). To date, no specific mutations in mtDNA have been found to be associated with PD, even though two mutations have been associated with parkinsonism: a point mutation in 12S rRNA was found in a family with parkinsonism, deafness, and neuropathy (*16*), and the G11778A mutation in the mtDNA encoding the ND4 subunit of complex I was found in a family with parkinsonism associated with Leber's optic neuropathy (*17*). Recently, mutations in the nuclear encoded gene, DNA polymerase γ (POLG), which is responsible for mtDNA replication, were reported in families with parkinsonism associated with progressive external ophthalmoplegia and multiple mtDNA deletions (*18*). Finally, specific mtDNA polymorphisms or haplotypes have been correlated with PD. Human mtDNA exhibits region-specific variations in indigenous populations. Haplotypes J and K were found to reduce the incidence of the disease by 50% in patients of European ancestry and the haplotype cluster UKJT also exhibited a 22% decrease in risk of PD. Both these haplotypes are associated with mild uncoupling of mitochondria, allowing adaptation to colder climate, increasing heat generation and reducing ROS production (*19*).

PD genes and Parkinson's disease

Recently several rare forms of inherited PD have been recognized. Inherited mutations in at least five genes associated with familial parkinsonism demonstrated that mutations in a single gene product could lead to SN neurons degeneration and clinical manifestation of parkinsonism. Studies on the cellular and physiological functions of these gene products has helped elucidate the pathogenic mechanism underlying the selective degeneration of dopaminergic neurons in PD. Interestingly, at least three of the genes associated with the familial form of PD —parkin, DJ-1, and PINK1— have been linked to mitochondria and oxidative stress-related survival pathways.

Even if such mutations account for a minority of cases of PD, it is reasonable to think that a similar mechanism might underlie the inherited and sporadic forms of the disease. A common site of action for these mutated gene products seems to be the mitochondria. A description of the genes associated with parkinsonism and their involvement in mitochondria pathophisyiology is presented below.

Parkin

The *parkin* gene encodes a 465 amino acid protein containing an N-terminal ubiquitin-like domain, a central linker region, and a C-terminus containing two Really Interesting New Gene (RING) domains separated by an in-between RING domain (*20*). Mammalian parkin is primarily localized in the cytoplasm of post-mitotic cells, but a fraction appears to associate with the outer mitochondrial membrane (*21*). In proliferating cells, parkin localizes to mitochondria and enhances mitochondrial biogenesis (*22*). Parkin functions as an E3 ubiquitin protein ligase by targeting misfolded proteins to the ubiquitin proteasome pathway for degradation (*23*). Mutations in the *parkin* gene are a major cause of autosomal recessive early-onset PD (*20, 23, 24*). The most accepted pathogenic mechanism of action for parkin mutations is that mutated parkin is no longer able to degrade its unique subset of target proteins, and these proteins subsequently accumulate in neurons. However, parkin-critical substrates remain to be determined. Recently it has been suggested that parkin is a neuroprotective protein crucial for neuronal survival (*25*). Consistent with this, parkin over-expression is protective in several cellular models of apoptosis (*21, 26, 27*). It has been reported that post-translational modification of parkin, due to covalent modification by dopamine quinones, impairs its ubiquitin E3 ligase activity and compromises its protective function (*28*). Analysis of *Drosophila* mutants that are deficient in an orthologue of parkin reveal striking mitochondrial pathology (*29*). Wild-type parkin also prevents mitochondrial swelling, cytochrome c release and caspase activation in response to cytotoxic insults, and these functions are abrogated when parkin is mutated (*21*). In flies, age-dependent dopaminergic neurodegeneration and motor impairments are observed due to expression of human mutant parkin but not wild-type parkin,

suggesting a toxic gain-of-function mechanism (*30*). Consistent with this, parkin knockout mice display little or no alterations in dopaminergic neurons (*31-33*).

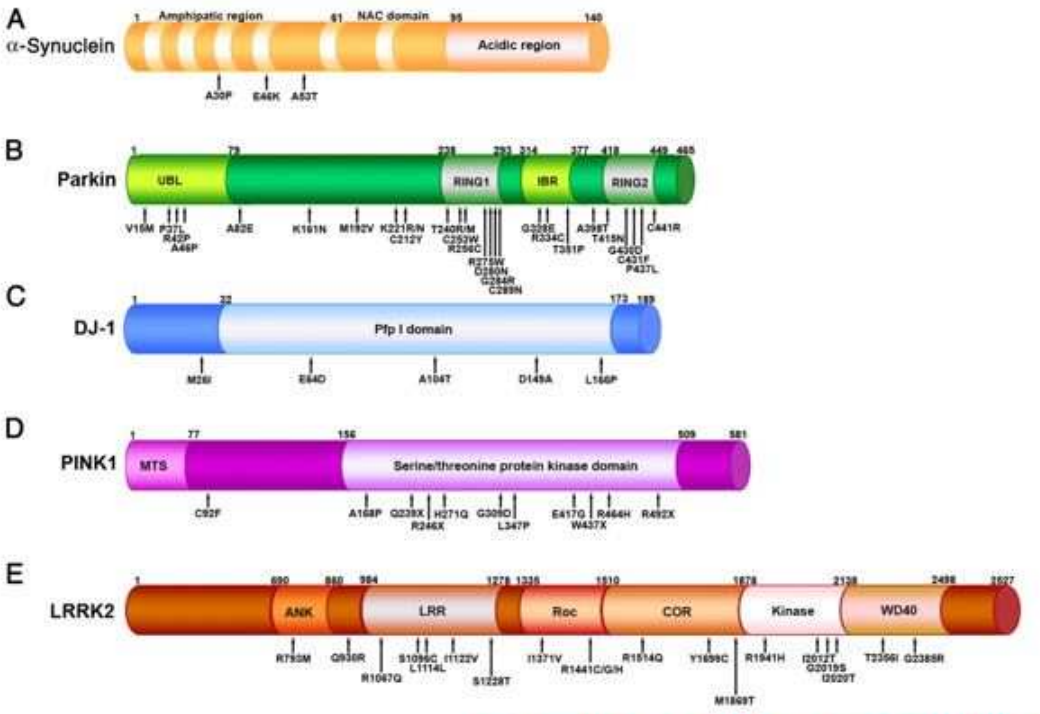

Figure 3 Models of human PD-associated proteins.

(A) -Synuclein is a 140 amino acid protein belonging to a family of related synuclein that includes - and -synuclein. It has an N-terminal amphipatic region containing six imperfect repeats with a KTKEGV consensus sequence, a hydrophobic central region that contains the non-amyloid- component (NAC) domain, and a highly acidic C-terminal tail containing several phosphorylation sites. (B) Parkin is a 465 amino acid protein that functions as an E3 ubiquitin ligase. It contains an N-terminal ubiquitin-like (UBL) domain that binds to RPN10 subunit of the 26S proteasome system, a central linker region, and a C-terminal RING domain comprising two RING finger motifs (RING1 and RING2) separated by an in-between-RING (IBR) domain. (C) DJ-1 is a highly conserved 189 amino acid protein that is ubiquitously and abundantly expressed in most mammalian tissues and belongs to the DJ-1/ThiJ/PfpI superfamily. (D) PINK1 is a highly conserved 581 amino acid protein that is ubiquitously expressed. It localizes to the mitochondria via an N-terminal mitochondrion-targeting motif (MTS). Furthermore, it shares sequence similarity with Ca^{2+}/calmodulin-dependent protein kinase I and contains a catalytic serine/threonine kinase domain. (E) LRRK2 is a 2537 amino acid complex multi-domain protein that consists of a ankyrin-repeat region (ANK), an N-terminal leucine-rich repeat domain (LRR), a GTPase Roc domain (Roc) followed by associated C terminal of Roc (COR), a mitogen-activated kinase kinase kinase domain, and C-terminal WD40 repeat (approximately 40 amino acid repeats that form a -propeller structure that might serve as a rigid scaffold for protein interactions). Approximate positions of missense mutations causing PD are indicated with arrows. (from Mandemarks W. et al., *J. of Cell Sci.*2007)

DJ-1

Loss-of-function mutations in the DJ-1 locus are associated with rare forms of autosomal recessive early-onset parkinsonism (*34*). DJ-1 is a homodimeric, highly conserved protein of 189 amino acids, ubiquitously expressed in a variety of mammalian tissues including brain (*35*). Familial PD-linked mutations in DJ-1 are considered to cause dopaminergic neuron degeneration through loss-of-function mechanism consistent with a recessive inheritance. Many lines of evidence suggest that DJ-1 functions as an antioxidant protein. Oxidative stress leads to an acidic shift of DJ-1's isoelectric point by oxidation of its Cys106 residue (*36*). Also, given its propensity to undergo auto-oxidation to eliminate H_2O_2, it may function as a scavenger of reactive oxygen species (ROS) (*36, 37*). Mouse models lacking DJ-1 developed age-dependent motor deficits, hypokinesia and dopaminergic dysfunction with no neuronal loss (*38, 39*). Nigrostriatal dopaminergic neurons in these mice show increased vulnerability to the parkinsonian toxin MPTP via an unknown mechanism (*40*). Consistent with these findings DJ-1 over expression appears protective against a number of oxidative toxic insults (*36*). DJ1 mitochondrial localization (*41*) and hypersensitivity of DJ-1 KO mice to mitochondrial toxins like MPTP (*40*) provides substantial evidence on its role in mitochondrial and oxidative stress-mediated neurodegeneration. Moreover a link to age-dependence in sporadic PD is further supported by oxidative inactivation of DJ-1 due to aging in flies (*42*).

PINK1

Discovery of PINK1, a mitochondrial serine/threonine kinase, mutated in some rare familial forms of PD, provided a clear link between mitochondrial dysfunction and neurodegeneration in PD. Further analysis confirmed that PINK1 protein accumulates within the intermembrane space of mitochondria (*43*). *In vitro* studies suggest that over expression of wild type PINK1 can prevent mitochondrial cytochrome c release and subsequent apoptosis and this function is abolished in familial PD-linked PINK1 mutants (*44*). This is consistent with

increased vulnerability of dopaminergic SH-SY5Y cells to the mitochondrial toxins rotenone and MPTP following PINK1 suppression by siRNA (*45*) or due to expression of PINK1 mutants (*46*). *In vivo* loss of PINK1 function due to its inactivation by siRNA or expression of PINK1 mutants leads to muscle and dopaminergic neuron degeneration as a consequence of mitochondrial dysfunction in flies (*47*). Interestingly, this phenotype was rescued by over expression of parkin. These data strongly suggest a genetic pathway with parkin functioning downstream PINK1 and implicate both parkin and PINK1 in the regulation of mitochondrial physiology and survival in flies (*47-49*). Mutations at the phosphorylation sites of the mitochondrial protease HtrA2/Omi have been found in sporadic forms of PD moreover it has been shown in vitro the PINK ability to phosphorylate HtrA2/Omi (*50*), and is tempting to speculate the presence of PINK1 and HtrA2/Omi in the same survival pathway and that its impairment leads to PD.

α-Synuclein

α-synuclein is a natively unfolded presynaptic protein of ~14-kDa that is believed to play a role in synaptic vesicles recycling, storage, and compartmentalization of neurotransmitters through its association with vesicular and membranous structures (*51-53*). Structurally, α-synuclein consists of an N-terminal amphipathic region, a hydrophobic middle region (containing a non-amyloid-β component domain) and an acidic C-terminal region. Three missense mutations in *α-synuclein* gene (A53T, A30P and E46K) as well as genomic triplication of the α-synuclein gene region are associated with autosomal dominant PD (*54-57*). α-synuclein has a propensity to aggregate due to its hydrophobic non-amyloid-β component domain. The presence of fibrillar α-synuclein as a major structural component of Lewy bodies in PD suggests a role of aggregated α-synuclein in PD pathogenesis (*58*). Presently it is unclear whether accumulation of misfolded proteins that lead to LB-like inclusions are toxic or protective in PD (*59*). Mechanisms by which abnormal accumulation of α-

synuclein disrupts basic cellular functions and leads to dopaminergic neurodegeneration are being intensely studied. One of the earliest defects following α-synuclein accumulation *in vivo* is blockade of endoplasmic reticulum (ER) to golgi vesicular trafficking leading to ER stress (*60*). The link between α-synuclein and mitochondria is still unclear, however, several lines of evidence suggest that the protein can affect mitochondrial function. Due to the ability of α-synuclein to modulate synaptic vesicle formation and neurotransmitter storage it has been suggested that α-synuclein can mediate levels of cytoplasmic DA, and thus the rate of DA oxidation and ROS formation. Thus, α-synuclein may indirectly affect mitochondrial function through ROS formation in SNpc neurons. Additionally, administration of MPTP to mice over-expressing α-synuclein produces enlarged and deformed mitochondria that are not observed in the control mice (*61*). In addition, a recent study demonstrates that mice expressing human A53T α-synuclein developed mitochondrial pathology and mitochondrial dysfunction suggesting a crucial role for α-synuclein in modulating mitochondrial functions in neurodegeneration (*62*). Conversely, mice lacking α-synuclein are resistant to mitochondrial toxins like MPTP, 3-nitropropionic acid and malonate (*63*). Another mechanism by which mutant α-synuclein can induce neuronal toxicity is by increasing cytosolic catecholamine concentrations, which in turn leads to increased levels of oxidized DA metabolites (*64*, *65*).

LRRK2

Mutations in the leucin-rich repeat kinase 2 (LRRK2) cause autosomal dominant PD (*66, 67*). LRRK2 encodes a 2527 amino acids multidomain of 280KDa protein belonging to the ROCO protein family and encompasses a Rho/Ras-like GTPase domain, protein kinase domain of the MAPKKK family as well as a WD40 repeat domain and leuchine-rich repeat domain (*66, 67*). An additional domain C-terminal to the GTPase domain termed COR (carboxy-terminal of Ras) is of unknown function. Point mutations have been identified in almost all of the known domains and a single mis-sense allele of LRRK2, 2019S may be associated with 1-2% of apparently “sporadic” PD cases (*68-70*). Kinase

activity appears to be required for the toxicity of 2019S LRRK2 in tissue culture cell lines (*71*). The cellular and molecular mechanisms of LRRK2 toxicity remain to described, however a role for LRRK2 as a modulator in a mitochondrial-dependent cell death pathway has been suggested (*66, 72, 73*).

Taken together, these accumulating evidence derived from the toxic and genetic models of PD provide a strong link between mitochondrial dysfunction and oxidative stress in PD pathogenesis and for the selective vulnerability of SNpc dopaminergic neurons. It has been for long hypothesized that vulnerability of dopaminergic neurons to ROS-mediated oxidative damage is likely due to the presence of DA itself. For this reason is compelling to understand the regulation of cytoplasmic DA levels such as synthesis, release and metabolism in dopaminergic neurons, since deregulation in these critical steps can play a critical role in the dopaminergic neuron loss in PD.

DA synthesis and metabolism

DA is synthesized in the cytoplasm from the amino acid tyrosine. Dietary tyrosine is taken up into axon terminals by the large neutral amino-acid transporter (LNAA) and converted to L-DOPA by the cytoplasmic enzyme tyrosine hydroxylase (TH) in the rate-limiting step of catecholamine synthesis (*74*). L-DOPA is decarboxylated to DA by L-amino-acid decarboxylase (AADC) (*74*). Newly synthesized DA is rapidly packaged into synaptic vesicles by the vesicular monoamine transporter-2 (VMAT2) in a process ATP-dependent. Additionally, newly synthesized DA that is not packed into vesicles can be degraded to dihydroxyphenylacetic acid (DOPAC) through a two-step reaction catalyzed by mitochondrial monoamine oxidase (MAO) and aldehyde dehydrogenase (AD). Cytoplasmic DA inhibits the activity of TH via end-product inhibition (*75*).

Action potential-induced Ca^{2+} entry into the axon terminal triggers the release of vesicular DA into the synaptic cleft. DA released into the synaptic cleft can bind to either postsynaptic D1 and D2 receptors, or the presynaptic D2 auto-

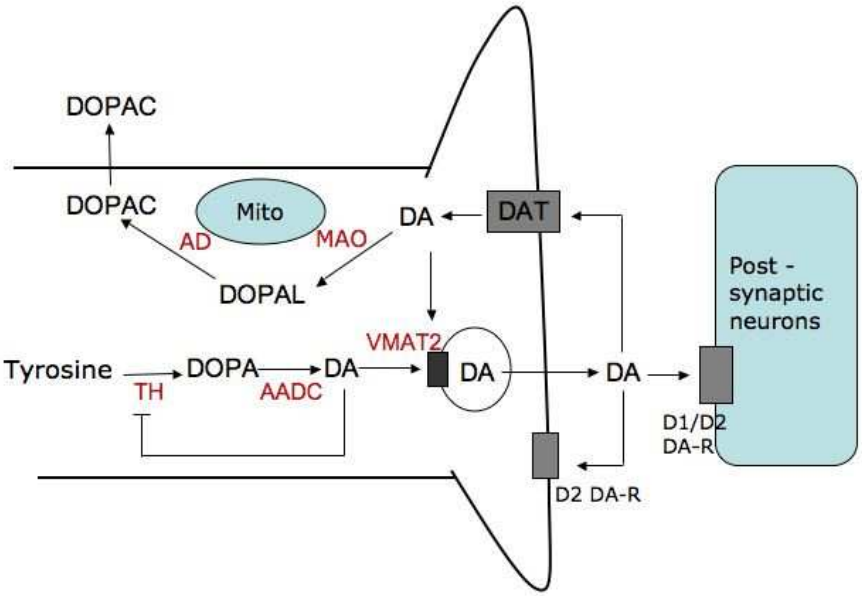

Figure 4 Schematic diagram of DA synthesis and metabolism in dopaminergic neurons.

Tyrosine is taken up into the axon terminal by the large neutral amino-acid transporter (LNAA) and converted to DOPA by tyrosine hydroxylase (TH). DOPA is decarboxylated to DA by L-aromatic amino-acid decarboxylase (AADC). Newly synthesized DA is packaged by vesicular monoamine transporter-2 (VMAT2) into synaptic vesicles for release. Alternatively, newly synthesized DA can be degraded by mitochondrial (Mito) MAO or can feedback to inhibit TH activity. DA released into the synapse can bind to post-synaptic D1 or D2 receptors (DA-R) or D2 auto-receptors (D2-R). Synaptic DA is removed via the dopamine transporter (DAT). Once again in the cytoplasm, DA can be repackaged into synaptic vesicles or metabolized by MAO and aldehyde dehydrogenase (AD) to DOPAC. DOPAC is removed from the axon terminal through diffusion.

receptor. Activation of presynaptic D2 auto receptors inhibits further DA synthesis. Synaptic DA is removed via the high affinity reuptake DA transporter (DAT) that recaptures a vast majority of synaptic DA (*76*). Recaptured DA can either be repackaged into synaptic vesicles for reuse, or metabolized to DOPAC by mitochondrial MAO and AD (*77*). Cytosolic DA can also be imported into lysosomes where it can auto-oxidize spontaneously to form the polymer neuromelanin (*77*). A loss of regulation of DA metabolism or storage can lead to an accumulation of DA in the cytoplasm. DA can give rise toxic species during its normal metabolism and/or if DA accumulates in the cytoplasm. For these reasons, the synthesis, storage, release, reuptake, and metabolism of DA are tightly regulated in dopaminergic neurons.

Intraneuronal oxidation of dopamine

DA metabolism by MAO-B and AD results in the formation of DOPAC and hydrogen peroxide (H_2O_2) (**Figure 4**). H_2O_2 can be scavenged by GSH or react with Fe^{2+} to form hydroxyl radical ($OH^{\bullet}$) by Fenton reaction. The MAO-B product DOPAL is a highly toxic metabolite (*78*) and mitochondrial impairment or AD inhibition can lead to its accumulation in dopaminergic neurons (*78*). Noteworthy in the context of PD is the fact that mitochondrial complex I is essential for DA metabolism since the byproduct of complex I (NAD^+), is a co-factors of AD. Impairment in complex I activity inhibit DA metabolism leading to an increase in cytosolic DA.

Cytosolic DA can undergo spontaneous or enzymatic metabolic pathways that give rise to cytotoxic molecules. This reaction leads to the formation of several cytotoxic reactive oxygen species (ROS): super oxide anions (O_2^-),

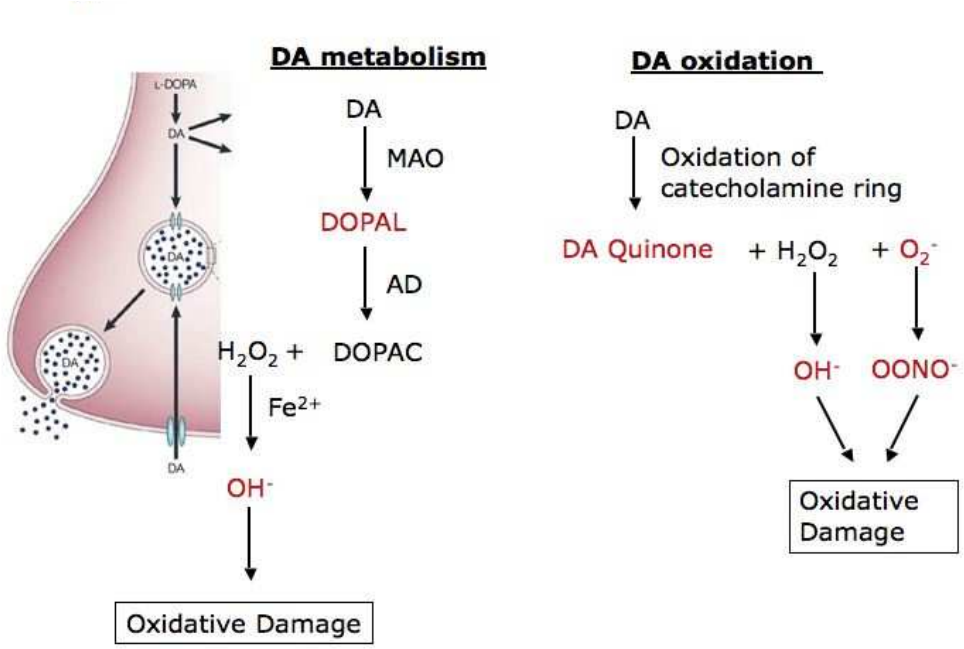

Figure 5 Byproducts of DA metabolism or DA oxidation are toxic for the neuron

DA enzymatic (left) and non-enzymatic (right) metabolic pathways. The enzymatic metabolism of DA results in the formation of DOPAC and a hydrogen peroxide (H_2O_2) molecule. H_2O_2 can be scavenged by glutathione (GSH) or react with iron to form hydroxyl radical (OH^-) through the Fenton reaction. DA auto-oxidation results in the formation of DA-quinone molecules and the reactive oxygen species H_2O_2 and superoxide (O_2^-). H_2O_2 can produce OH^- while O_2^- can react with nitric oxide to form peroxynitrite ($OONO^-$). ROS cause oxidative damage by reacting with lipids, DNA and proteins, and may ultimately result in neuronal death.

hydroxyl radicals ($OH^{\bullet}$), and dopamine quinones (DAQs)(*12*). This reaction is spontaneous in the cytoplasm and is enhanced under oxidative conditions (*79*) and in the presence of transition metals (*80*). Several enzymes can catalyze DAQ synthesis, however the sub cellular location and the extent to which these enzymes mediate this oxidative reaction are difficult to estimate. The enzyme tyrosinase can catalyze the oxidation of DA to DA-o-quinone (DQ) and from leukoaminochrome to aminochrome (AC) (*81, 82*), and tyrosinase's presence in brain tissues has been recently reported (*82*). Others enzymes present in the CNS also have the ability to produce these toxic quinones. The ubiquitous enzyme prostaglandin H synthase has been shown to oxidize DA to DA quinones via the peroxidase activity of the enzyme (*83*). Lipoxygenase and xanthine oxidase have also been shown to oxidize DA in presence of hydrogen peroxide (*84, 85*).

The toxicity of DAQs is related to their ability to react and covalently modify cellular molecules and macromolecules. Electrophilic DAQs have high affinity for free cysteine, reduced glutathione (GSH) and sulfhydryl groups of proteins to form cysteinyl-catechol conjugates (*85, 86*). Reduced glutathione (GSH) is the primary antioxidant defense against ROS-induced oxidative damage in the neurons. The antioxidant defense capacity of dopaminergic neurons can be overwhelmed by ROS and can also be impaired by depletion of GSH by DAQs. Interestingly, GSH levels within dopaminergic neurons are depleted in PD patients, suggesting that their ability to manage ROS is compromised in these cells (*87*). Free cysteinyl-catechol and GSH-catechol derivatives have been detected *in vivo* and are increased in the *sustantia nigra* of post-mortem brain from PD patients (*88*). In addition to a decrease in cellular antioxidant defenses, derivatives of GSH-DA conjugates have been found to be toxic to mitochondria (*89, 90*).

DAQs have been shown to bind covalently to protein cysteinyl residues *in vitro* and *in vivo* leading to the formation of 5-cysteyl-catechols (*12, 13, 83, 91, 92*). Sulfhydryl groups of cysteine are often found at the active site of proteins, and covalent modification by DAQs can lead to inhibition of protein function with toxic consequences for the cell (*92*).

So far it has been shown that, in the presence of tyrosinase, DA covalently modifies and inactivates TH (*93*), DAT (*94*), glutamate transporter (*95*) and

parkin (*28*). Cytosolic quinones can also enter the nucleus and induce DNA base modifications (*96*). Important for the pathogenesis of PD, DAQs can bind α-synuclein and stabilize protofibrils of α-synuclein (α-syn), which are hypothesized to be the toxic intermediates of α-syn fibril formation (*97*).

Statement of Purpose

The reasons for the specific loss of dopaminergic neurons in PD remain obscure. Based on the anatomy of this neurodegeneration, a toxic role for the neurotransmitter DA has been long hypothesized. DA in the cytoplasm can undergo spontaneous or enzyme-catalyzed oxidative reactions that give rise to several reactive toxic species including super oxide anion ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$) and reactive quinones (DAQs). For this reason the synthesis, storage, release, and metabolism of DA are tightly regulated in dopaminergic neurons. Impairments in DA storage or metabolism can lead to accumulation of DA in the cytoplasm with subsequent formation of toxic species. If ROS production exceeds the scavenging power of the cellular defense system, oxidative stress and oxidative damage can ensue. Additionally, the oxidized products of DA have been shown to bind and covalently modify proteins at the level of cysteinyl residues leading to impairment of protein function and consequent cellular damage. These products have been found *in vivo* and are increased in PD brains compared to age-matched control brains. Impaired DA storage resulting from α-synuclein mutations has been reported and this mechanism may contribute to the pathogenesis of some rare forms of PD.

The presence of these toxic reactive species in the cytoplasm of dopaminergic neurons can lead to several detrimental effects. The effect of the oxidized products of DA on mitochondria was chosen as the subject of the research that this dissertation comprises. The hypothesis that DAQs impaired mitochondrial functions was tested *in vitro* and cellular models systems. These toxic effects may contribute to the neurodegeneration of dopaminergic loss in PD and account for the specific vulnerability of these cells to oxidative damage.

Materials and Methods

Mitochondria Isolation

Rat brain mitochondria isolation

Brain mitochondria were prepared as described in Zoccarato *et al*. (2004) (*98*). Specifically, the cerebral cortices of two 6–7-week-old male Sprague-Dawley rats (300-350 g) were rapidly removed into 20 ml of ice-cold isolation medium (320 mM sucrose, 5 mM MOPS, and 0.05 mM EGTA, pH 7.4) and homogenized. The homogenate was centrifuged at 900 x *g* for 5 min at 4 °C. The supernatant was centrifuged at 8500 x *g* for 10 min, and the resulting pellet was resuspended in 1 ml of isolation medium. This was layered on a discontinuous gradient consisting of 4 ml of 6% Ficoll, 1.5 ml of 9% Ficoll, and 4 ml of 12% Ficoll (all prepared in isolation medium) and centrifuged at 75,000 x *g* for 30 min. The myelin, synaptosomal, and free mitochondrial fractions formed above the 6% layer, as a doublet within the 9% layer, and as a pellet, respectively. The pellet was resuspended in 250 mM sucrose and 10 mM K-MOPS, pH 7.2, and centrifuged at 8500 x *g* for 15 min before being resuspended in this last medium to 10–20 mg of protein/ml by the Gornall protein assay. Prior to the initiation of every experiment, respiration rates of the mitochondrial preparation were determined, and mitochondria were used for these studies when the ratio of state 3 respiration to state 4 respiration was determined to be between 3.5 and 7.0, signifying healthy, well-coupled mitochondria. with glutamate and malate as substrates.

Mouse liver mitochondria isolation

Liver mitochondria were prepared from liver homogenates by differential centrifugation as described in Costantini *et al*. (1995) (*99*). Specifically liver was readily removed from a male Winstar albino mice and placed in 50ml of ice-cold isolation medium containing (320 mM sucrose, 5 mM Na-HEPES and 0.05 mM EDTA, pH 7.4), and homogenized. The homogenized was transferred in two 50ml centrifuge tubes, balanced with ice-cold isolation medium and centrifuged at 700 x g for 6 minutes at 4 °C. The supernatant was transferred in two new tubes and centrifuged at 7000 x g for 6 minutes. The supernatant was discarded and the pellet re-suspended in 30ml of ice-cold isolation medium and centrifuged again at 7000 x g for 6 minutes. The pellet from this last centrifuged was gently re-suspended in 0.5-1ml of isolation buffer and the mg protein/ml was assessed by the biuret protein assay. The mitochondria preparation was keep at 4 °C and the respiration ratio was measured to assess the respiration control.

Biuret protein assay

In alkaline solutions ion copper (Cu^{2+}) interacts with the peptide bonds giving a "blue" complex. The amount of Cu^{2+}-proteins complex is estimated spectrophotometrically at 540nm and used to calculate the mg of mitochondrial proteins/ml of every mitochondrial preparation. In a 3ml cuvette were added 0.5ml deoxycholate (DOC) 1% and sub sequentially 20µl of mitochondria, 1ml mQ-H_2O and 1.5ml biuret solution. The blank is a cuvette containing all the reagents except the mitochondria. The cuvette were let warm in a beaker containing 70-80 °C water for 3 minutes and cool down to room temperature on ice. The absorbance of the sample respect to the blank was measured in a Perkin-Elmer Life Sciences 650-40 spectrophotometer at the wavelength of 540nm. The mg/ml concentration was calculated based on a standard curve made with increasing amount of bovine serum albumin (BSA) and freshly prepared for every new biuret solution. The biuret solution contains: for 500ml of solution, 4.5gr of Sodium Potassium Tartarate, 1.5gr of copper sulphate pentahydrate, 2.5gr

potassium iodide were dissolved in 200ml of 0.2M NaOH. The final volume of 500ml was reached adding distilled H_2O. The solution was kept at room temperature.

Measurements on Isolated Mitochondria

Mitochondrial respiration analysis

Mitochondria oxidized substrates in the matrix to extract electrons that flux through the four complexes of the mitochondrial electronic transport chain (ETC). The electron passage in the ETC ends with the utilization of these

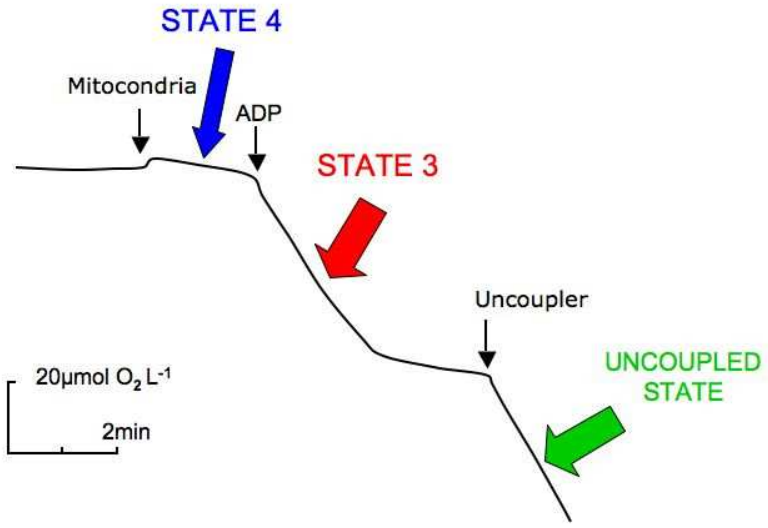

Figure 6 The three state of mitochondrial respiration in oxygraphic measurements

Mitochondrial respiration is assessed polarographically with a Clark oxygen electrode. To a medium containing oxidizing substrates for the ETC the mitochondria are added and the State 4 of the respiration, or resting state is measured. State 4 is defined as the respiration not coupled with ATP synthasys and it estimates the physiological state of the first four complex of the oxidative phosphorylation chain (OXPHOS). If ADP is added in the chamber the ETC ctivity is coupled with the ATP synthesis by the fifth complex of the OXPHOS, the F_1F_O ATP synthase complex. F_1F_O ATP synthase complex employ the proton gradient built by the ETC during the electron flux throught the first four complexes to synthesize ATP from ADP. Addition of a toxin that is able to dissipate the proton gradient lead to the uncoupling of the ETC from the complex V activity and ATP synthesis, an this state is called Uncoupled state where the oxygen consume is the highest. State 3/State 4 is called respiratory control ratio and for well-coupled mitochondria is between 5 and 12.

electrons by the complex IV to reduce molecular oxygen, O_2, to water. Measure of oxygen consumption by the mitochondria is used as index of the physiological state of the ETC of the mitochondrial preparation.

Oxygen consumption was measured polarographically with a Clark oxygen electrode in a closed 2-ml vessel equipped with magnetic stirring and thermostated at 25 °C. Experiments were started with the addition of 1mg mg mitochondria in 2 ml of respiration buffer containing 0.25 M sucrose, 10 mM Tris-MOPS, 1 mM Pi-Tris, 10 µM EGTA-Tris, pH 7.4. Two solutions were employed containing different oxidizing substrates for the first two complexes of the mitochondrial ETC. For complex I: 5 mM glutamate/2.5 mM malate were added to the medium, for complex II: 5 mM succinate-Tris as substrate for complex II plus 2 µM rotenone to completely inhibit complex I to avoid backward electron flux from complex II to complex I in the ETC, were added. Two distinct chambers were used for the two solutions. The measurement was started and the linear part of this trace (at least 200sec) was used to assess the State 4 (**Figure 6**) of the mitochondrial preparation. 100µM ADP (stock solution 0.1M) was added into the chamber with a Hamilton ™ syringe avoiding air bubbles. The measure was continuing until the trace returned parallel to the initial State 4. With addition of ADP the respiration is coupled with the phosphorylation of ADP to ATP by the last complex of the ETC, the F_1F_O ATPsynthase complex, or complex V. This respiration state is conventionally called State 3 and the State 3/State 4 ratio is used as index of the healthy state of the mitochondria in the preparation. Well-coupled and healthy mitochondria show a respiratory control State 3/State 4 between 5 and 12.

The maximal rate of oxidative phosphorylation (V_{ADP}) was measured after the addition in the chamber of 25µM 2,4-Dinitrophenol (DNP) to measure the uncoupled state. DNP is a molecule that dissipates the proton gradient, $\Delta\Psi$, built across the IMM between the matrix and the inter-membrane space by the complex I, II and IV of the ETC. In this state the electron flux among the four complexes of the ETC is no more coupled with the ATP production and the rate of oxygen consumption by the complex IV is the highest since the ETC try to restore the dissipated $\Delta\Psi$. Data are recorded as mV during the time of the experiment.

Data were analyzed as follow: the ngAt of molecular oxygen per mg of protein (ngAtO/mg) corresponding to 1mV in the oxyraph was calculated. The

average of all the initial values was calculated and the function (*function 1*), 1mV= [(485ng/ml) x ml in the chamber] / [average of starting point (mV) x mg of protein] was used. 485ngAtO/ml correspond to O_2 solubility at 25 °C. Titration was 485ngAtO/ml ~200mV in the oxygraph. For every sample the results were expressed in 485 ngAtO/ml multiplying the values expressed in mV for the results of the *function 1*. The slope of the curve in the linear part for all the three state was calculated and the results were expressed as 485ngAtO/[ml x min]. The program *Origin* ™ was used for the analysis of the data and the data are results of triplicate experiments.

Mitochondrial swelling

Mitochondrial swelling was followed as the change of light scattering of the mitochondrial suspension at 620 nm with a Perkin-Elmer Life Sciences 650-40 fluorescence spectrophotometer equipped with magnetic stirring and thermostatic control. Mitochondrial swelling is a consequence of the opening of the mitochondrial permeability transition pore (mPTP). The mPTP is a proteinaceous pore that is formed in the inner membranes of mitochondria (IMM) under certain pathological conditions such as traumatic brain injury and stroke. Permeability transition (PT) is an increase in the permeability of the mitochondrial membranes to molecules of less than 1500 Daltons in molecular weight and lead to mitochondrial swelling, the disruption of the IMM structure and the release in the medium of the pro-apoptotic factors normally located in the inter-membrane space. Even if are not known so far the structure and the components of the mPTP, nonetheless are well characterized conditions and molecules that induce the PT or inhibit it (**Figure 7**). Noteworthy is the ability of Ca^{2+} to induce PT and the molecule cyclosporine A (CsA) as one of the most powerful inhibitor of the mPTP.

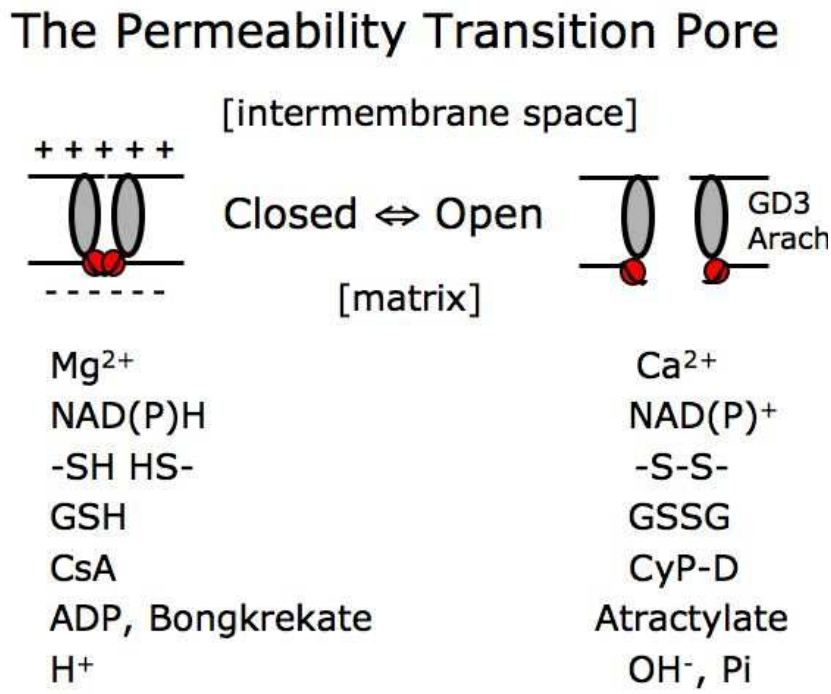

Figure 7 Conditions that induce or inhibit the PT.

Even if the structure of the mPTP has not been defined yet, several conditions and molecules are known to modulate mPTP conformation as inducer or inhibitors. A general reduced environment favorites the close conformation (-SH SH-, GSH) instead an oxidizing environment (-S-S-S, GSSG) promote opening of the mPTP. Noteworthy is the action of the molecule CsA, a specific and powerfull inhibitor of the PT, due to its interaction with the protein cyclophyllin D. Cyclophyllin D is so far the only PT modulator identified for sure. The ability of calcium ions to induce PT is also well characterized. Mitochondria are able to import calcium into the matrix by the Ca^{2+} uniporter and this is one of the cellular way to buffer cytoplasmic amount of Ca^{2+}. When Ca^{2+} reach a treshold in the mitochondrial matrix it triggers the PT by interaction with still unidentified sites.

Mitochondria (0.5mg/ml) were incubated in 2 ml of medium containing 0.25 M sucrose, 10 mM Tris-MOPS, 1 mM Pi-Tris, 10 µM EGTA-Tris, , 5 mM glutamate/2.5 mM malate or 5 mM succinate-Tris plus 2 µM rotenone. For every mitochondrial preparation was found a Ca^{2+} concentration that is not able to induce the PT *per se'* but "sensibilize" the mPTP. 30µM $CaCl_2$ were added (stock 10mM) to 1mg of mitochondria after 1min and the absorbance was registered for 10 minutes. Different amounts of calcium were tested to find the concentration that is slightly under the amount that induces PT. In this condition is easier to

reveal if the molecule tested is an inducer or not of the PT since the mPTP opening is faster. Usually the Ca^{2+} used were 50-55µM. Once found the [Ca^{2+}] that "sensibilize" the mPTP the swelling measures were carried out as follow: in 2ml of medium were added 1mg of mitochondria and the recording of the absorbance was started. After 1 minute the [Ca^{2+}] previously found was added. After 1 minute were added DA and tyrosinase to generate DA quinines (DAQs) or other compounds specified in the figure legends in the *Results* Chapters of this thesis. When CsA (0.8µM) was used it was added in the medium before addition of mitochondria (**Figure 8**).

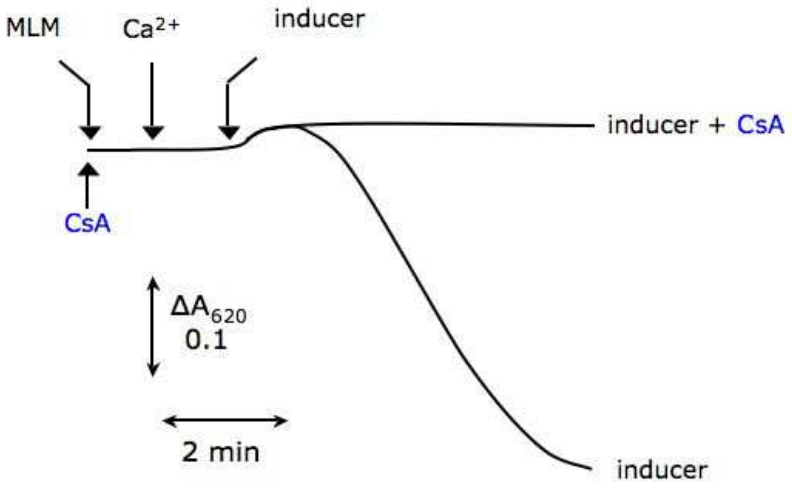

Figure 8 Explicative trace of a swelling experiment

The decrease of absorbance of a mitochondrial suspension containing an inducer of the PT was followed at the spectrophotometer. When the inducer triggers the opening of the mPTP the IMM becomes permeable to sulutes <~ 1500Da and the mitochondria start to swell. The increase size of mitochondria lead to a decrease decrease of light scattering detectable at the spectrophotoeter. To verify that this fenomenon is due to the induction of the PT the experiments are repeated in the presence of the inducer and the specific inhibitor of PT CsA.

Calcium Retention Capacity (CRC) experiments

Extra-mitochondrial calcium concentration was monitored fluorimetrically (Hitachi, F4500 or Ocean Optics SD2000 spectrofluorometer) using Calcium Green-5N (excitation–emission: 505–535 nm). The incubation medium contained 0.25M sucrose, 10mM Tris-MOPS, 1mM Pi-Tris, 10µM EGTA-Tris, , and 1µM

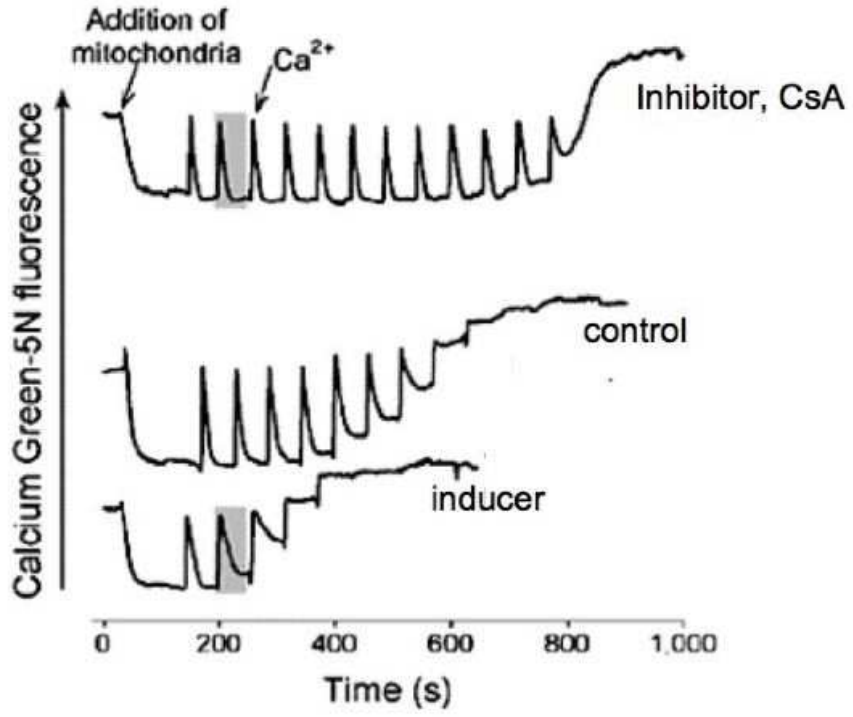

Figure 9 Example of Calcium Retention Capacity (CRC) experiments.

Mitochondria are incubated in the presence of the fluorescent probe Calcium Green 5N (1µM). Calcium Green 5N is able to detect the Ca^{2+} present in the medium but not the one that is in the mitochondria. To this solution aliquots of Ca^{2+} (10-20µM) are added (central trace in the Graph). Upward deflection of the traces reflects increases in extra-mitochondrial Ca^{2+} and downward deflections reflect mitochondrial Ca^{2+} uptake. Ca^{2+} is rapidly taken up by the mitochondria, and extra-mitochondrial Ca^{2+} levels rapidly returned to baseline until an aliquot of Ca^{2+} exceeded the mitochondrial Ca^{2+} retention capacity and the mitochondria released their calcium by opening the permeability transition pore (mPTP). If an inducer is present in the solution (Lower trace) the aliquots of Ca^{2+} that have to be added before inducing the mPTP are less than in the control, because the inducer "sensitize" mitochondria to the PT. That this phenomenon is due to the mPTP opening is verify repeating the experiments in presence of the inducer and the inhibitor of the PT cyclosporin A (CsA, upper trace). If CsA increase the CRC of the mitochondria in presence of the inducer it can be conclude that the inducer produce the calcium release by the opening of the PTP.

calcium green-5N. As oxidizing substrates were used 5 mM glutamate/2.5 mM malate or 5 mM succinate-Tris plus 2 µM rotenone to completely block the backward electron flux through complex I. Final volume was 2 ml, pH 7.4, 25 °C. All the experiments were started with the addition of 0.5 mg/ml of mitochondria. Calcium pulses (10µM) were then added at 1 min intervals until a Ca^{2+}-induced mitochondrial Ca^{2+} release was observed (**Figure 9**). Calcium retention capacity (CRC) was taken as the total amount of Ca^{2+} accumulated by mitochondria prior to the Ca^{2+} pulse triggering Ca^{2+} release. This value represents a reliable index of the threshold [Ca^{2+}] required to open the PTP in the whole mitochondrial population studied.

Pyridine Nucleotide Assay

Mitochondria (6mg, 0.5mg/ml) were incubated 10min in presence of different amount of DA and the enzyme tyrosinase to generate DAQs in the standard medium containing 0.25 M sucrose, 10 mM Tris-MOPS, 1 mM Pi-Tris, 10 µM EGTA-Tris 5 mM succinate-Tris plus 2 µM rotenone and 0.8µM CsA. To eliminate the colored quinines from this medium, mitochondria were spun 6min at 6000 x g in a benchtop centrifuge at 4 C. The mitochondria were then gently resuspended in 3 ml of the medium and the pyridine nucleotides were extracted from treated and untreated mitochondria. To 3ml of medium containing The untreated mitochondria were processed as the same manner than the DAQ-exposed mitochondria. To the mitochondria resuspended in 3ml of medium 0.6ml of 1M EtOH-KOH were added and the suspension was kept 30min at room temperature (RT). The samples were then cooled on ice for 10min and 1ml of 0.5M trietathanolamine (TEA)/K_3PO_4 were added and the suspension let 10min at RT. The samples were centrifuge 10min at 18000 x rpm in a Sorvall centrifuge, rotor SE12, and the supernatant transferred in new tubes. The supernatant was then spun again 45min at 30000 x g and the supernatant collected and kept on ice. The amount of NAD(P)H was assessed with two enzymatic assay at the fluorimeter (Hitachi, F4500) as described in Beatrice *et al*. 1984. NAD(P)H have a characteristic absorption peak at 340nm that is absent in the oxidized species.

Settings for the fluorimeter were excitation-emission: 340nm-460, slit excitation-emission 15nm.

For NADH: to 2 ml of pyridine nucleotide extract were added 1µl of lactic dehydrogenase enzyme and 50mM Na-Pyruvate were added and the oxidation of NADH was followed, according to the reaction:

$$\text{Piruvate} + \text{NADH} + H^+ \Leftrightarrow \text{lactate} + NAD^+$$

(reaction catalyzed by lactic dehydrognase enzyme).

For NADPH: when the signal was stable 5mM of oxidized glutathione (GSSG) and 2µl of glutathione reductase enzyme were added and the oxidation of NADPH was followed according to the reaction:

$$\text{GSSG} + \text{NADPH} \Leftrightarrow \text{GSH NADP+}$$

(reaction catalyzed by the enzyme glutathione reductase).

A standard curve with known amounts of NADH and NADPH was made and employed to express the $\Delta\Phi$ of absorbance in µM NAD(P)H. Untreated mitochondria were considered 100% NAD(P)H amounts in the graphs. All the measures were repeated in triplicate and the data analyzed with the program *Origin* ™.

Complex I assay

Complex I activity was assessed in isolated mitochondria as described in Janssen *et al*. (2007) (*100*). In this assay dichloroindophenol (DCIP) is used as terminal electron acceptor. Complex I oxidizes NADH, and the electrons are used to reduce the artificial substrates decylubiquinone that subsequently delivers electrons to DCIP. DCIP reduction is followed spectrophotometrically at 600nm. The sensitivity of this method resides in the fact that other NADH-dehydrogenase are not able to reduce decylubiquinone. 965µl of medium containing 25mM potassium phosphate (KPO_4, stock solution 100mM, pH 7.8), 3.5g/L BSA (stock

solution 70g/L), 60µM DCIP (stock solution 14mM in dimethil sulphoxide, DMSO), 70µM decylubiquinone (stock solution 6mM in water) and 1µM anticycine-A (stock solution 10mM in DMSO), pH 7.8 was pre-incubated with 25µl mitochondria (0.5mg of mitochondrial proteins) at 37 °C for 3 minutes. The reaction was started with addition of 20µl of NADH (stock solution 10mM in KPO_4 buffer) and absorbance at 600nm was followed for 10 minutes every 30 seconds. 1µl of rotenone (stock solution 1mM in DMSO) was added and absorbance measured for 1 minute every 30 seconds. Complex I activity was expressed as U/mg protein, in which 1U complex I activity is equal to 1 µmol DCIP reduced per min. Rotenone sensitivity was used to assess the assay specificity. The experiments were repeated three times for every condition for statistical analysis.

Complex I immunocapture (IP)

Complex I from isolated liver mitochondria was IP with Mitoscience™ immunocapure antibody. To a mitochondrial membrane suspension at 5.5 mg/ml protein in PBS 1/10 volume of 10% lauryl maltoside (final concentration of 1%) was added, mixed and the suspension kept on ice for 30 minutes. The samples were centrifuge at maximum speed in a benchtop microfuge for 30 minutes. The supernatant was collected in new tubes. 5µl of solid beads cross-linked with the antibody against complex I were added to the supernatants and the mix allowed to turn overnight at 4 °C on a in a tube rotator. After the mixing step the beads were collected by centrifugation for 1 minute at 1,000 x g on a benchtop microfuges and the supernatant discard. The beads were washed with 100 volumes of PBS buffer for 5 minutes on gently agitation to remove the non-specifically bound proteins prior to elution.. Washing process was repeated twice. Elution was performed adding SDS-PAGE loading buffer and boiling 10min at 96 °C before loading the samples on a 15% pre-cast gel (Bio-Rad).

Cell Biology

Cell Cultures

SHSY-5Y human neuroblastoma cells (a generous gift of Dr. Tullio Pozzan, Dept. of Biomedical Sciences, Padova, Italy) were grown in Dulbecco's modified Eagle's medium supplemented with 2mM glutamine. The media were all supplemented with 10% fetal calf serum, 50 units x ml^{-1} penicillin, and 50µg x ml^{-1} streptomycin. Cells were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C in a Forma tissue culture water-jacketed incubator. Cells were seeded 10^6 for every well of a 6wells plate or 2 x 10^5/ml in flask with 15ml medium and let grow until 75-80% confluency before re-seeding, transfection or treatment.

Trasnsfection

SHSY-5Y cells were transfected with the Nucleofector™ technology (amaxa biosystems). Cells were passage 3-4 days before transfection. Adherent cells from a 75% confluence were washed with PBS and trypsin/EDTA was added to cells. Trypsinization was stopped with additional 7 ml warm DMEM an aliquot of cell suspension was used for count cells to determine cell density. $1x10^6$ cells per sample were centrifuged at 90xg for 10 min and the supernatant discarded completely. The pellet was resuspended 100µl Cell Line Nucleofector™ Solution V and 2µg plasmid DNA (in 1-5 µl H_2O or TE) was added. The sample was transferred into an amaxa certified cuvette, avoiding air bubbles while pipetting. The cuvette was inserted into the cuvette holder of the Nucleofector™ and the Nucleofector™ programs G-04 was used to transfect the cells for high transfection efficiency and analysis up to 24 hours.

Cell count

After treatment or before transfection cells were counted with a spelled hemocytometer. Hemocytometer is an etched glass chamber with raised sides that hold a quartz coverslip exactly 0.1 mm above the chamber floor. The counting chamber is etched in a total surface area of 9 mm^2 (see Figure 5). Calculation of concentration is based on the volume underneath the cover slip. One large square has a volume of 0.0001 ml (length x width x height; i.e., 0.1 cm x 0.1 cm x 0.01 cm). Before counting, cells were added with an equal volume of trypan blue [0.4% (w/v) tyrypan blue in PBS] to facilitate visualization of the cells and to determine live/dead count (dead cells are blue). A well-suspended mix of cells was notch at the edge f the hemocytometer and fill slowly by capillarity. The number of live and dead cells was count in the 4 outer squares and the cell concentration was calculated as follows:

Cell concentration per ml = Total cell count in 4 squares x 2500 x dilution factor.

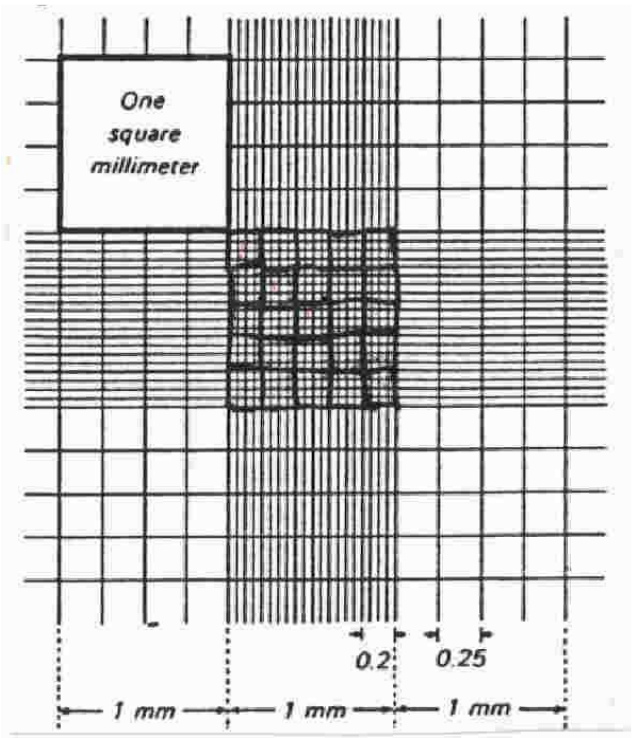

Figure 10 Dimensions of a hemacytometer.

Fluorescence Microscopy

One-hundred thousand cells were seeded onto 24-mm diameter round glass coverslips in 6-well plates and grown for 1 day. The coverslips were then transferred onto the stage of a Zeiss Axiovert 100TV inverted microscope equipped with a HBO mercury lamp (100 watts), and epifluorescence was detected with a 12-bit digital cooled CCD camera (Micromax, Princeton Instruments). Cells were incubated in Hanks' balanced salt solution without bicarbonate and allowed to equilibrate in the dark with 20 nM TMRM in the presence of 1.6 µM CsH or 1 µM CsA for 30 min at 37 °C prior to further additions. All the experiments were started with the addition of DA and tyrosinase (100U). Fluorescence images were acquired with a 560-nm dichroic mirror using a 40x/1.3 oil immersion objective (Zeiss), with excitation at 546 ± 5 nm and emission at 580 ± 15 nm. Exposure time was 80 ms, and data were acquired and analyzed with the MetaMorph Metafluour Imaging Software. Clusters of several mitochondria were identified as regions of interest, whereas background was taken from fields not containing cells. Sequential digital images were acquired every 2 min for 60 min, and the average fluorescence intensity of all the regions of interest and of the background was recorded and stored for subsequent analysis. Mitochondrial fluorescence intensities minus background were normalized to the initial fluorescence for comparative purposes.

Biochemistry

Electrophoresis and Western Blot

SDS-PAGE

Following determination of protein concentrations of mitochondria samples the desired amount of mitochondria were placed in a fresh centrifuge tubes and sun down at maximum rate in a benchtop centrifuge. The supernatant was removed and loading buffer containing 250 mM Tris base pH 6.8, 20% glycerol, 5% SDS, 0.01% bromophenol blue were added to samples to have a

final concentration of 5µg/µl. Samples were vortexed, boiled at 95°C for 10 min, and cooled to 4°C.

The desired stacking and running Acrylamide/Bis-Acrylamide gels were casted and run in a mini-vertical electrophoresis unit (SE250, Pharmacia) following Laemmli *et al.* (1970) (*101*). The chamber was filled with 125 ml of Laemmli running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). Mitochondrial protein samples were loaded into the wells within the gel. The volume of each sample loaded was adjusted to ensure loading of equal concentrations of protein. A standard containing proteins of known molecular weights was loaded into the tenth lane. Mitochondrial proteins were separated by applying a 100 mV current in the *stacking* gel and 150mV in the *running* gel.. Electrophoresis was stopped when the lightest band of the protein standard reached the bottom of the gel. Proteins were then transferred to 0.45-µm nitrocellulose membranes (Fisher Scientific) by electrophoresis at 30 mV for 12 h.

Blue Native-PAGE

Blue-Native PAGE was performed in a mini-vertical electrophoresis unit (SE250, Pharmacia) as described in Wittig *et al*. (2006). This technique is used to

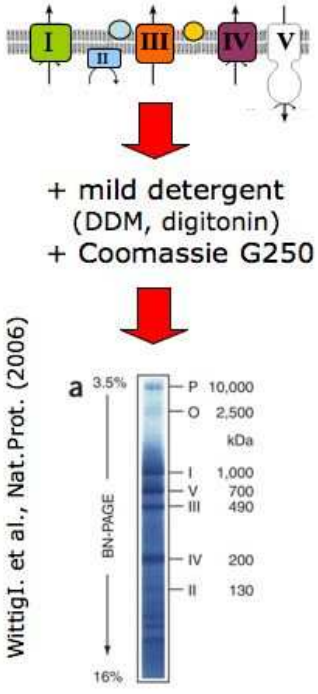

Figure 11 Separation of DDM-solubilized mitochondrial complexes by BN-PAGE

In BN-PAGE mitochondria are solubilized in the mild detergent DDM. Solubilized complexes are separated according to their masses on a linear 3.5-13% acrylamide gradient gel for BN-PAGE.

isolate mitochondrial complexes in their native and active form. In the present research this technique was used to separate ^{14}C-labelled proteins of the ETC complexes and detected by autoradiography.

The 3.5-13% acrylamide gradient gel was casted as in Table 1.

Table 1 3.5-13% BN-PAGE gradient gel

	Stacking gel	Gradient Separation gel	
	3.5% acrylamide	4% acrylamide	13% acrylamide
Acryl Mix (30%, 37,5/1-Acylamide/Bis Acrylamide	0.44ml	1.5ml	3.9ml
Gel Buffer *	2ml	6ml	5ml
Glycerol	-	-	3g
Water	3.4ml	10.4ml	3ml
Total volume	6ml	18ml	15ml
10% APS	10µl	10µl	50µl
TEMED	1µl	1µl	10µl

*** Gel Buffer:15M Aminocaproic acid, 150mM Bis-Tris pH 7.0**

Mitochondria pellet (400µg proteins) were solubilized 10min at RT in extraction buffer (EB), containing 0.75M aminocaproic acid, 50mM Bis-Tris pH 7, 10% nDodecyl-β-maltoside (12.5µl /100µl EB). The sample was centrifuge 30min at higher speed in a benchtop centrifuge and the supernatant transferred in a new tube and 5% w/v coomassie brilliant blue G-250 in 0.5M Aminocaproic acid (6.3µl/100µl EB) were added right before sample loading. Gel was placed in the chamber of a mini-vertical electrophoresis unit (SE250, Pharmacia) and the lower chamber was filled with Anode buffer containing 25mM Imidazole pH 7.0. The running was start with the Cathode buffer B containing 50mM Tricine, 7,5mM Imidazole pH 7.0, 0.02% Coomassie blue G-250 and after the blue running front has moved about one third of the total running distance the Cathode buffer was replaced with the Cathode buffer 1/10 containing 0.002% Coomasie blue G-250.

Running conditions were 100V until the sample has entered the gel and 50V overnight and until the end of the run. All the run was performed at 4 °C.

In-gel digestion of Proteins Separated by Polyacrylamide Gel Electrophoresis

Proteins were separated with SDS-PAGE and gel rinsed with water. Gloves were worn in every passage of the preparation to avoid cheratines contamination of the gel. The bands of interest were excised with a clean scalpel making sure to cut as close as the edge of the band as possible. The bands were chopped into cubes of ~1cm^3 and transferred into a clean microcentrifuge tube. Gel particles were washed with water for 5 min and spun down to remove the liquid. Acetonitrile (3-4 volumes of the gel pieces) was added for 10-15 min until gel pieces shrunk and they became white and stick together. The samples were sun and liquid remove. The gel particles were then dried in a vacuum centrifuge. To reduce protein10mM dithitreitol/0.1M NH_4HCO_3 was added enough to cover the gel pieces and incubate 30 min at 56 °C. Gel particles were the spun down and the excess of liquid removed. Acetonitrile was added until el pieces shrunk again and replaced with 55mM iodoacetamide/0.1M NH_4HCO_3. The samples were let 20 min in the dark at RT. Iodoacetamide solution was then removed and gel pieces washed with 150-200µl of 0.1M NH_4HCO_3 for 15 min. el pieces were spun down and liquid remove. Acetonitrile was added again until gel particles shrunk and then removed after centrifugation. Gel particles were dried in a vacuum centrifuge.

Gel particles were dehydrated with 10µl of digestion buffer containing 50mM NH_4HCO_3 and 40-50ng/µl of trypsin. The samples were let 30-40 min on ice checking after 15-20 min if the liquid was all absorbed by the gel pieces and eventually more buffer was added. The excess of supernatant was removed and 5-25µl of buffer without trypsin was added to cover the gel pieces. Digestion was continued ON at 37 °C. After ON digestion the sample was spun down and 1-2µl aliquot was used for MALDI analysis.

For ES MS/MS analysis the tryptic peptides were extracted from gel particles. 20-15µl of 25mM NH_4HCO_3 and samples incubated at 37 °C for 15 min with shacking. Gel particles were spun down and acetonitrile (1-2 volumes) were added. Samples were incubated 15 min at 37 °C with shaking, then spun down and the supernatants were collected in new tubes. To the gel particles 40-50µl of

5% formic acid was added and samples vortexed for 15 min at 37 °C. Gel particles were spun down and the supernatant pulled together with the one already collected. Extract were then dried in a vacuum centrifuge.

Activity Stain for F_1F_o ATPsynthase complex

In this assay the ATPase activity of the F_1F_o ATPsynthase complex is assessed as described in Zerbetto *et al*. (1997) (*102*). The complex is separated by BN-PAGE in its active form and both the dimmer and the monomer of the complex can be resolved. In presence of ATP, F_1F_o ATPsynthase complex idrolizes ATP to ADP + Pi. Inorganic phosphate reacts with Pb^{2+} and Pi_2Pb precipitates in the gel in correspondence of the F_1F_o ATPsynthase complex band.

In the BN-PAGE a Cathode buffer without Coomassie blue -250 was added at half of the run to ameliorate detection of the bands of the active stain. Once run until the end gel was rinsed with water briefly and a buffer containing 35mM Tris, 270mM glycine pH 7.4, 14mM $MgSO_4$, 8mM ATP and 0.2% (w/v) $PbNO_3$ was added (20ml for a minigel). The gel was kept at RT until the bands of the active stain appeared and picture at different time point of the reaction were kept for comparison. Staining was stopped with water.

RESULTS

Part I: Permeability of mitochondria to DAQ and mitochondrial proteins modifications.

Introduction

One pathological hallmark of Parkinson disease (PD) is the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (*103*). The hypothesis that the neurotransmitter DA, specifically expressed by this type of neurons, can play, besides its fundamental physiological functions, a cytotoxic role that can contribute to the neurodegeneration of SNpc dopaminergic neurons in PD was considered in this research. In particular, the toxic effects of the oxidized products of DA on mitochondria were studied in detail. .

DA is synthesized in the cytoplasm and rapidly sequestered by the VMAT-2 transporter into synaptic vesicles where is maintained in the reduced state by the low pH (*104, 105*). If DA synthesis, storage of metabolism is impaired DA can accumulate in the cytoplasm of dopaminergic neurons. Cytoplasmic DA can undergo to spontaneous oxidation in presence of molecular oxygen (*91*). This reaction leads to the oxidation of the catechol ring of DA and the formation of several reactive oxygen species (ROS) as super oxide anion ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$) and DA quinone molecules (DAQs) (*12*). The oxidative reaction of DA involves multiple steps of oxidation and spontaneous rearrangements of the intermediates as depicted in **Figure 12**. ROS can increase oxidative stress and if the scavenging systems are overwhelmed, oxidative damage can lead to cell death. Besides toxic role of ROS, growing evidence account for a detrimental role of the oxidation products of DA. Due to their electrophilic nature DAQs are highly reactive species toward cellular nucleophiles as the sulphydryl groups of free cysteines, glutathione (SH. Moreover, their ability to react and bind covalently to cysteinyl residues of proteins both *in vitro* and *in vivo* has been

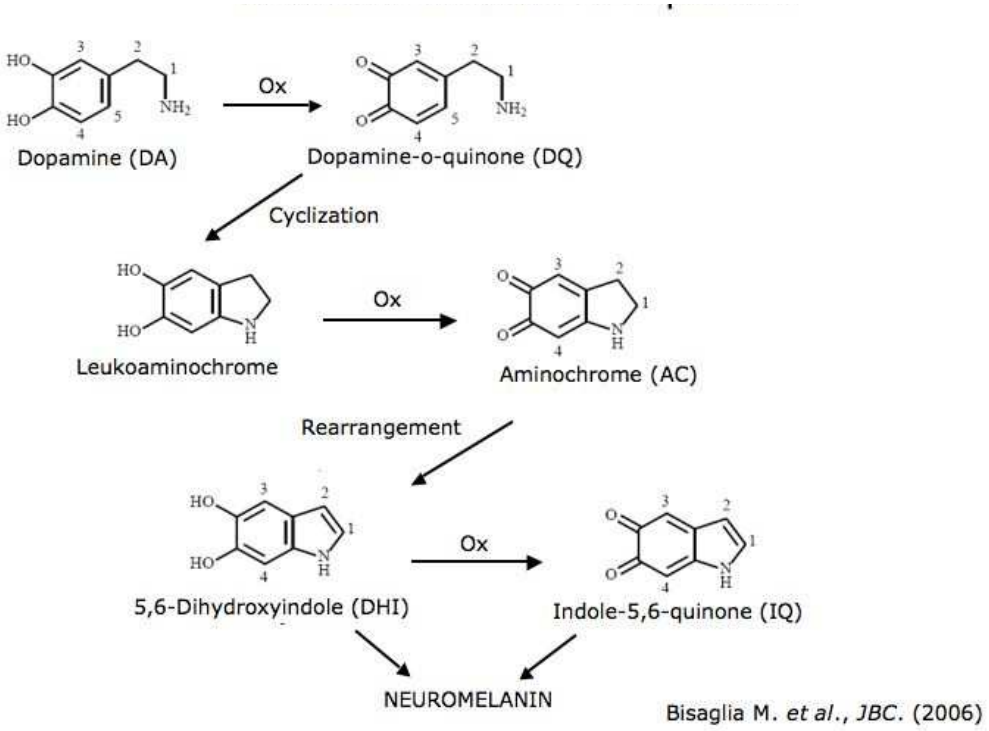

Figure 12 The oxidative pathway of DA that leads to neuromelanine synthesis.

In the initial reaction DA is oxidized to dopamine-o-quinone (DQ). Neuromelanin is synthesize in the lysosomes that express the Vescicular monoamine transporter-2 (VMAT2) to import DA. Nonetheless DA oxidation is spontaneous in the cytoplasm and strongly favored at alkaline pH. Moreover several enzymes, found in the brain, can catalize this reaction as tyrosinase (Ty, Sanchez-Ferrer *et al*. 1995), Prostaglandin H synthase (Hastings *et al*. 1995), lipoxygenase (Blarzino *et al*. 1999), xanthine oxidase (Foppoli *et al*. 1997). In the lysosomes the pathway to neuromelanin proceed with the spountaneous cyclization of DQ to leukoaminochrome, this reaction is favored at lower pH and considered the rate-determinin step for the subsequent oxidation reaction of leukoaminochrome to Aminochrome AC). Aminochrome spountaneously rearrange to 5,6-dihydroxyndole (DHI) which can be oxidize o Indole-5,6-quinone (IQ) and polymerize to form neuromelanin

reported (*28, 92, 94, 106-108*). The reaction between dopamine-o-quinone and the amino acid cysteine is depicted in **Figure 13**. Because these residues are often located at the active site of a protein covalent modifications result in an impairment of protein function with potentially deleterious effects on the cell, as described for the PD-gene product *parkin* (*92*). In addition to protein, the oxidized products of DA can react and covalently modify DNA (*109*). Several evidence account for a toxic effect of DAQs on mitochondria. The ability of DAQs to inhibit mitochondrial respiration and in particular

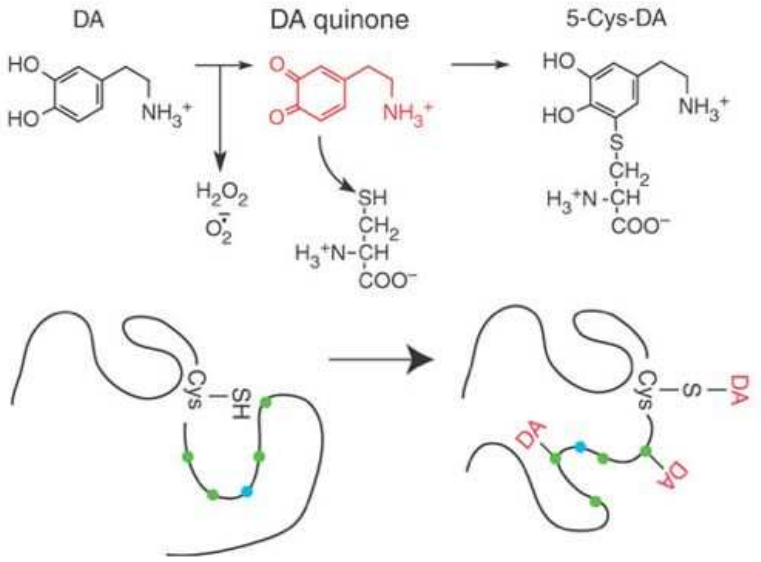

Figure 13 DAQs react and covalently modify cysteine residues of proteins

Oxidation of DA leads to the formation of DA quinone molecule and ROS as super oxide ion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). H_2O_2 can react with iron abundant in the SN to form hydroxyl radical (OH^-) through Fenton reaction. DA quinones are highly electrophiles and easily react with cellular nucleophiles as GSH, free cysteine or cysteine residues of proteins. With cysteine the C5 of the quinone ring react with the thinly of cysteine to give a 5-cysteynil-catechol molecule. Since cysteine residues are often found in active sites of proteins DA quinone-modification can lead to structure and function alteration of proteins. (from LaVoie *et al*. 2003)

their toxicity toward complex I has been reported (*110-112*). DAQs are also able to induce the permeability transition (PT) in brain mitochondria (*110*). Recently, their ability to induce the electron leakage from the mitochondrial electronic transport chain (ETC) at the level of complex I, increasing the amount of ROS normally produced by mitochondria has been reported (*113*). Interestingly this is the same mechanism claimed for the toxicity of the PD-inducing toxin rotenone (*11*)

Since in PD mitochondrial dysfunctions seem to play a central role in the onset or progression of the pathogenesis of PD, the toxic effects of the oxidized products of DA on mitochondria is a intriguing aspect to clarify and their mechanism of interaction with the mitochondria organelle is subjects of this chapter.

Statement of purpose

The overall aim of the research that comprises this dissertation is to enlighten toxic mechanisms of DA quinones toward mitochondria that contribute to the degeneration of dopaminergic neurons in PD. The experiments described in this chapter utilizes *in vitro* and cellular models approaches to assess the ability of DAQs to enter the mitochondria and covalently modify mitochondrial proteins. The attempt to identify two DAQs-modified proteins is also reported.

To generate DAQs the mitochondria were incubated with DA and the enzyme tyrosinase (Ty). Ty is able to catalyze the two steps of the oxidation of DA to melanin, in particular, the first oxidation that generate the dopamine-o-quinone (DQ) and the oxidation of leukoaminochrome to aminochrome (AC) (See also **Figure 12**).

Once proven the ability of DAQs to cross the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), the capacity of DAQs to bind covalently and modify mitochondrial proteins was assessed by autoradiography techniques. Finally the attempts to identify two DAQs-modified proteins with mass spectrometry analysis (MS) and the functional consequences of these modifications are reported.

Results

DAQs enter the mitochondria

Several evidences in literature show the ability of the oxidized products of the neurotransmitter DA to exert toxic effects toward the mitochondria such as swelling, decreased activity of the electron transport chain (ETC) and altered mitochondrial potential (*89, 110-115*).

To further characterize the toxic interaction of DAQs toward mitochondria the ability of DAQs to cross both the outer and the inner mitochondrial membranes (OMM and IMM) was examined. The model selected was the non-synaptosomal fraction of mitochondria purified from rat brain. To be able to track

the presence DA inside mitochondria ^{14}C-DA was used and scintillation or autoradiography experiments were carried out. The oxidizing agent used to generate DAQs from DA was the enzyme mushroom tyrosinase (EC 1.14.18.1, from Sigma). In the medium both complex I and complex II substrates (glutamate-malate and succinate, respectively), were present, to mimic a more physiological situation. Once exposed the mitochondria to DAQs for 10 minutes the medium was removed by filtration of mitochondria through silicone and the mitochondria washed three times to remove the non-specifically bound DA and DAQs. Titration experiments of several DA amounts and incubation times were carried out. The amount of radioactivity accumulated in mitochondria was measured by scintillation.

Data from these experiments (**Figure 14**) show that DAQs accumulate fast in the mitochondria where they become 35 times more concentrated than in the medium. DA itself is unable to enter the mitochondria, highlighting that the active, and more probable toxic, species are the oxidized products of the neurotransmitter.

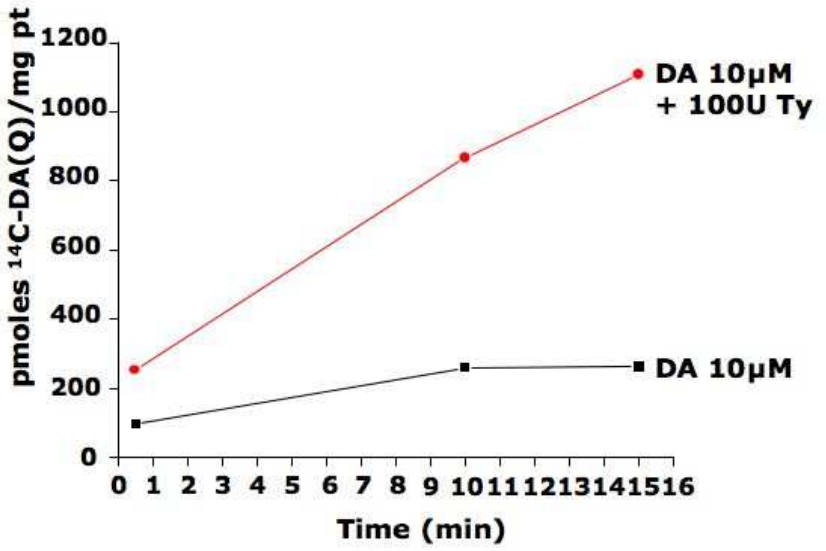

Figure 14 DAQs but not DA enter the mitochondria.

Rat brain mitochondria were incubated with ^{14}C-DA or ^{14}DA and tyrosinase as described in the *Materials and Methods*. Samples at subsequent time points were taken and the DAQs accumulated were detected by scintillation. Radioactivity in the samples is expressed as pmoles of ^{14}C-DA(Q)/ mg of proteins. As shown in the graph DAQs accumulate fast in the mitochondria on the contrary DA remains excluded.

To test further the ability of DA quinones to cross the IMM mitochondria were treated with the mild detergent digitonin. Digitonin is able to remove the outer mitochondrial membrane (OMM) generating the mitoplasts. Mitoplast are mitochondria of which OMM has been removed and only the IMM and matrix remain. Then the radioactivity accumulated in the mitoplast was measured. DAQs accumulate also in the mitoplast, suggesting the ability of these compounds to cross both OMM and IMM. (**Figure 15**). The mechanism by which the DAQs cross the two membranes is not clarified so far, since an active transport seems unconvincing, even if it cannot be excluded, and passive diffusion would be hindered by the negative charge of the quinone compounds.

To test if the entrance of DAQs in mitochondria depends on the energetic state of the organelle, and therefore the $\Delta\Psi$ among the IMM, DAQs-accumulation in the presence or absence of substrate were compared.

No significant differences in the accumulation of the DAQs based on the

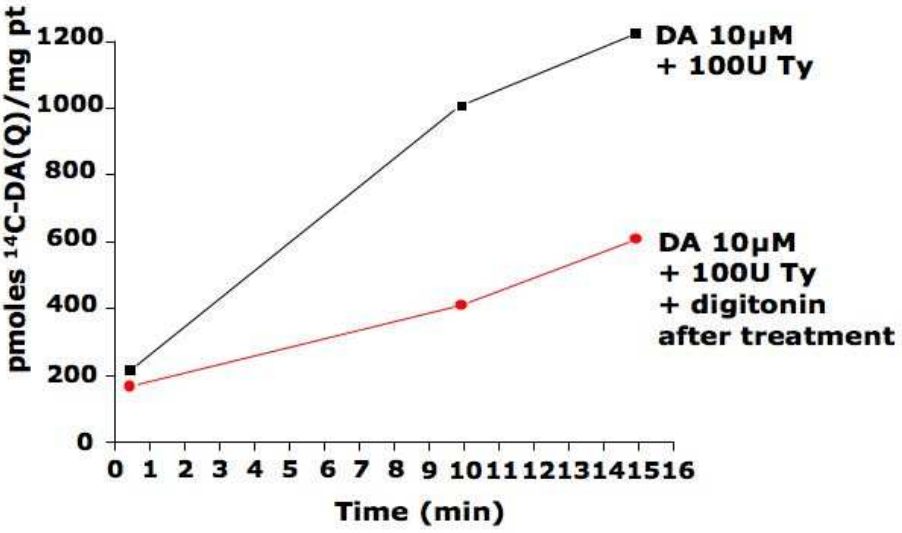

Figure 15 DAQs can cross both the outer and the inner mitochondrial membrane

Brain rat mitochondria were purified as described in *Materials and Methods*. To test if the oxidized products of DA can cross both the outer and the inner mitochondrial membrane (OMM, IMM) the OMM was removed with the mild detergent digitonin. Radioactivity corresponding to ^{14}C-DAQ accumulated in the mitochondria or in the digitonin-generated mitoplast was measured by scintillation experiments. As shown in the graph DAQs can cross both mitochondrial membranes and accumulate in either in mitochondria and mitoplast. Radioactivity in the samples is expressed as pmoles of ^{14}C-DAQ/ mg of proteins.

energetic state of the ETC were detectable (**Figure 16**). This result leads to the exclude an active transport of DAQs into mitochondria, since an active transport requires energy not available in absence of substrates. Interestingly, $\Delta\Psi$ seems not influence the entrance of the negative charged DA-quinones molecules into the mitochondria.

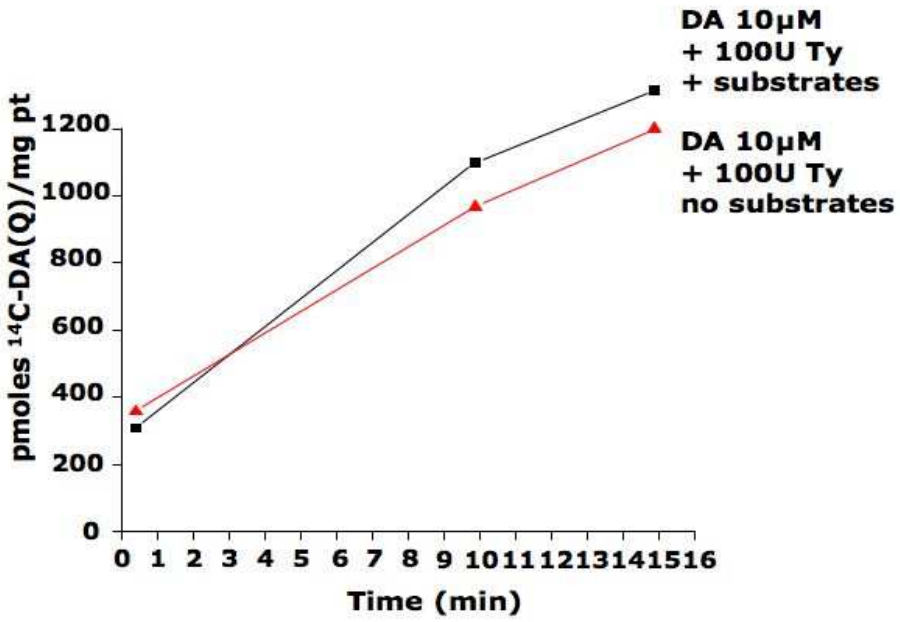

Figure 16 Energetic state of mitochondria does not influence DAQs entrance.

Rat brain mitochondria purified as described in the *Materials and Methods* and incubated with ^{14}C-DA and tyrosinase to generate DAQs. Radioactivity accumulated in the presence or absence of oxidizing substrates in the medium was compared. Radioactivity in the samples is expressed as pmoles of ^{14}C-DAQ/ mg of proteins. As shown in the graph no significant differences are detectable between the two condition, excluding the energetic state of mitochondria as a important factor for DAQs entrance.

DAQs but not DA modify mitochondrial proteins

The ability of DAQs to bind and covalently modify mitochondrial proteins was investigated. Rat brain mitochondria were purified and exposed to ^{14}C-DA alone or ^{14}C-DA and tyrosinase to generate DAQs. At the end of the treatment the medium was removed by centrifugation of mitochondria and mitochondrial proteins separated by gel electrophoresis. The radioactivity incorporated in

mitochondrial proteins was detected by scintillation (**Figure 17**). Results from these experiments show that only in presence of tyrosinase radioactivity is detectable in the mitochondria proteins.

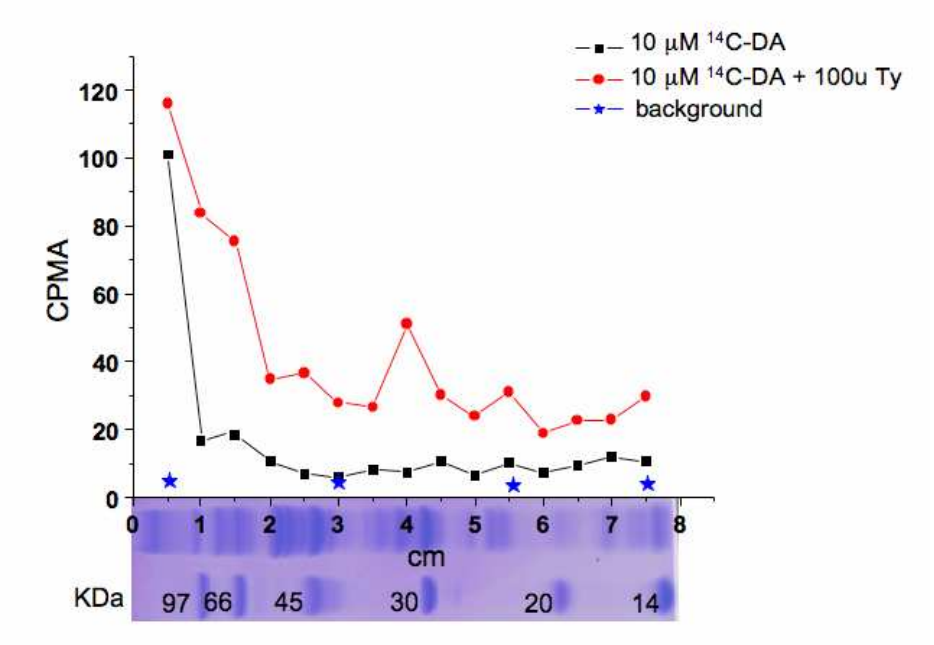

Figure 17 DAQ but not DA modify mitochondrial proteins.

Rat brain mitochondria were incubated with ^{14}C-DA or ^{14}C-DA and tyrosinase. After 10 minutes of incubation mitochondria were spun and washed three times with fresh buffer. Proteins were extracted in loading buffer and isolated by gel electrophoresys. Every lane was cutted in 16 segments of 0.5cm each and the radioactivity present in the segments was detected by scintillation.

In addition fluorography experiments were carried out to investigate the ability of DAQs to modify mitochondrial proteins. In these experiments the ^{14}C-DAQ- modified proteins were revealed by autoradiography of X-rays exposure of the mitochondrial proteins separated by SDS-PAGE. Data from these experiments show that DAQs can bind and modify several mitochondria proteins and two bands slightly above the 30kDa molecular weight are the most reactive (**Figure 18**).

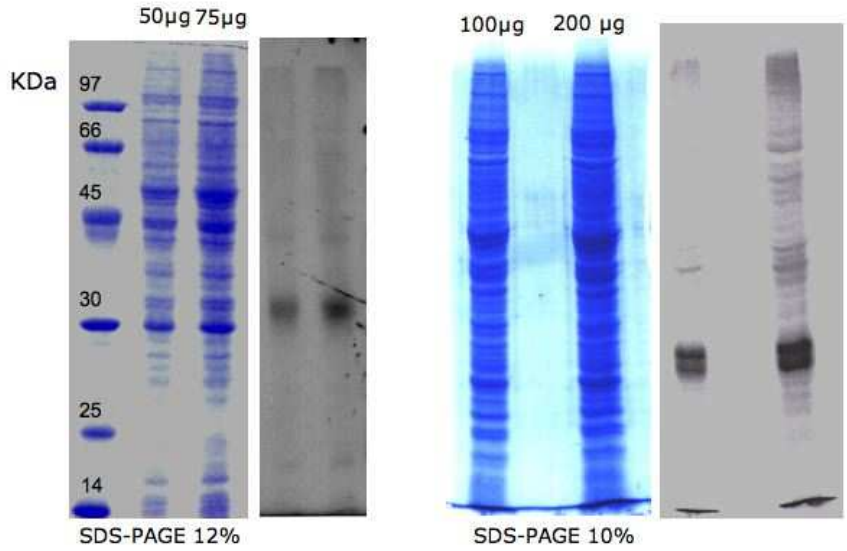

Figure 18 DAQ modify mitochondrial proteins

Purified mitochondria from brain, incubated with 10µM ^{14}C-DA (1µCi) in the presence of the enzyme tyrosinase (100U) for 10 minutes. After the treatment the mitochondria were filtered trough silicone and washed three times with fresh buffer to remove the non specifically bound DA. Several amount of mitochondrial proteins (50µg-75µg-100µg-200µg) were separated by gel electrophoresis (coomassie stain, on the left) and the dried gel exposed to X-ray film to detect the labeled bands (autoradiogram on the right respect the coomassie). Several proteins are detectable in the autoradiogram and two bands slightly above the 30KDa molecular weight are hightly reactive.

MS analysis of two proteins modified by DAQs

A Mass Spectrometry (MS) approach was employed to identify the proteins highly reactive with DAQs. The two bands on the SDS-PAGE were excised and processed for MS analysis. For the upper band the sequence identified after passive elution was not unambiguous. A MALDI-TOF analysis of the Q-V/I/F-L/E/A-E-D/G-I-K-Q/A/E-F/V-V/G peptide was necessary and three different proteins were identified in the band: the voltage-dependent anion-selective channel protein 1 (VDAC-1), Slc25a3 protein and Phosphate carrier protein, mitochondrial precursor (PTP) (Solute carrier family 25 member 3). For the lower band the γ-subunit ATP synthase complex was unambiguously identified from the ATLKDITRXL peptide.

The γ subunit of F_1F_o ATP synthase protein complex is a structural component of the complex V of the oxidative phosphorylation chain in the inner mitochondrial membrane. Complex V synthesizes ATP from ADP using the electrochemical potential made by the complex I, II and IV of the ETC. In particular γ subunit is the stator that connect the functional F_1F_o part of the complex, facing the matrix, with the membrane-anchored part of the complex. Structural analysis of the γ-subunit from the crystallographic structure of the F_1 component of the F_1F_o ATP-synthase complex (*116*) reveals that a cysteine residue (Cys 78, PDB 1OHH) is present in the protein facing the matrix space (**Figure 19**). Based on the well reported capacity of DAQs to react with cystein residues in the proteins, this residue can be a reasonable candidate for DAQs attack.

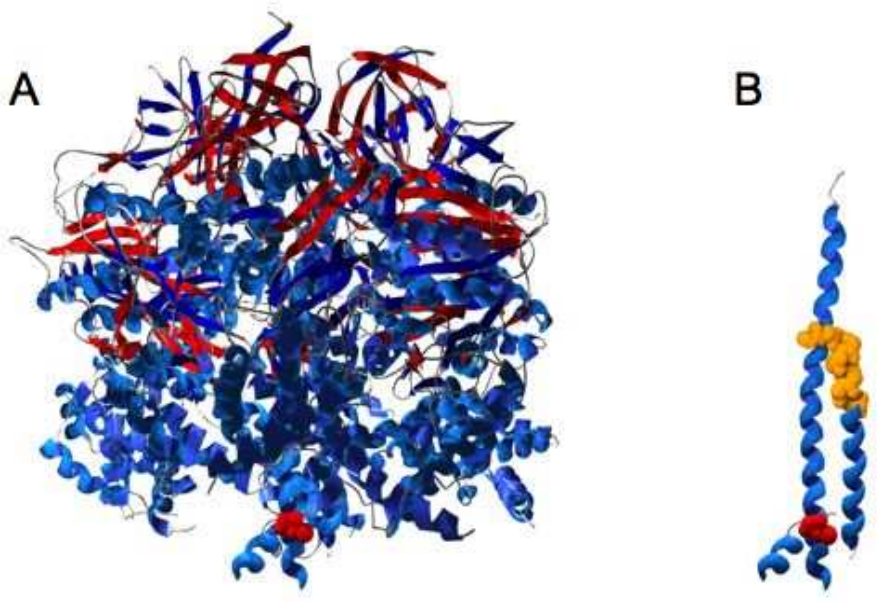

Figure 19 Structure of the F_1-ATPsynthase complex and the γ subunit

The structure of the matrix-protruding part of the ATP synthase complex from hearth bovine mitochondria (Ref 116) is shown (**A**). γ subunit is shown on the right (**B**). γ subunit is the "shaft" that connects the F_o component of the complex embedded in the IMM with the F_1 component that protrude into the mitochondrial matrix. A cysteine residue (Cys78, PDB 1OHH) is highlighted (red balls) and can be a target for the attack of DAQs.

VDAC1 is not one of the proteins modified by DAQs.

To evaluate the role of VDAC-1 in DAQs modification we repeated the experiment with mitochondria purified from Vdac-$1^{-/-}$ mice (kindly provided by the laboratory of Prof. Paolo Bernardi at the Department of Biomedical Science of the University of Padova). If VDAC-1 was the target of DAQs what was expected was a disappear of the modified band in the autoradiogram of mitochondria exposed to ^{14}C-DAQ extracted from Vdac1 KO mice.

Results from this experiment shown that the modification pattern from Vdac-$1^{-/-}$ mitochondria is undistinguishable from the wild type. (**Figure 20**). These result lead to exclude VDAC-1 as one of the target of the oxidized products of dopamine and further analysis are necessary to identity the others DAQ-modified proteins and the functional consequences of these modifications.

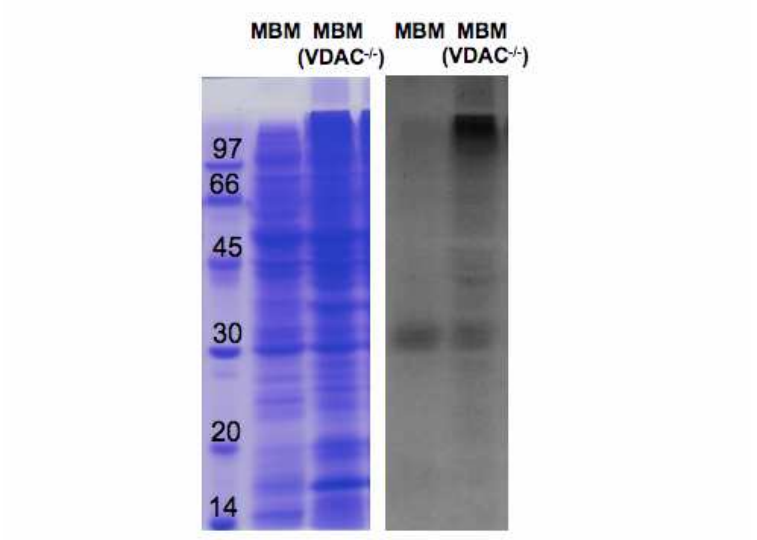

Figure 20 Vdac1 KO mice show the same pattern of modifications of WT mice

Mouse brain mitochondria from wt and Vdac$^{-/-}$ mice were purified by differential centrifugation. Mitochondria (0.5mg/ml) were incubated in 2 ml of medium containing 0.25 M sucrose, 10 mM Tris-MOPS pH 7.4, 1 mM Pi-Tris, 10 µM EGTA-Tris, , 5 mM glutamate/2.5 mM malate. Mitochondria were treated for 10 minutes with 10µM ^{14}C-DA in presence of 120U tyrosinase. The protein were extracted in loading buffer and separated by SDS-PAGE. The pattern of protein modification by ^{14}C-DAQ was compared with protein treated in the same way from wt animals

Independent experiments show that the γ subunit of the F_1F_o ATP synthase complex is modified by DAQs.

To verify the ability of DAQs to bind to the γ subunit of the F_1F_o ATP synthase a proteomic approach was employed. Blue Native-PAGE technique was used to isolate the native complex V from mitochondria exposed to ^{14}C-DAQ (**Figure 21**). The several subunits of the isolated complex V were then separated by SDS-PAGE, and the DAQs-modified proteins were identified by fluorography of the gel. Results from these experiments confirm the binding of the oxidized products of DA to the γ subunit of the F_1F_o ATP synthase.

These data confirm the finding that the γ-subunit of the F_1F_o ATP synthase complex is target of DAQs reactive species and lead to exclude that possibility of contaminants in the MS experiments.

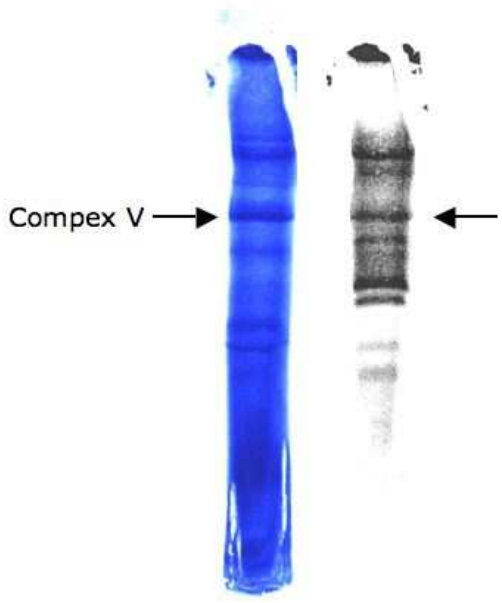

Figure 21 DAQs react and covalently modify complex V of the ETC

Mouse liver mitochondria were purified as described *in Materials and Methods*. Mitochondria were incubated with 10μM ^{14}C-DA (1μCi) plus Ty to generate DAQs, and the five complexes of the OXPHOS were separated by BN-PAGE. The dryed gel was exposed to X-ray to reaveal the DAQ-modified complexes. Data from these experiments show that complex V is modified from DAQs. An upper band, corresponding to complex I is also detectable in the autoradiogram.

Unaltered activity in the DAQs-modified F_1F_o ATP synthase complex .

To assess if the Complex V modifications at the γ subunit by DAQs are able to induce alterations in the enzyme activity an in gel activity assay for the ATPase function of the complex V was carried out. The five complexes of the mitochondria were isolated in the native and active state by Blue Native PAGE. The ATPase activity of DAQs-modified and wild type Complex V was assessed with an in-gel assay. The ability of the F_1F_o ATP synthase complex to hydrolize ADP was tested. ATP complex V cans idrolyze the molecule to ADP and Pi inorganic. Pi reacts with Pb^{2+} ion to give the Pi_2Pb that precipitates on the ATP-synthase band in the gel. lead-phosphate. The activity of the isolated complex can then be measured by densitometry analysis. The results from these experiments do not show a decrease of the complex activity for the DAQs modified complex respect to the control (**Figure 22**).

Nuclear Magnetic Resonance techniques were employed to investigate the ATP synthase activity of the DAQs-modified F_1F_o ATP synthase. With this technique was possible to follow the ATP synthesis in the intact mitochondria exposed to DAQs. As for the ATPase activity, also for the ATP synthase activity

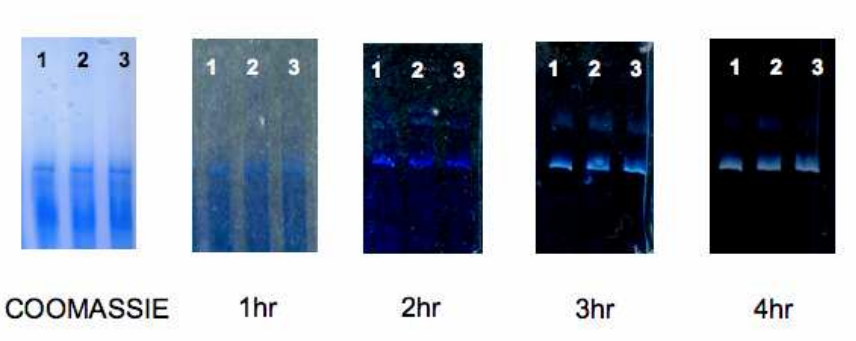

Figure 22 Complex V modified by DAQ at the γ subunit retain its F_1F_o ATPase activity

Activity staining was performed on Complex V isolated from mitochondria incubated with DA and tyrosinase. Lane 1: control, 50μg proteins; lane 2: 50μg proteins from mitochondria exposed 10 minutes to 20μM DA plus 120U tyrosinase. To test if the latter oxidation products of DA show a different effect on ATPase activity DA and Ty were preincubated 5 minutes and after mitochondria were incubated with this mix for 10 minutes. 50μg of proteins from this sample were loaded in lane 3.

was not possible to detect a significative differences between DAQs-modified complex V activity respect to the control.

Further studies are necessary to investigate the consequences of DAQs-modification of γ subunit of the F_1F_o ATP synthase complex. It is now clearly established that dimerization of the F_1F_o ATP synthase takes place in the mitochondrial inner membrane. Interestingly, oligomerization of this enzyme seems to be involved in cristae morphogenesis. As they were able to form homodimers, subunits 4, ε, and γ have been proposed as potential ATP synthase dimerization subunits. The hypothesis that DAQs-modified F_1F_o ATP synthase can alter mitochondrial morphology of the cristae is under study.

Conclusions

The present studies have shown that the oxidized products of the neurotransmitter DA can enter the mitochondria crossing both the outer and the inner membranes of the organelle. Moreover these reactive species are able to bind and covalently modify several mitochondrial proteins. The ability of DAQs to modify proteins at sulphydryl residues of proteins has been extensively reported. Moreover several evidences of the ability of DAQs to bind and inhibit cytosolic proteins as TH, DAT and parkin are present in literature. Evidence of DAQs ability to enter and modify mitochondrial proteins is a crucial step in the study of mitochondrial impairment in DA neurons in PD. This study has lead to the identification of the γ subunit of the ATP synthase complex as one of the protein modified by DAQs. Anyway assays to test the activity of modified ATP synthase complex failed to shown impairments in ATP synthesis and hydrolization activity of complex V. Further experiments are required in order to understand if the binding of DAQs to the γ subunit of the ATP synthase complex can impair other functions of complex V, as e.g. the structural role of complex V in the formation of the cristae of the IMM. Moreover investigation to identity the other DAQs-modified proteins in mitochondria are necessary and they can shed light on the specific vulnerability of DA neurons and the mitochondrial dysfunction in PD.

Part II: Pathological effects of oxidized products of dopamine in mitochondria.

DAQs induce mitochondrial dysfunctions

Mitochondria are key regulator of cell survival and death. A major role for mitochondrial dysfunctions has been claimed in the pathogenesis of Parkinson's disease (PD). Mitochondrial dysfunction have been also assessed in PD patients, and in particular defects in mitochondrial complex I of the respiratory chain has been shown in the SNpc of sporadic PD (*3*). This defects seems to be present also in other tissue of PD patients, since several evidence are reported for mitochondrial impairment also in platelets and muscles (*117*).

A role of the neurotransmitter DA in the oxidative stress that lead to dopamine neurons cell death in PD has been suggested for long time. As already described in this thesis, the neurotransmitter DA can give rise to reactive species in its normal metabolism or undergoes to spontaneous or catalyzed oxidative reactions if accumulated in the cytoplasm of the cell. These reactive species can lead to oxidative damage if not efficiently removed by antioxidant system of the cell.

Previous studies on mitochondrial functions following exposure to oxidized products of DA have shown that DAQs are able to induce several mitochondrial damages as inhibition of mitochondrial respiration, increase of ROS production and induction of apoptosis (*89, 110-115*). Interestingly the DAQs ability to induce the mitochondrial PT in brain mitochondria has been also shown (*110*).

Mitochondrial permeability transition (PT) is an increase in the permeability of the mitochondrial membranes to molecules of less than 1500

Daltons in molecular weight. PT results from opening of mitochondrial permeability transition pores (mPTP) (*118-120*). The mPT pore is a proteinaceous pore that is formed in the inner membranes of mitochondria under certain pathological conditions such as traumatic brain injury and stroke. Induction of the permeability transition pore can lead to mitochondrial swelling and cell death and plays an important role in some types of apoptosis (*121-124*). The MPTP was proposed by Haworth and Hunter in 1979 and has since been found to be involved in, among other things, neurodegeneration, a process that results in damage and death of neurons (*125*). MPT is frequently studied in liver cells, which have especially large number of mitochondria. *In vitro* PT is accompanied by depolarization, matrix swelling, depletion of matrix pyridine nucleotides (PN), outer membrane rupture and release of intermembrane proteins, including cytochrome c (*123, 126*).

Statement of purpose

PT is claimed as one of the pathway of beginning for the mitochondrial apoptosis. In PD the apoptotic pathway seems to be highly activated (*7, 8*). The ability of DAQs to induce PT can account as one of the way that lead to the massive loss of dopaminergic neurons in PD. Of great interest is then to investigate the mechanism by which DAQs induce PT on mitochondria. As shown in chapter I of this thesis DAQs have the ability to cross both mitochondria membranes, enter the mitochondrial matrix, and covalently modify several mitochondrial proteins.

The structure of the mPTP is hotly debated and the proteins that constitute or regulate the PT are still not defined. Nonetheless several conditions and molecules that modulate the mPTP conformation open/close are well characterized as well as protocols to study the PT phenomenon (*See also*: Materials and Methods, Mitochondrial Swelling) .

An analytical study on the ability of DAQs to induce PT in mitochondria and the investigation of mechanism of its induction were carried out and the results are explained in this chapter.

Results

DAQs Induce swelling in mitochondria

The characterization of the mechanism of DAQs induction of the permeability transition (PT) in mitochondria was studied in collaboration with the laboratory of the Prof. Paolo Bernardi at the Department of Biomedical Sciences of the University of Padova.

Two types of experiments were used to evaluate the functional effects of DAQs-mitochondrial swelling and modification of the calcium retention capacity (CRC). Experimentally swelling of mitochondria can be followed at the spectrophotometer as a decrease of light scattering of the mitochondrial suspension due to the increase of mitochondria dimensions (*127*). The other experimental protocol extensively used to study the induction of PT by DAQs is the calcium retention capacity (CRC) experiment. Mitochondrial Ca^{2+} is controlled by a transport cycle driven by the proton pumps of the respiratory chain. The transport cycle is mediated by the Ca^{2+} uniporter, the Na^{+}/Ca^{2+} antiporter and Na^{+}/H^{+} antiporter of the inner membrane. Under physiological conditions, mitochondrial Ca^{2+} controls key regulatory dehydrogenises in the mitochondrial matrix. Under pathological conditions associated with cellular ATP depletion and oxidative stress, mitochondrial Ca2+ triggers opening of the PT pore (*122*). This is possible because Ca^{2+} binds to and activates Ca^{2+} binding sites on the matrix side of the mPTP. The presence of free radicals, another result of excessive intracellular calcium concentrations,
can also cause the mPT pore to open.

The amount of Ca^{2+} that mitochondria can store under different solution conditions can be measure in the CRC experiments. In this fluorimetric assay

mitochondria are resuspended in presence of a fluorescent probe Calcium green-5N that cannot permeate into the mitochondria. Small amounts of calcium are then added and Ca^{2+} is sequestered by the functional mitochondria through the Ca^{2+} uniporter. When the amount of calcium in the matrix reach a threshold of excitotoxicity the mPT pore open with subsequent release of the calcium in the medium. The fluorescent signal of the probe becomes then visible. Analysis of the amount of calcium added to the mitochondria in different conditions allows defining the Calcium Retention Capacity of mitochondria. A decrease of CRC of treated mitochondria respect to the control can be read as an ability of the studied compound to induce PT. Verification of this hypothesis can be carry on by repeating the CRC experiments in the presence at the same time of the studied compound and CsA. CsA is a powerful inhibitor of PT pore opening binding the matrix protein cyclophillin D. Genetic inactivation of genes encoding putative pore components have conclusively demonstrated the role of matrix cyclophilin D as the mitochondrial receptor for the desensitizing effects of cyclosporin A (*118*). Nevertheless the mechanism by which CsA keeps the mPTP in the close conformation by binding cyclophillin D is still not fully understood.

Results from these experiments allow concluding that DAQs induce the opening of the PTP in a dose dependant manner between 20-200μM DA (**Figure 23**). Moreover, the CRC experiments confirmed that DAQs are an act as inducers of PT decreasing the CRC (**Figure 24**). For both these protocols controls experiments with the specific inhibitor of the PTP Cyclosporine A (CsA) where carried out. CsA is able to reverse the effect of DAQs on the PTP opening and these results confirm that the mechanism by which DAQs induce the swelling of mitochondria is opening the permeability transition pore.

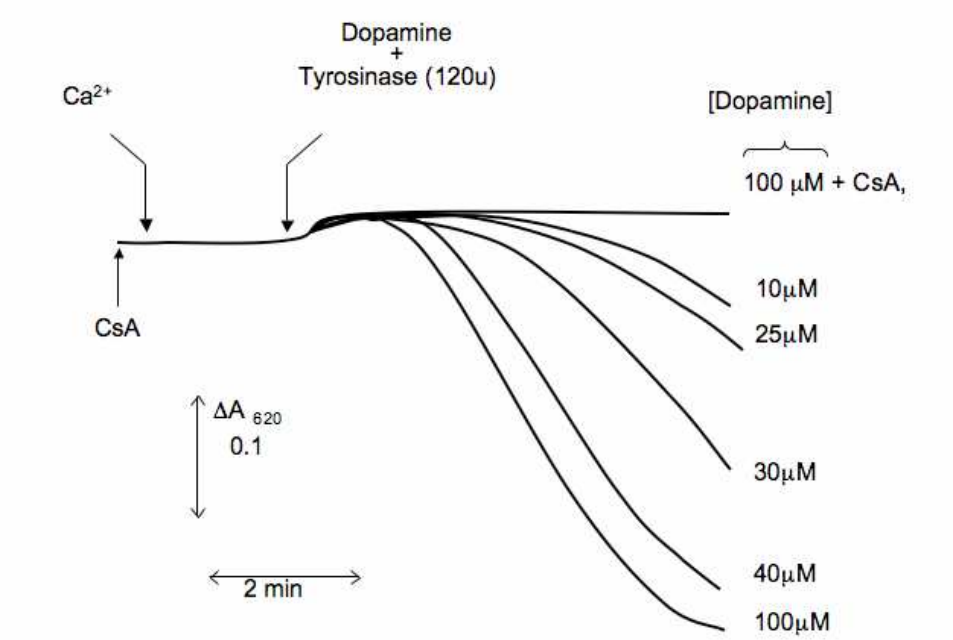

Figure 23 DAQs induce swelling in mitochondria

Mouse liver mitochondria (0.5mg/ml) were incubated with increasing amount of DA in presence of the enzyme tyrosinase. The decreasing in absorbance, correlated to the mitochondrial swelling, was followed spectrophotometrically at 620nm. Results from these experiments show that DAQs are able to induce the PT in mitochondria in a dose-dependant manner. To prove that this effect was due to the mitochondrial permeability transition pore (mPTP) opening, the same experiments were repeated in presence of the mPTP inhibitor CsA. As shown in the picture for the 100μM DA trace, CsA is able to completely revert the effect of DAQs.

The early oxidized products of DA are more toxic compared with the late ones.

The reaction of DA oxidation includes several steps that lead to melanin as final product (*81*). Of great interest was to define which among the several intermediates of the oxidation reaction has the more significant effect toward the induction of mitochondrial permeability transition. The laboratory in Padova invested a great deal of work in the chemical characterization of the several oxidation products of dopamine. In the presence of the enzyme tyrosinase dopamine is oxidized to dopamine-o-quinone (DAQ) that spontaneously

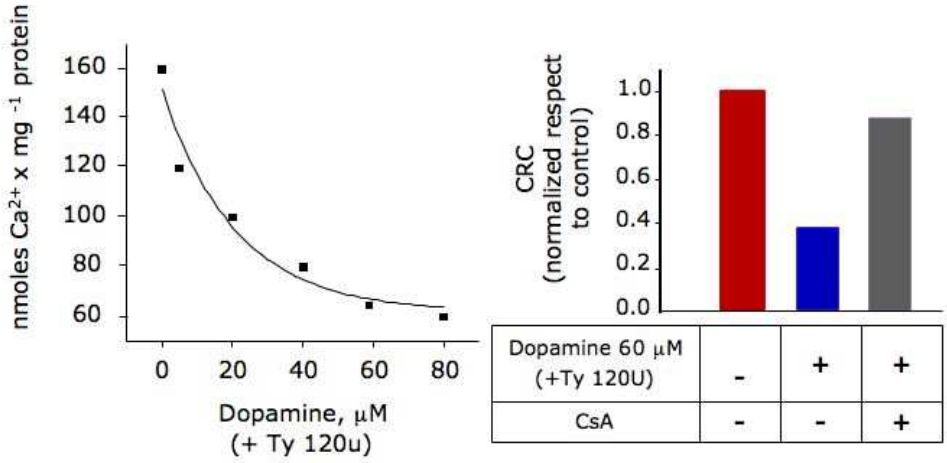

Figure 24 Calcium retention capacity of mouse liver mitochondria treated with DA and tyrosinase.

Mouse liver mitochondria (0.5mg/ml) were incubated in the presence of the fluorescent probe calcium green 5N, Calcium green 5N is fluorescent in presence of calcium and is excluded from mitochondria. Mitochondria are able to store Ca2+ in the matrix through the calcium uniporter. When Ca2+ reaches a threshold of toxicity it activates some still unknown sites triggering the mPTP opening and Ca2+ is released in the medium. In this protocol the amount of calcium that mitochondria can import in the presence of different amounts of the PT inducer DAQs is measured. Small mounts of calcium (10µM) are added every min until the PT happens with release of calcium in the medium visible at the fluorimeter as an increased of fluorescence of the probe calcium green 5N. The graph on the left shows that between 10 and 80µM DA (+120U tyrosinase) the calcium retention capacity of mitochondria decrease in a dose-dependant manner. The same experiments were repeated in the presence of the mPTP inhibitor CsA and results (on the right) show that CsA protect mitochondria from DAQs toxicity.

ciclyzates to form the molecule leukoaminochrome. This catechol molecule can be oxidized by tyrosinase to the quinone aminochrome (AC) that in a spontaneous reaction rearrange to form the 5,6-dihydroxyindole (DHI). DHI can be oxidized again by tyrosinase to indole-5, 6-quinone that combine with DHI to form the polymer melanin (*81*).

The complexity of this reaction and the high reactivity of some of the intermediates hinder the possibility to isolate these molecules and maintain them stable in the physiologic buffers in order to single out the individual effect on mitochondria. The strategy chosen to highlight the reactivity of the several

compounds was to incubate mitochondria with the products of subsequent time points of the oxidative reaction, and compare the swelling induction effect.

Results from these experiments show that the earlier products of the DA oxidation are more effective to induce the PT than the later ones. Since in the reaction mix different products are present at the same time the identification of the specific reactivity for the different products have to be investigated (**Figure 25**).

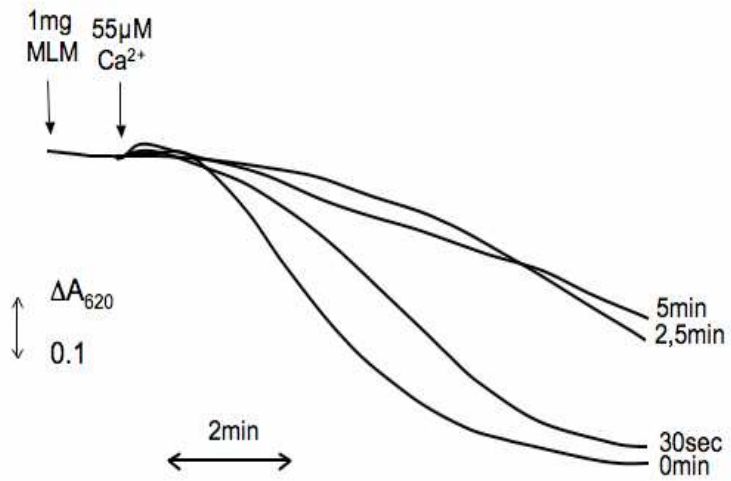

Figure 25 Mitochondrial swelling at different time point of the DA oxidation reaction: the early products are more toxic than the late.

Mitochondria purified from mouse liver (0.5mg/ml) were resuspended in medium containing glutamate and malate as oxidizing substrates for the ETC. Different time points of the oxidation reaction of 60µMDA plus tyrosinase were prepared separately and added to the mitochondria solution and the decreasing in absorbance at 620nm, corresponding to mitochondria swelling, was followed at the spectrophotometer. Results from these experiments show that the early products of DA oxidation reaction are more effective than the late ones to induce PT in liver mitochondria. I these conditions the first oxidation is catalized by the enzyme Ty and the spontaneous cyclization of the first product dopamine-o-quinone to leukoaminochrome is the rate limiting step for the formation of the second quinone aminochrome. Since cyclization is favored by acidic condition and the pH of the medium is 7.4, the dopamine-o-quinone, seems to be the most reactive species

DAQs toxicity involved the pyridine nucleotide pool.

In Costantini et al., 1996, authors describe two separate sites on the pore that contains vicinal thiols (*128*). These thiols groups regulate PTP functions through their redox state. Thiols/disulfide conformation on these residues depend on the redox state of the matrix pool of pyridine nucleotide for one of site and on the glutathione redox state for the other site (*128*). Authors show that selective alkylation of this two sites with monothiol-binding compound makes possible to point out if a molecule induce PT with a mechanism that involve redox state of PN or GSH pool.

Based on the reported high capacity of DAQs to react with the free thiols of GSH this protocol was employed to investigate the PT induction mechanism of DAQs. The experiments were carried out as explained in *Materials and Methods* of this thesis. The results show that DAQs ability of induce PT is reversed if the thiols groups in equilibrium with the PN pool are blocked with the alkylating reagent N-ethylmalemide (NEM) instead no differences are noticed if the thiols in equilibrium with GSH pool are blocked (**Figure 26**). To further investigate the nature of the interaction between DAQs and the pyridine nucleotides pool, experiments with the molecule β-idroxybutirrate (BOH) where carried out. This molecule is able increase the amounts of NADH in the matrix via the reaction catalyzed by the enzyme acetate dehydrogenise. Acetate dehydrogenase use BOH as oxidizing substrate to produce acetate and reduce NAD^+ to NADH. Swelling experiment where performed to test the protective effect of the BOH and results from these experiments show that if the PN pool is maintained in the reduced state mitochondria are protected from DAQs toxicity (**Figure 27**). These interesting results let to the hypothesis that DAQs induce PT altering (directly or indirectly) redox state of the PN pool in the mitochondrial matrix. The redox state of PN pool has been prove to be one of the factors that affect the PTP state, in particular oxidation of PN induce the pore opening (*129*). With these premises the state of PN after mitochondria incubation with DAQs was assessed.

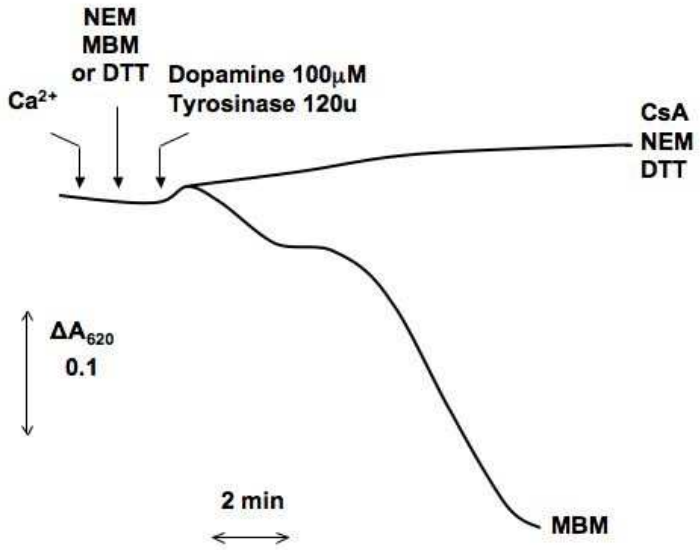

Figure 26 DAQ induce swelling through alteration of the pyridine nucleotide (PN) pool in mitochondria.

Mitochondria from mouse liver (0.5mg/ml) were incubated in medium containing glutamate and malate as oxidizing substrates. An amount of Ca^{2+} under the threshold necessary to trigger mPTP opening was added to accelerate mPTP opening by subsequent treatments. Alkylating reagents as monobromobimne (MBM) or N-ethylmaleimide (NEM) or reducing agent dithiotrehytol (DTT) were added after 1 minute. DA (100μM) and tyrosinase (120 units) were added to the solution after another minute and absorbance at 620nm was followed at the spectrofotometer. Results from these experiments reveal that if mitochondria are preincubated with the alkylating agen NEM, DAQs are not able to induce the PT. This result suggest that DAQs can induce PT with a echanism that involve thiols alkylation and in particular the thiols in equilibrium with the pyridine nucleotide (PN) pool in the matrix seem involved in this mechanism.

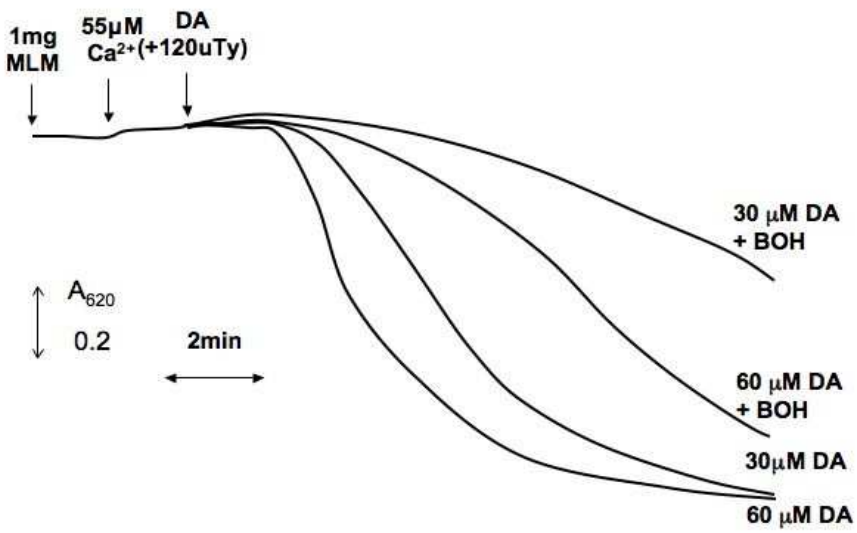

Figure 27 The maintenance of PN pool in the reduced state protects toward DAQ-induced mitochondrial swelling

Mitochondria from mouse liver (0.5mg/ml) were incubated in medium containing glutamate and malate as oxidizing substrates. An amount of calcium under the threshold that trigger PTP opening was added after 1 minute to accelerate PT in subsequent treatment. 30µM or 60µM DA were added to the mitochondrial suspension together with the enzyme tyrosinase (Ty, 120 units). β-idroxybutirrate (BOH) was added when indicated and the decrease of absorbance at 620nm corresponding to mitochondrial swelling was followed spectrofotometrically. Results from these experiments show that if NADH is maintained reduced by BOH presence, itochondria are protected from DAQ-induced swelling, reinforcing previous data of an involvement of PN oxidation in DAQ-induced PT in mitochndria.

DAQs oxidize the pyridine nucleotide in mitochondria.

Experiments to evaluate the redox state of PN pool after DAQs treatment of mitochondria were carried out. As explained in Materials and Methods section the pyridine nucleotides were extracted from mitochondria after DAQs treatment and an enzymatic assay was performed to estimate the amount of NADH and NADPH separately. Data from these experiments demonstrate that NADH in mitochondria exposed to small amounts of DAQs (20µ DA) is oxidized of 40% respect to the control (**Figure 28**).

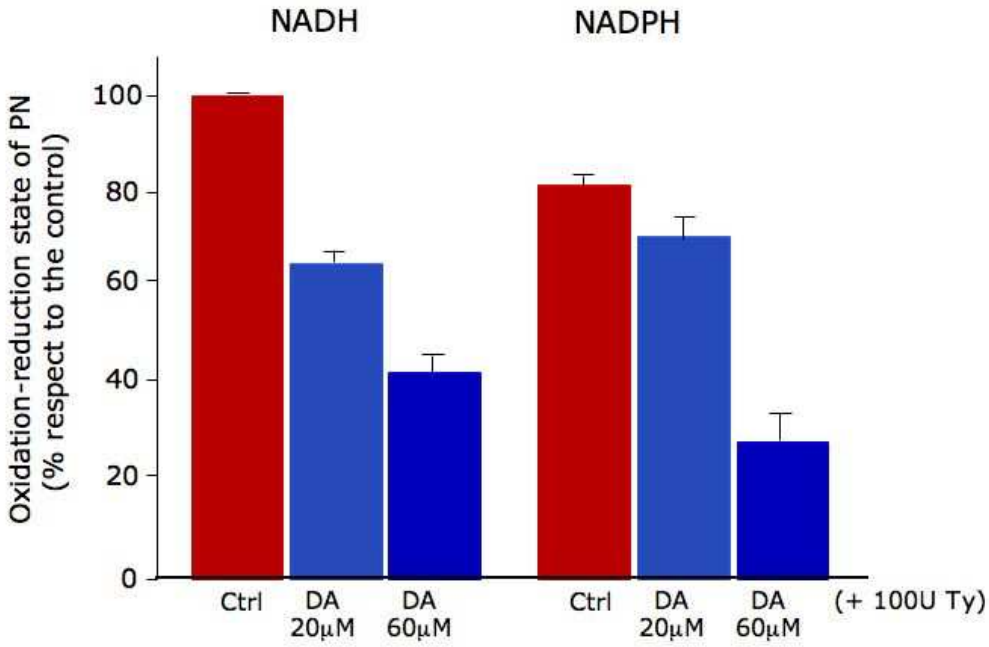

Figure 28 DAQs oxidize the pyridine nucleotides in isolated mitochondria.

Mouse liver mitochondria were purified and exposed to DAQs as described in *Materials and Methods*. The amounts of reduced NADH and NADPH were assessed and compared to the untreated controls. Results from these experiments show that DAQs are able to decrease the reduced amounts of pyridine nucleotide in mitochondrial matrix. Further studies are necessary to establish if this oxidation is due to a direct redox reaction between the electrophiles DAQ and the reduced NAD(P)H or to other mechanism.

These results show a toxic effect of DAQs toward mitochondria and lead to hypothesize a mechanism by which DAQs induce the PT, since an oxidation of the PN pool is recognized as inducing factor of mPTP opening (*129*). The oxidation of PN can also lead to others detrimental effect in mitochondria, among them an impairment of mitochondrial respiration, since PN are the electronic donors of the complex I of the ETC. Further studies on this aspect of the toxicity of DAQs in mitochondria can highlight the consequences of DAQs-induced PN oxidation in mitochondria.

To point out if DAQs can directly react with NADH *in vitro* experiment were carried out (**Figure 29**). The reaction between DA and DAQs with NADH was followed specrophotometrically. The oxidation of NADH can be detected by the decrease of the signal at 340nm since NADH has a characteristic peak of

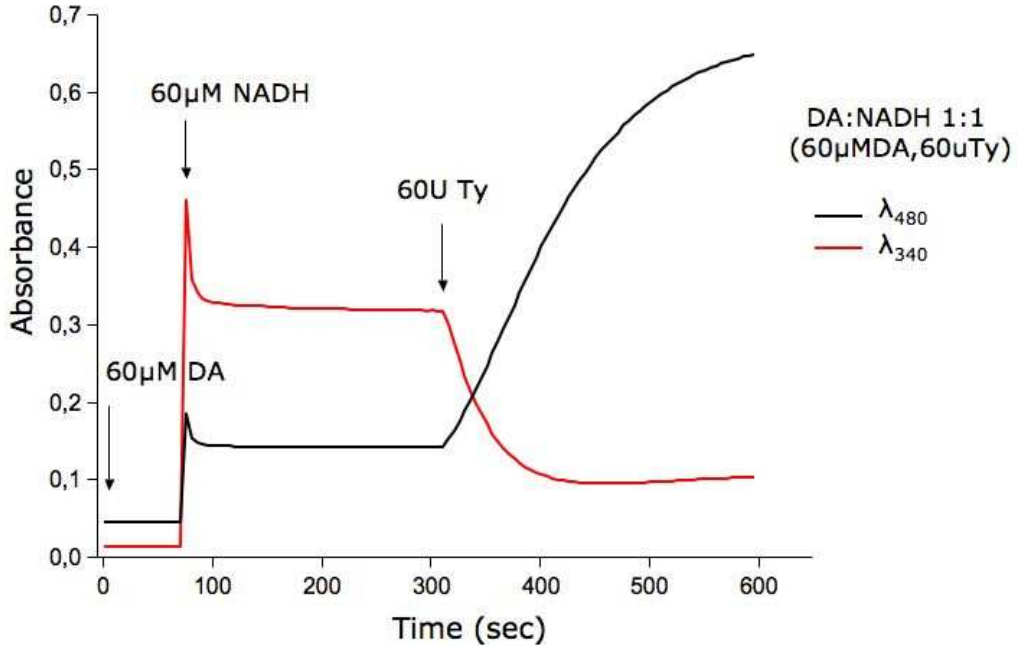

Figure 29 DAQs oxidize NADH *in vitro*

The reaction between DAQs and NADH was studied spectrophotometrically *in vitro*. Oxidation of NADH to NAD^+ was followed at 340nm were NADH but not NAD^+ has a characteristic absorption peak. Dopamine-o-quinone formation was followed at 480nm. As shown in the spectra above DA and NADH do not react instead when tyrosinase (Ty) enzyme is added in the cuvette the dopamine-o-quinone formation is accompanied by NADH oxidation, sign of a redox reaction between the electrophiles DAQs and the reduced NADH. These data suggest one possible meccanism of DAQs toxicity in mitochondria since NADH is one of the oxidizing substrates of complex I of the ETC and its depletion has been shown to induce PT. Both these events are detectable in mitochondria exposed to DAQs

absorption at 340nm and NAD^+ does not. Results from these experiments (Fig) show that as DAQs is formed (signal at 480nm) NADH is rapidly oxidized (signal 340nm) claiming for a direct redox reaction between DAQs and NADH.

DAQs depolarize mitochondria a cellular model.

Since all the previous experiments were performed in *in vitro* systems, was of great interest to explore if DAQs have the ability of induce PT in a cellular model. To probe this toxic effect of DAQs could contribute to understand the unknown mechanism of dopaminergic neurons loss in PD. PTP opening leads to

mitochondrial membrane depolarization and this effect was followed in a neuroblastoma SH-SY5Y cell line exposed to DA and tyrosinase. Mitochondrial membrane potential was measured based on the accumulation of tetramethylrhodamine methyl ester (TMRM) as described in the Materials and Methods section of this thesis. Results from these experiments show that DAQs induce depolarization of mitochondria *in vivo* (**Figure 30**). To prove that this effect is due to PTP opening SHSY-5Y cell were exposed to DAQs in presence of the PTP inhibitor CsA. CsA protects mitochondria from DAQs toxicity confirming that the oxidized products of DA can be toxic toward mitochondria *in vivo*.

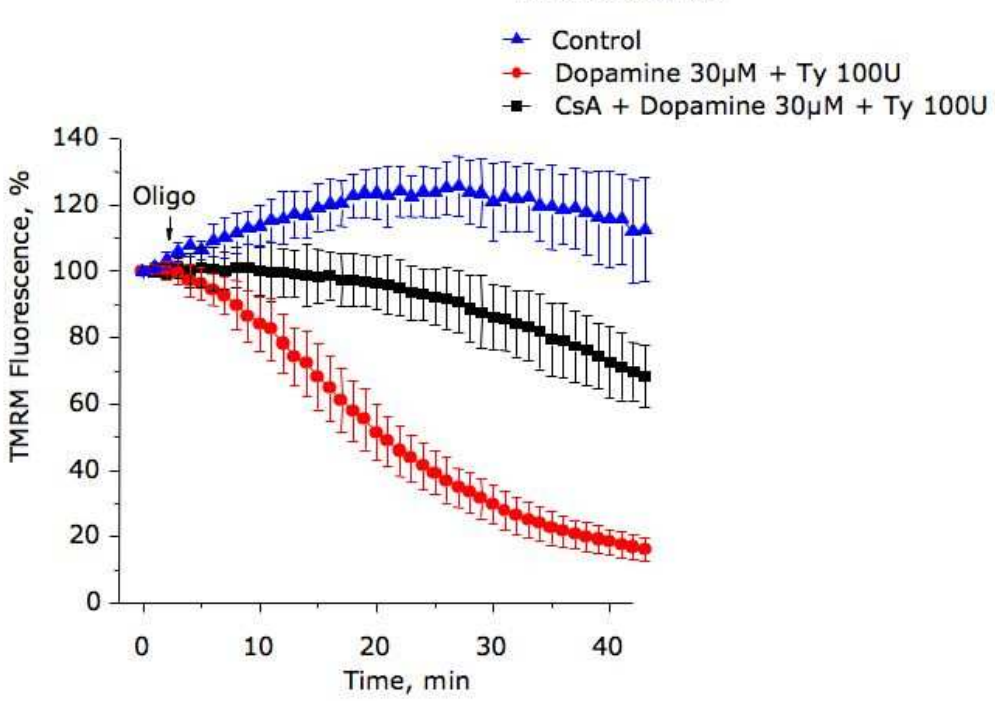

Figure 30 Changes of mitochondrial TMRM fluorescence induced by DAQs in SHSY-5Y neuroblastoma cells.

Neuroblastoma cell line SHSY-5Y were loaded with the fluorescent probe tetramethylrhodamine methyl ester (TMRM) and studied as described in the *Materials and Methods*. Oligomycin (Oligo) was added in every experiment to avoid depolarization due to the F_1F_o ATPase complex. Cluster of mitochondria (10-30) were chosen as region of interest. Cells were exposed to 30μM DA and 100U of the enzyme tyrosinase (Ty). The same experiments in presence of the inhibitor of the mPTP CsA were carried out. Sequential digital images were acquired every 2 min, and the average fluorescence intensity of all relevant regions was recorded and stored for subsequent analysis. Results from these experiments show that DAQs depolarize mitochondria *in vivo* and that CsA protects at least in part, mitochondria from depolarization suggesting that DAQs-induce depolarization is due to induction of mPTP opening.

Conclusions

A critical role of mitochondrial dysfunction and oxidative damage has been claimed in the pathogenesis of neurodegenerative diseases as Parkinson's disease (PD), Alzheimer's disease, Huntington's disease. Historically mitochondria dysfunction has been associated with PD based on the observation that accidental exposure to the environmental toxin 1-methyl-4-phenyl-1,2,3,4-tetrathydropyridine (MPTP) results in development in parkinsonian syndrome (*10*). MPTP is a weak inhibitor of mitochondrial complex I and its toxic mechanism seems to involve oxidative damage by increase of ROS production and DA oxidation (*8*). Moreover chronic exposure to the pesticide rotenone, another complex I inhibitor reproduce the main pathological hallmarks of PD as loss of dopaminergic neurons in the substantia nigra, α-synuclein positive inclusions and the characteristic motor symptoms in mice (*9*). Mitochondrial dysfunction has been reported in the SN of PD patients and in other tissues outside the brain (*3*, *117*) .

In the scenario of a global mitochondrial dysfunction in PD what becomes interesting is to investigate what makes dopaminergic neurons more vulnerable to a mitochondrial deficiency. A role for DA as endogenous toxin has been claimed for long. Deregulation of DA synthesis, storage or metabolism can lead to DA oxidation in the cytoplasm with accumulation of toxic products as ROS and DA quinones. Taken together these evidence lead to the hypothesis that a mitochondrial chronic defect or protein mutation can be exacerbated in dopaminergic neurons by presence DA and its toxic metabolites.

The research presented in this chapter was focused on the characterization of the toxic effects of the oxidized products of DA on mitochondria. DAQs are able to cross both mitochondria membranes and covalently modify several mitochondria proteins (Part I of this thesis). The pathological consequences, if any, of this interaction were investigated. Previous evidence report the ability of DAQs to induce PT in brain and liver mitochondria (*110*). This result was the starting point for the analytical characterization of the ability of DAQs to induce PT in liver mitochondria. Swelling experiments were carried out testing several conditions of substrates and DA concentrations. CRC experiments were also

performed to quantify DAQs induction effect. The results presented in this chapter show that the oxidized products of the neurotransmitter DA can induce the permeability transition (PT) in a dose dependant manner between 5-100μM DA in presence of tyrosinase enzyme as oxidizing agent of DA. On the contrary in the same conditions DA or tyrosinase alone are not able to induce PT. CsA, a specific inhibitor of PT, completely reverts DAQs effect proving that DAQs-induced mitochondrial swelling is due to opening of the mPTP. Calcium retention capacity experiments confirmed the PT induction ability of DAQs and allowed to quantify the effect, in Ca^{2+} nmol that the treated mitochondria are able to store before mPTP opening.

The mechanism by which DAQs induce PT was investigated and alteration of the redox state of matrix pyridine nucleotide pool of mitochondria was shown. Oxidation of NAD(P)H is one of the known mechanism to induce PT(*129*). Finally the ability of DAQs to induce PT in a cellular model was shown. Since PT has as final consequence the disruption of OMM and release of the pro-apoptotic factors that resides in the intermembrane space, these data lead to the hypothesis that DAQs-induced PT can contribute to the loss of dopaminergic neurons in PD. Taken together, these data provide an additional biochemical mechanism by which DAQs may affect cell function, and support the idea that mitochondrial damage is an important determinant in DAQs toxicity.

Part III: Complex I and Parkinson's disease

Complex I

Complex I (reduced nicotinamide adenine dinucleotide–ubiquinone oxido-reductase, or NADH:ubiquinone oxidoreductase) is the first complex of the electron transport chain (ETC) in mitochondria. Among all the others four complexes of the ETC it has the most complicated structure and genetics. It is the largest complex of the respiratory chain and its relative molecular mass is ~980 000 Da. In mammals, it is composed at least of 45 subunits (identified so far), of which 7 are encoded by the mitochondrial genome (mtDNA) and the remaining by the nuclear genome(*130*). Complex I catalyzes the transfer of electrons from NADH to coenzyme-Q_{10} or ubiquinone via a flavin mononucleotide cofactor and iron sulfur centers. The pathway of electrons occurs as follows: NADH is oxidized to NAD^+, reducing flavin mononucleotide to $FMNH_2$ in one two-electron step. The next electron carrier is a Fe-S cluster, which can only accept one electron at a time to reduce the ferric ion into a ferrous ion. In a convenient manner, $FMNH_2$ can be oxidized in only two one-electron steps, through a semiquinone intermediate. The electron thus travels from the $FMNH_2$ to the Fe-S cluster, then from the Fe-S cluster to the oxidized Q to give the free-radical (semiquinone) form of Q. This happens again to reduce the semiquinone form to the ubiquinol form, QH_2. Complex I also has proton-transporting activity over the inner mitochondrial membrane to the intermembrane space, indeed during this process, four protons are translocated across the inner mitochondrial membrane, from the matrix to the intermembrane space. This creates a proton gradient that will be later used to generate ATP from ADP through oxidative phosphorylation. . Complex I is one of the main sites at which premature electron leakage to oxygen occurs, thus being one of main sites of production of a harmful free radical called super oxide.

The great complexity of mammal complex I in comparison with other complex I from different species has made hypothesize that it can exerts supplementary functions. Fontaine et al. have suggested that the electron flux through complex I regulate the mitochondrial PTP (*131*), since when respiration is supported by complex I substrates the PTP opened at much lower calcium loads than with complex II and IV substrates. This effects was directly related to the rate of electron flow through complex I and not to membrane potential or the redox status of pyridine nucleotides in the matrix. Moreover rotenone, a specific inhibitor of the electron flux through complex I, delays PTP opening and increased CRC in mitochondria (*132*). Involvement of complex I in cell death pathways has also been claimed, based on Cao's laboratory observation that a mammal complex I subunit, GRIM19, is released in the cytoplasm of apoptotic cells (*133*).

A deficiency in complex I is the most frequently encountered cause f mitochondrial disease and mutation in several nuclear DNA-encoded subunits cause devastating neurological disorders as Leigh syndrome or Leber's Hereditary Optic Neuropathy (LHON) (*134*) . Milder defects of complex I has also been claimed from long time to play a central role in several neurodegenerative diseases as Parkinson's disease, Alzheimer's disease and Huntington's disease.

Complex I is impaired in PD

Several lines of evidence point to an inhibition of complex I activity in PD. The first evidence of complex I impairment in PD was reported in 1983 by Langston and colleagues (*10*) when they identified 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a side-product of the synthesis of an heroin analog, as the cause of development of acute, severe and permanent Parkinsonism in several drug addicts in northern California. MPTP is a pro-toxin that enters the brain and is then metabolized by monoamine oxidase B to 1-methyl-4-phenylpiridinium ion (MPP^+). MPP^+ is accumulated in dopaminergic neurons and being an excellent substrate of the dopamine transporter (DAT) and in these neurons accumulates in mitochondria where inhibits the ETC at the level of complex I (*8*).

Other evidences stand for an inhibition of complex I in idiopathic PD. Shapira and colleagues reported defects in complex I and reduced glutathione in the substantia nigra of PD patients (*3*). Complex I deficits seems affect also tissue outside the brain, several studies have reported reduced complex I activity in the platelets of people with PD (*135-138*). Others studies with cells without mtDNA (ρ0 cells) fused with platelets from PD patients (cybrid) show that the defect in complex I activity can be stably transferred to ρ0 cells (*4, 139*). These studies strongly suggest that complex I defects in PD might be due to mtDNA mutations but efforts so far to identify these mutations have been unsuccessful. Upon these findings a systemic defect of complex I in idiopathic PD has been hypothesized

The in vivo rotenone model of Parkinson's disease is one powerful tool to help to clarify this question (*9*). Rotenone is a high affinity inhibitor of complex I used as organic pesticide. Moreover rotenone extreme lipophilic nature allows it to cross easily biological membranes and enter the brain without specific transporters. Systemic administration of rotenone in rats cause chronic and uniform inhibition of complex I in several organs like brains, kidney hearth and liver. Anyway after some weeks selective degeneration of neurons in the SN occurred. Moreover many of the dying neurons have large cytoplasmatic inclusions containing alpha-synuclein and ubiquitin as Lewy bodies in PD. Dopaminergic degeneration is accompanied by marked increase in oxidative protein damage only in affected brain region similar to which reported in PD. Finally rotenone-infused animals developed symptoms of Parkinsonism including bradikinesia and rigidity. A crucial implication of this work is that dopaminergic neurons in the substantia nigra have intrinsic sensitivity to complex I defects, since are the ones that die while complex I is uniformly inhibited throughout the brain (and the rest of the body). Since the degeneration occurred at level of complex I inhibition that don't significantly impair respiration, the mechanism of rotenone toxicity is not related to a bioenergetic deficit. The mechanism of rotenone toxicity is an increased oxidative stress and oxidative damage of mitochondria and the whole cell (*11*). Other experimental models of PD also suggest oxidative damage as a key mechanism of dopaminergic degeneration (*8*).

Under physiological conditions complex I is one of the main sites at which premature electron leakage to oxygen occurs, thus being one of main sites of

production of a harmful free radical called super oxide. Partial inhibition of complex I greatly increased the ROS production by complex I (*11*). Interestingly it has been shown that the single subunit rotenone-insensitive NADH-quinone oxidoreductase (Ndi1) of *Sacharomyces cerevisiae* mitochondria can restore NADH oxidation in complex I-deficient mammalian cells. The Ndi1 enzyme is insensitive to complex I inhibitors such as rotenone and 1-methyl-4-phenylpyridinium ion, known as a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Injection of recombinant adeno-associated virus carrying the Ndi1 gene into the vicinity of the substantia nigra in rat resulted in expression of the Ndi1 protein in the entire substantia nigra. Resistance to the deleterious effects caused by rotenone in these animals has recently been shown (*111, 114, 115, 140, 141*).

What is still unclear is why a systemic defect as the increased production of ROS by the rotenone-inhibited complex I lead primary to dopaminergic neurons loss sparing others neuron kinds and cell types. One of the hypotheses for the intrinsic sensitivity of dopaminergic neurons of the substantia nigra is related to the presence of dopamine (DA) itself. Dopamine neurons are believed to exist in a constant state of oxidative stress and dopamine itself can undergo to spontaneous or catalyzed oxidative reactions that give raises several toxic reactive species as previously described in this thesis (Results, Part I and II of this thesis).

Presence of DA can be the reason why a mild defect in ETC and in particular at the level of complex I that increase oxidative stress has deleterious effects in dopaminergic neurons where DA can undergoes to oxidation and generates several toxic species.

Interestingly evidence in literature suggest a direct toxic role of DAQs toward complex I, as dose-dependent inhibition of complex I in disrupted mitochondria and in a neuroblastoma cell line (*111, 114, 115*). Moreover has been shown that increasing the amount of ROS normally produced by complex I in rat brain mitochondria exposed with DAQs (*113*)

Two hypothesis could be formulated for a toxic effect of the oxidized products of DA toward complex I: first DAQs can increase the electron leakage from complex I to oxygen and then increase the reactive oxygen species normally produced by complex I, secondly DAQs can impair complex I functions

covalently bind and modify protein subunits of the complex leading to a loss of structure and function of proteins and the entire complex.

Statement of purpose

The toxic effects of the oxidized products of DA toward complex I was investigated. Several approach were pursued to characterize the interaction between DAQs and complex I. The ability of DAQs to inhibit mitochondrial respiration was tested by oxygraphic measures and a specific assay for complex I activity was set. The ability of DAQs to bind and covalently modify complex I subunits was also investigated. The toxic activity of DAQs was also compared to the one of the specific inhibitor of complex I rotenone and the competition between DAQs and rotenone to bind complex I subunits was compared.

Dopaminergic cells expressing the rotenone-insensitive complex I subunit Ndi1 was used to point out the specificity of DAQs toward complex I (*11*). If cells expressing Ndi1 subunit are resistant or protected from DAQs toxicity too, is possible to hypothesize parallel effects of DAQs and rotenone toward mitochondrial complex I.

Results

DAQs inhibit mitochondrial respiration.

NADH and NADPH are the electrons donor of the first complex of the oxidative phosphorilation chain in mitochondria. As DAQs are able to oxidize the pyridine nucleotide pool in a redox reaction (Results, Part II, of this thesis), is reasonable to hypothesize that this effect leads to an impairment of the mitochondrial respiration. Data present in literature (*110*) show the faculty of DAQs to inhibit the state 3 of mitochondrial respiration. During mitochondrial respiration, molecular oxygen is reduced to water by complex IV (cytochrome oxidase complex), in the last step of the electronic transport chain.

Experimentally, mitochondria respiration is assessed by oxigraphic measurement of the decrease of molecular oxygen dissolved in a mitochondria suspension. Conventionally mitochondria respiration is divided in three states. State 4, or resting respiration, is defined as oxygen consumption of isolated mitochondria in the presence of oxidizing substrates and in the absence of ADP. State 3, or active respiration, is ADP-stimulated respiration in presence of oxidizing substrates. The uncoupled state is defined as a condition in which the rate of electron transport can no longer be regulated by an intact chemiosmotic gradient, and is measured after addition of an uncoupler agent as 2,4-dinitrophenol (DNP) or carbonyl cyanide p-[rifluoromethoxyl]-phenyl-hydrozone (FCCP). DNP and FCCP act as proton ionophore binding protons on one side of the membrane and being fat soluble, drifting to the opposite side where they lose the protons, dissipating the chemiosmotic gradient and leaving the electron transport system unhibited. Oxygraphic measurements were carried out to evaluate the respiration capacity of mitochondria. Accurate precautions were taken to remove the enzyme tyrosinase and the non-reacted DA from the samples. Since tyrosinase requires molecular oxygen as co-substrate in the oxidation of its substrates removal of tyrosinase is crucial to avoid measurement of oxygen consumed in the tyrosinase oxidative reaction.

Data from these experiments show that after incubation with DAQs all the three states of mitochondrial respiration are inhibited. In particular state 3 and the uncoupled state are almost equally inhibited leading to the hypothesis that complex V is not inhibited by DAQs reinforcing previous findings of this thesis. These findings do not permit to define which among the first four complexes of the ETC is more affected by DAQs toxic effect respect to the others. Specific assays for every complex of the ETC were planned to investigate at which point of the mitochondrial respiration DAQs exert their inhibitory effect and the results for the complex I are reported in the next paragraph (**Figure 31**).

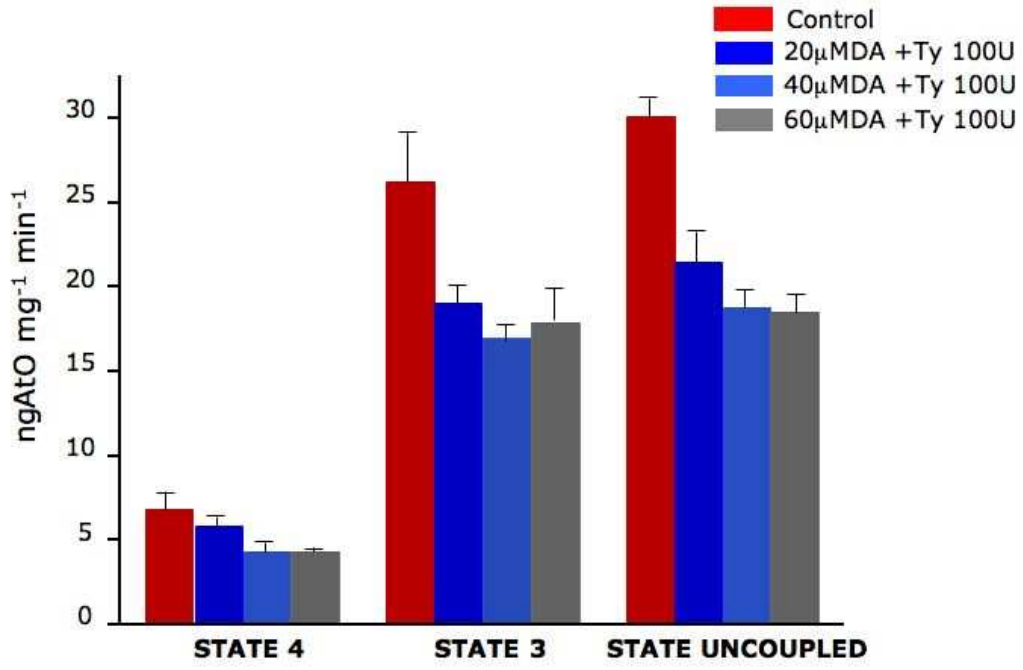

Figure 31 DAQ inhibit mitochondrial respiration

Mouse liver mitochondria were exposed to DA (20-60μM) and tyrosinase (Ty, 100 units) for 10 minutes as described in *Materials and Methods*. The medium contained glutamate and malate as oxidizing substrates and CsA (2μM) to inhibit mPTP opening and subsequent mitochondria depolarization. The three state of mitochondrial respiration were assessed by oxygraphic measurements with a Clark electrode chamber. Sate 4 was measured in absence of ADP. State 3 after ADP addition and the Uncoupled State after DNP addition. Non-reacted DA and Ty were removed by centrifugation before starting the respiration since Ty employs molecular oxygen for its oxidizing reaction. Results from these experiments show that DAQs are able to inhibit all the three states of mitochondrial respiration. State 3 and the Uncoupled state are inhibited almost at the same amount. This result leads to exclude the complex V as one of the target of DAQs inhibition.

DAQs inhibit Complex I of the electronic transport chain in mitochondria.

Since DAQs inhibit mitochondrial respiration, experiments to point out the functional state of complex I after DAQs treatment were carried out. A specific assay for complex I was employed (*100*) and the activity of the complex from mitochondria exposed to increasing amount of DAQs was measured. In this assay reduction of 2,6-dichloroindophenol (DCIP) by electrons accepted from

decylubiquinol was measured as described in the *Materials and Methods*. Decylubiquinol is reduced by complex I after oxidation of NADH.

Results from these experiments show an inhibitory effect of DAQs treatment on complex I activity (**Figure 32**). Specific precautions were taken to remove unreacted DA and the enzyme tyrosinase before starting the measurements. This solution was necessary since NAD(P)H is readily oxidized by DAQs in a redox reaction (Results, Part I of this thesis). After removal of both the

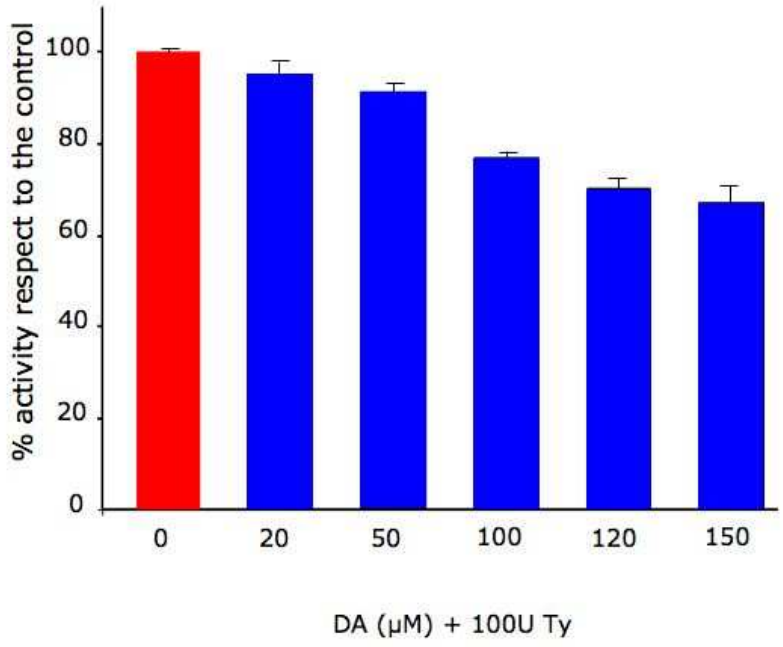

Figure 32 DAQs inhibit Complex I

A sensitive and specific assay for mitochondrial complex I activity was employed to test the inhibitory effect of DAQs on the first complex of the ETC. This method is based on measuring 2,6-dichloroindophenol (DCIP) reduction by electrons accepted from decylubiquinol reduced after oxidation of NADH by complex I. 0.5mg/ml of mitochondria were exposed to DA (0-150μM) and tyrosinase (Ty, 100U) to generate DAQs for 10 minutes. Mitochondria were gently spun down and resuspnded in 965μl of reaction buffer containing 25mM KPO_4, 3.5g/L BSA, 70μM Decylubiquinone, 60μM Dichloroindophenol (DCIP), 1μM Antimycin A. The solution was let equilibrate for 3-5min at 37 °C and the reaction was started by adding 20μl of 10mM NADH solution. Absorbance at 600nm was measured every 30 seconds for 10 minutes. 1μl of rotenone 1mM was added and absorbance was measured every 30 seconds for 5 minutes. The data are expressed respect the control and the non specific DCIP reduction, if any, was detected after addition of rotenone and subtracted to every measure.

non-reacted DA and the enzyme tyrosinase, pyridine nucleotide were added to the mitochondria, then these data refer to a direct impairment of the complex activity non-dependent on the oxidizing substrates deficiency. As shown in the Fig 3.2 DAQs impairment of complex I in mitochondria exposed to DAQs is detectable. Concentrations of DA between 10-150μM were tested and the effect is dose dependent.

Since at 40μM DA State 3 and the Uncoupled State of respiration are inhibited almost at 50% and complex I is not as much inhibited it has to be concluded that there is at least another point between complex I and complex IV of mitochondrial ETC where DAQs exert their inhibitory effect. Further experiments are necessary to identify which one(s) are these steps.

DAQs directly modify complex I subunits.

Several evidence in literature point out the ability of DAQs to directly react with proteins and covalently modify them (*81, 93-95, 110, 142*). These modifications can lead to protein structure and function modification that result in protein impairment. The ability of DAQs to directly modify complex I subunits was thus assessed. Complex I from mitochondria incubated with ^{14}C-DAQ was purified by immunocapture (see *Materials and Methods*) and the several subunits isolated by SDS-PAGE. The ^{14}C-DAQ-modified subunits were then detected by autoradiography of the Western blot.

As shown in the picture (**Figure 33**) 3-4 bands are clearly detectable. These bands correspond to the subunits of Complex I modified by the binding of the DAQs. Further investigations to identify these bands are necessary.

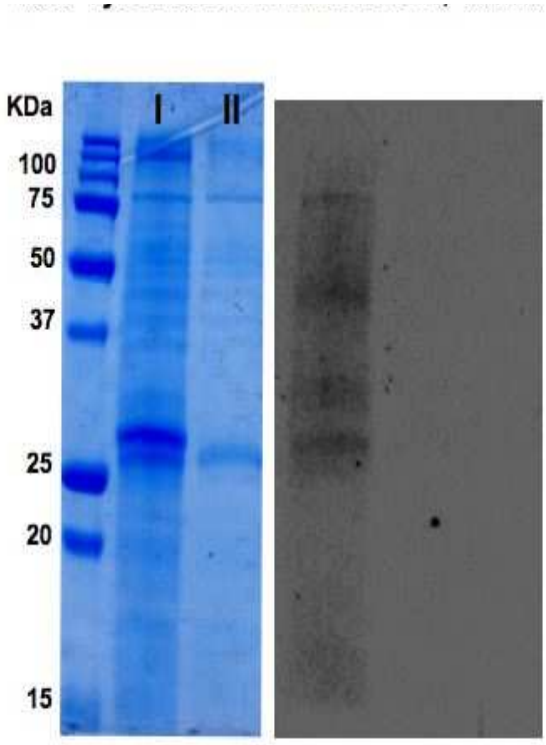

Figure 33 Autoradiogram of Complex I isolated by Immunoprecipitation from mitochondria treated with 14C-DA and tyrosinase.

Liver rat mitochondria were purified as described in *Materials and Methods*. Lane I: Mitochondria (5mg) were then resuspended in medium containing glutamate and malate as oxidizing substrates and exposed 10 minutes to 20μM ^{14}C-DA, (1μCi), and tyrosinase (120 units) to generate DAQs. At the end of the treatment mitochondria were lysed and complex I immunocaptured following manufacture directions. Complex I subunits were then separated in a precast 12% gel by SDS-PAGE and the proteins transferred overnight in a PVDF membrane by Western blot. The membrane was the exposed to X-ray film and the autoradiogram developed 21 days later.
Lane II as lane I, plus 2mM rotenone during DAQs tratment for 10 minutes. The Autoradiograms show that DAQs are able to react with at least three subunits of complex I instead rotenone completely revert this interaction.

Rotenone-DAQs competition

Previous results shown in this research outline that the rotenone, a specific complex I inhibitor, exacerbates the toxic effect of DAQ in mitochondria. To test if the rotenone molecule and the oxidized products of DA compete for the same sites of complex I and delineate their mechanism of interaction with the complex, a competitive assay was carried out. Mitochondria were incubated with a large amount of rotenone (2mM) and after ^{14}C-DA and tyrosinase was added for 10

minutes. The medium was then removed after centrifugation of mitochondria and the mitochondria washed.

Complex I from this sample was isolated by immunocapture and the subunits separated by SDS-PAGE. The proteins were transferred to a membrane by Western blot and the membrane exposed to an X-ray film to detect the band modified by the binding to DAQ.

The absence of detectable bands in the autoradiogram (**Figure 33** lane II) can suggest a direct competition between the molecules and a inability of DAQ to bind to the complex I subunits sites already bound to rotenone molecules or a conformation change in complex I structure due to rotenone that make the DAQ binding not possible. This interesting result open new prospective of research in both rotenone mechanism of complex I inhibition and DAQ modification ability of complex I subunits.

Comparative experiments of DAQs and rotenone toxicity were then planned to figure out if the toxic mechanism of these compounds is comparable *in vitro* and *in vivo*.

Cells resistant to rotenone show a decrease vulnerability to DAQs toxicity.

To further investigate if DAQs toxicity depends on interaction with complex I, the effect of DAQs in cells expressing the rotenone-insensitive single subunit NADH dehydrogenase of *Saccharomices cerevisiae* (Ndi1) was analyzed. This single subunit acts as a "replacement" for the entire complex I in mammalian cells (Seo et al 200, Bai *et* al 2001, see Rotenone toxicity is caused by complex I inhibition in Todd paper). This system was used to assess that rotenone toxicity depended on direct interaction with complex I in previous findings from Greenamyre's laboratory (*11*). In this system, electrons from complex I substrates (e.g., glutamate) are shunted through Ndi1 into downstream portions of the ETC, thereby allowing mitochondrial respiration to bypass complex I inhibition. Since cells expressing Ndi1 subunit are resistant to rotenone toxicity rotenone effects can be entirely explained by the inhibition of complex I. This system was then chosen to investigate the toxic effects of DAQs. If DAQs exert their toxic affect

toward complex I, cells expressing the Ndi1 subunit will be protected from DAQs toxicity. Cells expressing Ndi1 subunit were treated with DAQs and the cells availability after the treatment was assessed (See *Material and Methods*). Interestingly cells expressing the rotenone-resistant subunit show an increased resistance to DAQs toxicity too (**Figura 34**). This finding proves that DAQs exert toxic effects from their interaction with complex I and if it is bypassed DAQs are less toxic. Is tempting to speculate that DAQs exert their toxic effects on complex I in a similar way as rotenone does. If this hypothesis is correct DAQs have the ability to increase the electrons leakage from complex I increasing the production of ROS and the oxidative damage. Evidences in literature support this speculation. The ability of DAQs to increase the amount of H_2O_2 produced by mitochondria in

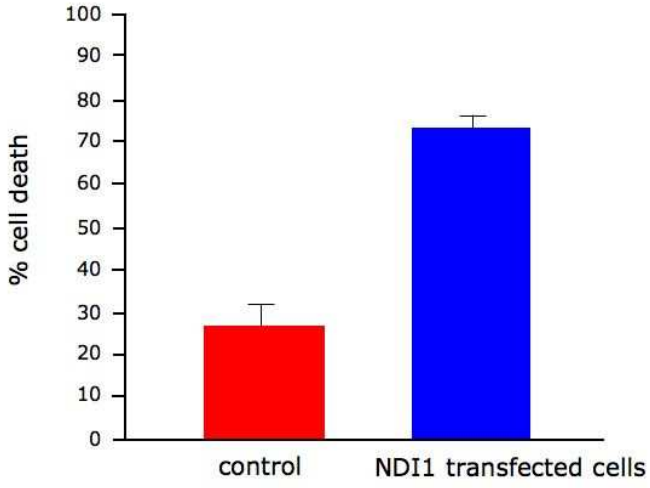

Figure 34 Cells expressing the rotenone-insensitive Ndi1 subunits are protected from DAQs toxicity.

Neuroblastoma SHSY-5Y cells were transfected with a NDI1 gene expressing plasmid as described in the *Materials and Methods*. The efficiency of the transfection was assessed at the microscope before starting the treatment. Cells were exposed to DA overnight and the cells viability was assessed at the microscope. Four fields were considered and the % of cell death is referred to the untreated control. As shown in the graph above the Ndi1 expressing cells are ore resistant to DAQs toxicity than the untreated cells. Ndi1 is a rotenone-insensitive single subunit NADH dehydrogenase of *Saccharomices cerevisiae*. These data suggest that one of the mechanism of DAQs toxicity can be similar to the rotenone with the specific inhibition of complex I and the increase of electrons leakage from this site.

presence of complex I oxidizing substrates has been shown, on the contrary the effect is not detectable if complex I is bypassed with succinate, a complex II substrate (*113*). Nonetheless cells expressing Ndi1 subunit are not fully protected from DAQs toxicity their detrimental effect does not result solely from complex I impairment. This result is expected from the high reactivity of DA oxidized products and also from previous results from this thesis.

Conclusions

The research presented in this chapter show the ability of the oxidized products of DA to inhibit mitochondrial respiration and the inhibitory effect at the complex I level was studied further. The inhibition of the ETC was assessed by oxygraphic measurements and the results show that all the three states of mitochondrial respiration are inhibited after exposure to DAQs. Since the State 3 and the uncoupled State are equally inhibited (**Figure 31**) it can be excluded at specific inhibition at the level of complex V of the mitochondria ETC. State 3 is the respiration in presence of oxidizing substrates and ADP, so is an index of the respiration coupled with ATP synthesis instead the Uncoupled State is the maximum rate of the respiration not coupled with ATP synthesis, that is an index of the function of the four complex of the ETC. Among them, the effect of DAQs toward the first complex was investigated more in detail. Previous results from this research suggest a possible inhibitory effect of DAQs on complex I can derived from their ability to oxidized the pyridine nucleotide in mitochondrial matrix (**Figure 28**) since complex I catalyzes the transfer of electrons from the pyridine nucleotide NADH to ubiquinone. Results from these experiments lead to conclude that his mechanism, even if present, is not the only way of DAQs toxicity. Cells expressing the rotenone-insensitive single subunit NADH dehydrogenase of *Saccharomices cerevisiae* (Ndi1) were a powerful tool to investigate DAQs toxicity toward complex I. In this system NADH is still oxidized by Ndi1 subunit but what is missing is the site of action of the complex I inhibitor rotenone. In Ndi1-expressing cells DAQs are less toxic than in the control, suggesting that besides the oxidation of the pyridine nucleotide there must

be another mechanism by which these cells are protected from DAQs toxicity. Is tempting to speculate that the mechanism by Ndi1 cells are protected from DAQs is the same they are protected from rotenone. Rotenone exerts its toxic effect binding to complex I and increasing electron leakage from complex I, heightening the amounts of ROS produced by complex I in mitochondria. There are already evidence in literature that mitochondria exposed to DAQs increase the amount of H_2O_2 produced respect to the control (*113*). In addition preincubation of mitochondria with rotenone prevent DAQs to bind and covalently modify DAQs subunits (**Figure 33**) suggesting that there can be a competition for the same site(s) between these two species. Further investigation are necessary to clarify the toxic mechanism of DAQs on mitochondria nonetheless evidences of their effects to complex I is clearly suggested herein. Complex I is impaired in the dopaminergic neurons of PD patients and studies in PD brain show a great increase of oxidative stress in dopaminergic neurons. The results describe in this chapter show a possible way of mitochondrial impairment and complex I inhibition due to the oxidized products of DA.

Discussion

The research that comprises this dissertation concerns the toxic effects of DA oxidation products on mitochondria. Further characterization of this detrimental interaction may shed light on the still unknown mechanism of dopaminergic neuron loss in Parkinson's disease (PD). Based on studies using human PD brains, animals models of PD, and *in vitro* cellular models, three mechanisms seem to be primarily involved in the neurodegeneration of dopaminergic neurons in PD: 1) increased oxidative stress, 2) mitochondrial dysfunction (i.e. decrease of ATP production, Ca^{2+} mishandling and increase of electrons leakage from ETC with consequent ROS production), and 3) impairment of the ubiquitin-proteasome system with consequent accumulation of misfolded proteins in the cytoplasm of neurons. Several factors have been shown to induce neurodegeneration of dopaminerigc neurons, invoking one or more of these mechanisms. It is particularly noteworthy that mitochondrial dysfunction can exacerbate each of these pathogenic mechanisms. Additionally, since none of these proposed pathogenic mechanisms adequately explains why dopaminergic neurons are selectively vulnerable in PD, further characterizing the effects of DA on mitochondria is paramount.

A combination of genetic susceptibilities and environmental factors seems to play a central role in PD pathogenesis. In the last ten years, mutations in seven genes have been reported to cause rare familial forms of PD. While these genetic forms of PD represent a small portion of total PD cases, they highlight pathogenic pathways that may be involved in all cases of PD. Of particular interest to this dissertation, is that all mutations in genes related to PD exert an effect on mitochondrial function.

Environmental toxins that inhibit mitochondrial complex I throughout the body (MPTP and the pesticide rotenone) induce PD in humans and are considered one of the factors involved in the development of sporadic forms of PD. What is still not understood is why a defect or impairment that affects all the cells in the

body (for the rotenone) has as prime consequence the degeneration of a specific family of neurons in the brain. However, similar to the genetic mutations, which give rise to PD, these environmental toxins highlight the importance of mitochondrial function and ROS-mediated oxidative stress in the death of dopamineric neurons.

Based on the anatomy of this neurodegeneration, a central role of the neurotransmitter DA in dopaminergic cell loss in PD has been for long proposed (*143*). DA is a highly reactive molecule that can undergo spontaneous or enzymatic catalyzed oxidative reactions in the cytoplasm, leading to the production of highly reactive oxidized species. For this reason cytoplasmic DA is normally stored in synaptic vesicles or readily metabolized to less toxic products by cytosolic enzymes. If the synthesis or metabolism of DA is impaired, DA can accumulate in the cytosol and give rise to several toxic reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and DA quinones. Pathological events that lead to DA catabolism/metabolism impairment can lead to accumulation of ROS and DA quinone formation, increasing oxidative stress in dopaminergic neurons. If cellular antioxidant systems that scavenge these toxic species are overwhelmed, growing ROS production can induce cytotoxicity and consequent cell death. Moreover, growing evidence suggests a toxic role specifically for the DA quinone species. DA has a catechol ring that can be oxidized to a quinone. Quinones are highly electrophilic and readily react with cellular nucleophiles. It has been reported that DA quinones are able to react with glutathione, and covalently modify proteins at cysteine residues *in vitro* and *in vivo (28, 94, 106, 108)*. Since cysteine amino acids are often found in the active sites of proteins, binding of such residues by DA quinones can lead to protein modification and impair function. DA quinones have been reported to modify and inhibit several proteins, including tyrosine hydroxylase (TH) (*108*), dopamine transporter (DAT) (*94*) and parkin (*92*). Moreover, DAQ-modification inhibits α-synuclein fibrillation process and leads to accumulation of the more toxic protofibrils species (*97*).

Evidence suggests that an alteration of DA metabolism occurs in PD with consequent accumulation of DA in the cytoplasm (*144, 145*). Several mechanisms have been suggested that lead to increased cytoplasmic DA, such as mitochondrial

dysfunction and a resulting decrease in ATP. This decrease in ATP production can affect several ATP-dependent cellular processes, including the active transport of DA molecules into synaptic vesicles (*14*). Other process that can inhibit the transport of DA into vesicles have been reported, including a toxic interaction between mutated α-synuclein and the synaptic vesicles (*146*) or MPTP exposure (*147*). Moreover, free cysteinyl-catechol and GSH-catechol derivatives have been found to be increased in post-mortem PD brains compared to age-matched controls (*85, 109*). This growing body of evidence supports the theory that DA oxidation and protein modification by DA quinones can be one of the key explanations for why dopaminergic neurons are more vulnerable to insults in PD.

Based upon all these data, it was compelling to explore the possible mechanisms that define the effects of DA quinones on mitochondria. Evidence in the literature shows that the oxidized products of DA induce permeability transition (PT) in isolated brain mitochondria and inhibit mitochondrial respiration (*110*). In isolated and disrupted mitochondria, DAQs inhibit complex I of the ETC. This effect can be reversed by GSH but not by ROS scavengers such as catalase or α-tocopherol, suggesting that DAQs are the toxic species (*114, 115*).

In the research presented in this thesis, the toxic effects of DA quinones on mitochondria were investigated using different approaches. The ability of the oxidized products of DA to enter in the mitochondria was first investigated. The results from these experiments show that DAQs but not DA are able to enter mitochondria. The ability of DAQs to cross both the outer and inner mitochondrial membranes of mitochondria was assessed. Within the mitochondria, DAQs are able to react and covalently modify mitochondrial proteins. DAQ-modified mitochondrial matrix proteins such as the γ-subunit of F_1F_o ATP synthase and complex I subunits were found. Interestingly the γ-subunit of F_1F_o ATP synthase contains a cysteine residues (Cys 78) in a loop exposed to the matrix and this is a reasonable site for DAQs-binding and modification. Experiments to verify whether the activity of the DAQ-modified F_1F_o ATP synthase is impaired were carried out. An in-gel activity assay for the ATPase activity of the F_1F_o ATP synthase complex did not reveal significant differences when compared to the untreated complex. It is now clearly established that

dimerization of the F_1F_o ATP synthase takes place in the mitochondrial inner membrane. Interestingly, oligomerization of this enzyme seems to be involved in cristae morphogenesis. As they were able to form homodimers, subunit γ has been proposed as one of the potential ATP synthase dimerization subunits (*148*). The hypothesis that DAQs-modified F_1F_o ATP synthase can alter mitochondrial morphology of the cristae is currently under study.

The mechanism by which DAQs enter the mitochondria was then investigated. Results from these experiments have shown that DAQs enter the mitochondria in a way that is independent of mitochondrial $\Delta\Psi$. Experiments that assess the ability of DAQs to enter the mitochondria were carried out in the presence or absence of oxidizing substrates, however no significant differences could be detected. From these experiments it can be concluded that DAQs enter the mitochondria without an active transport, since absence of substrates for the oxidative phosphorylation doesn't alter their ability to enter the organelle.

DAQs were able to inhibit mitochondrial respiration and specific inhibition of complex I isolated from mitochondria exposed to DAQs was found. DAQ-modified complex I subunits were identified by autoradiography experiments with ^{14}C-DAQs and these modifications can account for complex I inhibition. Another possible mechanism of DAQ-induced toxicity at the level of complex I is suggested in Zoccarato et al. 2006 based on the capacity of the electrophilic DAQs to remove electrons from complex I and increased ROS production (*113*).

These *in vitro* findings point to a toxic role for the oxidized products of the neurotransmitter DA on mitochondria, a scenario that is likely to occur if DA accumulates in the cytoplasm of the dopaminergic neurons. Impaired storage or metabolism of DA has been suggested in PD resulting from mitochondrial impairments and decreased ATP production (*143*) or α-synuclein mutations (*13*). Mitochondrial ETC dysfunction can lead to increased oxidative stress, and a decrease of ATP production. Both these phenomena can lead to further deregulation of DA synthesis, storage, and metabolism, thus reinforcing a vicious cycle.

These results are also **important for understanding the specific vulnerability** of DA neurons in PD, since this type of neuron selectively

expresses DA. Some dopaminergic neurons (in other regions of the CNS) are spared in PD. This suggests that other mechanisms can be involved in this cell loss. One possibility is that injury to SNpc neurons triggers compensatory mechanisms in surviving neurons that increase DA release (*149*) and metabolism, ultimately increasing DA oxidation as a negative side effect.

The ability of DAQs to induce permeability transition (PT) was demonstrated in detail. PT is a phenomenon due to the opening of a proteinaceous pore in the internal mitochondrial matrix (IMM) that makes the IMM permeable to solutes with masses below ~1.5kDa. A consequence of this transition is the loss of structure of the cristae of the IMM and swelling of the mitochondria. The final outcome is the disruption of the outer mitochondrial membrane and the release into the cytosol of the pro-apoptotic factors that reside in the intermembrane mitochondrial space.

Data from this thesis confirm previous findings (*110*) that the oxidized products of DA induce PT in isolated mitochondria. Liver mitochondria were chosen to characterize the DAQs ability to induce PT. The choice of this system is based on practical reasons because PT transition in liver is better characterized, the effect is easier to measure and the system is sensitive to cyclosporin A (CsA) inhibition. The results from these studies show that DAQs are able to induce PT in a dose-dependent manner at concentrations among 10 to 150μM. Concentrations higher than 200μM were not tested since they are not considered physiologically relevant. As expected, the same concentrations reduce the calcium retention capacity of mitochondria, likewise in a dose-dependent manner. Both of these effects are completely reversed when the specific inhibitor of the PT, CsA, is added to the solution. These results let to conclude that the effect of DA quinones is related to the opening of the PT pore.

Time-course experiments of DA oxidation reactions were carried out to compare the reactivity of several products that arise from DA oxidation. The earlier oxidation products exhibit the greatest ability to induce PT, suggesting that the first quinone (dopamine-o-quinone) or the aminochrome formed are the most reactive. Kinetic analyses of the DA oxidation process reveal that at physiological pH the dopamine-o-quinone rearrange to form leukoaminochrome in kinetic very fast (*81*). In the presence of –SH instead the electrophile attack and formation of

the 5-*s*-cystenil-catechol is favored several times respect the internal cyclization. If –SH are not present the most reactive species seems to be the aminochrome, due to its higher stability (*81*).

Interestingly, if the mPTP is kept in the closed conformation by the addition of CsA, the various DAQs are equally able to enter the mitochondria. Furthermore, the pattern of protein modification is the same regardless of whether the mPTP is open (by Ca^{2+} action) or closed. Taken together these findings suggest that DAQs are able to enter the mitochondria before PT induction and only once inside the organelle DAQs are able induce PT.

The mechanism of DAQ-induced PT was then investigated. Even if the structure and composition of the mPTP is not yet defined, several conditions are known to induce PT or to maintain the pore in the closed conformation. The pyridine nucleotides pool, [NAD(P)H/NAD(P)$^+$], and glutathione (GSH/GSSG) pool in the reduced state, promote the closed conformation, on the contrary, under normal physiological (oxidizing) conditions the probability of PT induction increases. The redox state of the pyridine nucleotide pool of mitochondria that were exposed to DAQs was then estimated. Results from these experiments show that DAQ exposure causes the oxidation of the pyridine nucleotide pool in the mitochondrial matrix in a dose-dependent manner. These results suggest a possible mechanism of toxicity of DAQs in the mitochondria that can lead to a great variety of toxic effects. Pyridine nucleotide oxidation can directly affect the activity of the ETC, particularly at the level of complex I since NADH is oxidized by complex I in order to extract the electrons that flux through the ETC to create the proton gradient that is used by the F_1F_O ATP synthase complex to produce ATP from ADP. Additionally, the reduced state of the pyridine nucleotide pool is required to scavenge ROS that are normally produced by the mitochondrion. Mitochondria are the primary cellular site of ROS production, primarily due to the electron leakage from the ETC, and for this reason several mechanism of ROS scavenging are present in mitochondria. Reduced GSH, which is present in the mitochondrial matrix in high amounts is one of the most important of these antioxidant defenses. To maintain glutathione in the reduced state (GSH), NADPH is oxidized by the enzyme glutathione reductase, meanwhile reducing GSSG to GSH. If the pyridine nucleotide pool is oxidized, then the pool of

reduced glutathione cannot be regenerated, and this important antioxidant defense is impaired, allowing for oxidative damage to the mitochondria. Lastly, the reduced state of the pyridine nucleotide pool is one of several conditions that keep the mPTP in the closed conformation, preventing PT. Under oxidizing conditions, the oxidized pyridine nucleotide pool induces PT, and this effect is shown to play a central role in DAQ-induced PT.

The ability of DAQs to induce PT transition in a cellular model has also been shown. These results suggest a possible mechanism of cellular damage induced by DAQs. As mentioned above, one consequence of PT is the disruption of IMM structure and the subsequent disruption of the OMM with the release of the pro-apoptotic factors that reside in the intermembrane space. The apoptotic pathway seems to be highly activated in PD (*7*, *8*) and cell death as a consequence of DAQ-induced PT could account for the specific vulnerability of DA-containing neurons in PD.

Another mechanism by which DAQs exert their toxic effects was presented in the Part III of this thesis. DAQs are able to inhibit the principal function of mitochondria, the ETC. In particular, the inhibitory activity of DAQs on the first complex of the ETC was shown. Previous findings suggest that DAQ-mediated inhibition of complex I is exerted by increasing of electron leakage from the complex I, leading to increased ROS formation. This mechanism accounts for the toxic effects of the environmental PD toxin, rotenone. Rotenone is a specific inhibitor of complex I that has been shown to induce neurodegeneration of dopaminergic neurons and is widely used to recapitulate PD in animals. Rotenone toxicity is due principally to its ability of inhibit complex I and cause increased ROS production (via electron leakage from this site) rather than by inducing a mitochondrial bioenergetic defect with decreased ATP production, since cellular toxicity still occurs when concentrations of rotenone that don't alter ATP production are used.

The toxicity of rotenone and DAQs was compared employing the Ndi1-cellular model. In this model, neuroblastoma cells SH-SY5Y expressing the rotenone-insensitive complex I subunit (Ndi1) were exposed to DAQs. Results from these experiments show that the presence of Ndi1 subunits protects cells from DAQs toxicity. Ndi1's protective effect against rotenone relies on its ability

to oxidize NADH and maintain the electron flux through the ETC because it does not have the rotenone binding site, thereby preventing rotenone from inducing electron leakage from this complex and a resultant increase ROS production. Since Ndi1-expressing cells are protected from the toxic effects of DAQs as well, a possible mechanism of DAQ toxicity could be increasing ROS production in mitochondria, in particular at complex I level.

One of the biochemical hallmarks of the PD brain is the significant increase in oxidative modification to cellular macromolecules. These oxidative end products include protein carbonyls, lipid peroxides, and oxidized DNA. Alterations in DA catabolism and metabolism have long been claimed as one of the factors that contribute to dopaminergic neurons loss in PD. If DA accumulates in the cytoplasm, it can spontaneously undergo oxidation, resulting in DAQ formation. Indeed, the presence of DAQ-modified proteins is increased in the SN of PD brains.

Data from this thesis suggest a possible mechanism of DAQ toxicity that involves mitochondria. DAQ can enter mitochondria and induce oxidative damage by oxidizing the pyridine nucleotide pool in the mitochondrial matrix. Inhibition of complex I promotes electron leakage and ROS formation, covalently modifying and impairing the function of mitochondrial proteins, and inducing PT, ultimately leading to cell death.

In conclusion, data presented in this work describe the toxic effects of the oxidized products of the neurotransmitter DA on mitochondria both *in vitro* and *in vivo*. The effects concern mitochondrial respiration, which leads to decreased production of ATP as a final outcome with resulting energetic impairment for the whole cell. DAQs are also shown to induce PT, which represents one of the triggers of the mitochondrial pathway of cell death. Since in DAminergic neurons the oxidation of cytoplasmatic DA occurs naturally, pathological conditions that increase the accumulation of DA in the cytoplasm, such as an impairment in DA storage and/or metabolism, can lead to increased formation of DAQs and their downstream toxic effects.

In PD, the reason for the specific loss of dopaminergic neurons remains elusive. In both sporadic and genetic forms of PD it is likely that mitochondrial impairment underlies the vulnerability dopaminergic neurons of SNpc. Therefore,

the research described in this dissertation details a mechanism that may render SNpc DA neurons specifically more vulnerable to oxidative stress and ROS-mediated oxidative damage, and neurodegeneration in PD. The contribution of the neurotransmitter DA to oxidative stress represents a valid explanation for the peculiar vulnerability of these neurons in PD and also represents a logical therapeutic target for protecting these neurons.

Several strategies have been developed to protect dopaminergic neurons from DA-induce toxicity. One tricky issue in developing therapeutic solution for PD patients is constituted by the fact that the severe motor symptoms that characterize PD are alleviated increasing presence of DA neurotransmitter in the striatum of PD patients. This goal is achieved by somministration of the DA precursor L-DOPA or by inhibitor of the metabolic pathway of DA (i.e. inhibitors of mitochondria MAO). These strategies, even if the most commonly used so far, have detrimental side effects due to the increase of DA-derived oxidative stress.

Over the past few years several efforts has been spent to develop antioxidants that decrease oxidative stress in dopaminergic neurons in PD.

The antioxidant efficacy of these molecules was increased considerably by targeting these compounds to mitochondria. This was achieved by covalent attachment of the antioxidant to lipophilic cation (*150*). Due to the large mitochondrial potential, these cations accumulate several folds within mitochondrial matrix protecting them from oxidative damage. Among these molecules the natural antioxidant ubiquinone was modified with the lipophilic tri-phenylphosphonium cation (MitoQ) and the small aromatic-cationic peptide (SS-peptides Szeto-Schiller-peptides) seem the most promising. These compounds are now tested for their potential therapeutic properties in animal model of PD (MPTP mice) and they have reveled significant protection of striatal dopaminergic neurons decreasing ROS production from mitochondria passing into the cytoplasm.

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