UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Biologia

SCUOLA DI DOTTORATO DI RICERCA IN BIOSCIENZE E BIOTECNOLOGIE

INDIRIZZO: BIOTECNOLOGIE

CICLO XXV

Dopamine and dopamine-quinones toxicity in Parkinson’s disease: cellular models revealing a possible role for superoxide dismutases

Direttore della Scuola : Ch.mo Prof. Giuseppe Zanotti
Coordinatore d’indirizzo: Ch.mo Prof. Giorgio Valle
Supervisore :Ch.mo Prof. Luigi Bubacco

Dottoranda : Rekha Cappellini

Content

Abstract 5

Riassunto 8

1. Introduction 11

1.1 Parkinson's disease 11

1.1.1 Clinical and pathological hallmarks 11

1.1.2 Treatments 13

1.1.3 Neuronal circuits implicated: the basal ganglia 14

1.2 Etiology of parkinson's disease 17

1.3 Genetics forms of PD 17

1.3.1 Genes associated with autosomal dominant PD 19

1.3.2 Genes associated with autosomal recessive PD 19

1.4 Sporadic Parkinson's disease 21

1.4.1 Neuroinflammation 21

1.4.2 Proteasome dysfunction and protein aggregation 22

1.4.3 Oxidative stress 24

1.4.4 Mitochondrial dysfunction 26

1.4.5 Dopamine 28

1.4.6 Apoptosis in PD 30

1.4.7 Cellular models in the context of PD 31

Aim of the thesis 33

2. Materials and methods 35

2.1 Molecular biology 35

2.1.1 Cloning of SOD1 and SOD2 sequence 35

2.1.2 Polymerase chain reaction (PCR) 36

2.1.3 DNA purification 37

2.1.4 DNA and vector enzymatic restriction 37

2.1.5 Vector dephosphorylation 38

2.1.6 DNA ligation 39

2.1.7 Bacterial transformation 41

2.1.8 Bacterial colonies screening 41

2.1.9 Plasmid DNA extraction 42

2.1.10 DNA quantification 43
2.1.11 Agarose gel electrophoresis 43
2.1.12 DNA sequencing 44
2.1.13 RNA extraction.. 44
2.1.14 mRNA retrotranscription 45
2.1.15 Amplification of dopaminergic markers 45
2.2 biochemistry.. 47
2.2.1 SDS-PAGE and western blotting 47
2.3 Cellular Biology 48
2.3.1 Splitting adherent cell lines by trypsinisation 48
2.3.2 Poly-lysine coating tissue culture vessels 48
2.3.3 Transient transfection (lipofectamine)... 48
2.3.4 Paraformaldehyde recipe 49
2.3.5 Hoechst Staining for Nuclear Fragmentation..................................... 49
2.3.6 Mitosox probe 50
2.3.7 Time-lapse imaging of superoxide anion production 50
2.3.8 Flow cytometry 50
2.3.9 Cell detachment method evaluation 51
2.3.10 Fluorophore staining... 52
2.3.11 Treatments.. 52
2.4 Statistical analysis 53
3. Results 55
3.1 evaluation of the cellular model.. 55
3.2 Evaluation of dopamine and dopamine-quinones citotoxicity 56
3.2.1. Evaluatation of apoptotic nuclei by Hoechst staining 56
3.2.2 Flow cytometry 62
3.3 Mitochondrial superoxide production 71
3.3.1 Time-lapse imaging 72
3.3.2 Flow cytometry 76
3.4 Activation of apoptotic cascade 82
3.4.1 Caspase 3 cleavage 82
3.4.2.Poly (ADP) ribose polymerase (PARP) cleavage 84
3.5 Superoxide dismutase 1 and 2 over expression 85

3.5.1 Cloning of SOD1 and SOD2 86
3.5.2 Transient transfection and cellular localization 89
3.5.3 western blot analysis of SODs over expression 90
3.5.4 SODs effect against dopamine toxicity 92
3.5.5 SODs effect against dopamine-quinones toxicity 93
4. Discussion and conclusion 95
5. References 103

Abstract

Parkinson's disease is a widespread neurodegenerative disorder that affect 2% of the population above the age of 60. The hallmark of the pathology is the preferential degeneration of the dopaminergic neurons in the *substantia nigra pars compacta* of the midbrain, and the presence of proteinaceous inclusions called Lewy bodies in the surviving neurons (Braak 2004). In 10% of the cases, the disease is linked to mutation on several genes, among them α-synuclein, DJ-1, PARKIN, PINK1 and LRRK2, but in the vast majority of the cases the aetiology is still unknown (sporadic PD) (Gwinn-Hardy 2002). *Post mortem* studies and in *vitro* and in *vivo* PD model have revealed a possible interconnection between genetic and sporadic PD, which involves both mitochondrial dysfunction and oxidative stress as central players in the pathogenesis of the disease (Gilgun-Sherki Y. et al. 2001, Mythri R. B. et al. 2011). Oxidative stress is a condition characterized by the inability of the cellular antioxidant defences to cope with the production of reactive oxygen species (ROS). This condition of unbalance between the production and the clearance of ROS causes irreversible damage to cellular components such as lipids, proteins and DNA, leading eventually to cell death (Lotharius et al. 2002). Among the enzymes implicated in the detoxification of ROS, are superoxide dismutases (SODs) that catalyze the dismutation of superoxide anion into molecular oxygen and hydrogen peroxide (Fridovich 1995). Since oxidative stress does not explain alone the selectivity death of dopaminergic neurons, the main working hypothesis is that dopamine itself could have a central role. Under physiological conditions, dopamine is synthesized in the cytosol and stored in synaptic vesicles by the action of Vesicular Monoamine Transporter (VMAT2) where it is stabilized by the low pH (Erickson, J. D 1992). If the amount of cytosolic DA exceeds the physiological concentration, DA is metabolized to the non-toxic metabolite 3,4-dihydroxyphenylacetic acid and hydrogen peroxide by the action of monoamine oxidase (MAO) and aldehyde dehydrogenase, or sequestered into lysosomes where it can auto-oxidize to form neuromelanin (NM). If not buffered by these pathways, cytosolic DA can be oxidized to DA-quinone (DAQs) (spontaneously or enzymatically), (Sulzer, D.,

2000, Elsworth, J. D. 1997), a reaction that also leads to the formation of ROS. On these premise, we evaluated two line of research using a cellular model for PD (SH SY5Y cell line): one concerning about the effect of dopamine and its oxidized forms on cellular viability, the second one on the potential role of superoxide dismutases (1 and 2) over expression.

From the use of different techniques we started to evaluate which kind of cell death pathway was activated by dopamine and DAQs. Looking for the presence of nuclear fragmentation, that is one of the later stages of apoptosis, we determined that both dopamine and DAQs induce cell death via apoptosis but the dopamine toxicity depends on its internalization by the action of the dopamine transporter (DAT), since the pre-treatment of cells with GBR 12909 (a DAT inhibitor) had a rescue effect. To confirm the apoptotic pathway we also evaluated another hallmark of apoptosis (one of the former stages of the apoptotic cascade): phosphatidil-serine externalization (PS) using ANNEXIN-V-FLUOS; a specific probe for PS. Using flow cytometry we confirm that both dopamine and DAQs induce cell death via apoptosis. Next we wanted to evaluate if dopamine and DAQs exert their toxicity from extracellular environment or they are required to enter in the cells. Treating cells with GBR12909, we demonstrate that dopamine needs to enter cells to exert its toxicity (since the treatment with the DAT inhibitor rescues cells from DA toxicity) while DAQs toxicity was not affected by this treatment leading to cell death. Since oxidative stress is one of the mechanisms that have been implicated in the pathogenesis of PD, and the chemistry of dopamine (auto-oxidation and enzyme-mediated oxidation) leads to the production of ROS, we evaluate the production of mitochondrial superoxide anion using a specific probe. The data demonstrate that only the auto oxidation of dopamine leads to the production of superoxide anion and dopamine is required to enter cell to exert its effect. To dissect more in depth the toxicity mechanism of both dopamine and DAQs, and since only cytosolic dopamine led to the production of mitochondrial superoxide anion, we asked if this two different oxidation processes activated different cell death pathways (the major are the mitochondrial one and the one mediated by death receptor) or not. From preliminary data we observed a marked difference in the activation of caspase 3 and the subsequent cleavage and inactivation of Poly (ADP) ribose polymerase

(PARP) due to DAQs treatment convincing us to proceed in the investigation of the possible differences between this different oxidation processes. The second part of the work was focused on the role for superoxide dismutases 1 and 2 against dopamine and DAQs cytotoxicity since previous data demonstrated a role in superoxide anion production and induction of cell death in the case of cytosolic dopamine. Over expression of both SOD1 and SOD2 revealed a protective effect against dopamine cytotoxity, while they were not able to counteract DAQs-induced cell death. In the present work the main working hypothesis was that oxidative stress induced by dopamine and its oxidized forms accumulation could have a central role in the specific dopaminergic cell loss in Parkinson's disease. The data obtained so far seems to highlights that dopamine and DAQs activates different apoptotic pathway that are superoxide anion-dependent for DA and superoxide anion-independent for DAQs. Since oxidative stress is considered one of the mechanism that interconnect genetic form and sporadic forms of the pathology and dopamine, in its oxidative chemistry, leads to the production of ROS, understanding which cell death pathways are activated and to which extent, is crucial to develop a therapy to counteract the start and the progression of the pathology. Data from the over expression of SODs demonstrate that compounds that counteract the production of superoxide anion (like SOD-mimetics that are currently used for other diseases) could have a protective role against the oxidative stress and the subsequent cell death condition induced by dopamine. Also compounds that block the activation of the apoptotic cascade induced by dopamine and DAQs could rescue cells from dying in this neurodegenerative disease.

Riassunto

La malattia di Parkinson è una diffusa sindrome neurodegenerativa che affligge circa il 2% della popolazione oltre l'età dei 60 anni. La caratteristica principale della patologia è la preferenziale morte dei neuroni dopaminergici della *substantia nigra pars compacta* del mesencefalo, e la presenza di inclusioni proteinacee chiamate Lewy body nei neuroni sopravvissuti. Nel 10% dei casi, la malattia è collegata a mutazioni su diversi geni, tra i quali α-synucleina, DJ-1, PARKIN, PINK1 e LRRK2, ma nella maggior parte dei casi (Parkinson sporadico) (Gwinn-Hardy 2002) l'eziologia è ancora sconosciuta. Da studi *post mortem* e da modelli in vitro e in vivo per il PD è stata rilevata una possibile connessione tra le forme genetiche e quelle sporadiche che implica sia la disfunzione mitocondriale e lo stress ossidativo come fattori centrali nella patogenesi della malattia (Gilgun-Sherki Y. et al. 2001, Mythri R. B. et al. 2011). Lo stress ossidativo è una condizione in cui le capacità antiossidanti della cellula non sono in grado di sopperire alla produzione di specie reattive dell'ossigeno (ROS). Questa condizione di sbilanciamento tra la produzione e la detossificazione dei ROS causa danni irreversibili ai componenti cellulari come lipidi, proteine e DNA, portando alla morte cellulare (Lotharius et al. 2002). Tra gli enzimi coinvolti nella difesa antiossidante delle cellule, le superossido dismutasi giocano un ruolo fondamentale poiché catalizzano la dismutazione dell'anione superossido in ossigeno molecolare e perossido di idrogeno (Fridovich 1995). Poiché lo stress ossidativo non spiega da solo la selettiva morte dei neuroni dopaminerigici, l'ipotesi è che la dopamina stessa abbia un ruolo chiave. In condizioni fisiologiche, la dopamina viene metabolizzata nel citosol e stoccata all'interno delle vescicole sinaptiche grazie all'azione del trasportatore vescicolare delle monoamine 2 (VMAT2) dove è stabilizzata dal basso pH (Erickson, J. D 1992). Se la concentrazione citosolica di dopamina supera quella fisiologica, essa viene metabolizzata ad acido 3,4-diidrofenilacetico e perossido di idrogeno grazie all'azione dell'enzima monoamina ossidasi (MAO) e aldeide deidrogenasi, o sequestrata nei lisosomi dove può auto ossidarsi a formare neuromelanina. Se non è metabolizzata in questi pathway, la dopamina può essere ossidata a dopamino-

chinoni (DAQs) (spontaneamente o enzimaticamente) (Sulzer, D., 2000, Elsworth, J. D. 1997)' una reazione che produce anche specie reattive dell'ossigeno (ROS). Con queste premesse, abbiamo valutato due linee di ricerca usando un modello in *vitro* per il PD (la linea cellulare SH SY5Y): la prima riguardante l'effetto della dopamina e le sue forme di ossidazione sulla vitalità cellulare, la seconda sul potenziale ruolo dell'over espressione delle superossido dismutasi (1 e 2).

Utilizzando differenti tecniche abbiamo iniziato a valutare quale pathway di morte cellulare veniva attivato dalla dopamina e dalle sue forme ossidate. Andando a valutare la frammentazione nucleare, uno degli ultimi stadi dell'apoptosi, abbiamo determinato che sia la dopamina sia i chinoni inducono l'attivazione di questo processo di morte. Per confermare il processo apoptotico, abbiamo valutato un altro marker: l'esternalizzazione delle fosfatidil-serine (PS): uno dei primi stadi di attivazione del processo apoptotico. con l'utilizzo di ANNEXIN-V-FLUOS, una sonda specifica per le PS. Mediante la tecnica di citoflurimetria abbiamo confermato che sia la dopamina che i chinoni attivano l'apoptosi. Successivamente abbiamo voluto valutare se la dopamina e i chinoni avevano effetti tossici a livello extracellulare o necessitavano di entrare nelle cellule. Pre-trattando le cellule con l'inibitore specifico del trasportatore della dopamina (GBR 12909) abbiamo dimostrato che la tossicità della dopamina è dipendente dal suo trasporto all'interno della cellula (in quanto il trattamento con l'inibitore ha avuto un effetto protettivo per le cellule), mentre i chinoni non lo necessitano. Poichè lo stress ossidativo è stato proposto come possibile meccanismo implicato nella patogenesi della malattia, e la chimica ossidativa della dopamina (auto ossidazione e ossidazione mediata da enzimi) porta alla produzione di specie radicaliche, abbiamo valutato la produzione dell'anione superossido (la prima specie radicalica prodotta soprattutto a livello del mitocondrio) usando una sonda specifica. I dati dimostrano che solo il processo auto ossidativo della dopamina porta alla produzione di anione superossido e che questo processo è dipendente dall'internalizzazione della dopamina a livello del trasportatore. Per comprendere maggiormente il meccanismo alla base della tossicità del processo di ossidazione e ossidazione mediate da enzima della dopamina abbiamo valutato altri marker di apoptosi. Poiché dai dati precedenti è stato visto che solo la dopamina citosolica

era in grado di indurre la produzione dell'anione superossido a livello del mitocondrio, abbiamo voluto verificare se i due processi ossidativi della dopamina, attivassero differenti pathway apoptotici (i prevalenti comprendo la via mediata dai recettori di morte, l'altro quella mitocondriale). Dati preliminari dimostrano una marcata attivazione della caspasi 3 e la conseguente inattivazione della poli (ADP) ribosio polimerasi in conseguenza al trattamento con i chinoni suggerendoci di investigare maggiormente sulle possibili differenze tra i diversi processi ossidativi della dopamina. La seconda parte del progetto si è focalizzata sul possibile ruolo delle superossido dismutasi 1 e 2 contro la tossicità indotta dalla dopamina e dai suoi prodotti di ossidazione. L'over espressione sia della SOD1 che della SOD2 hanno rivelato un effetto protettivo contro la produzione di anione superossido indotto dalla dopamina, mentre non hanno presentato alcun effetto contro la tossicità indotta dai chinoni rimarcando il ruolo dell'anione superossido nella tossicità indotta dalla dopamina. Nel presente progetto di dottorato, abbiamo valutato l'ipotesi che lo stress ossidativo indotto dalla dopamina e dalle sue specie ossidate possa avere un ruolo chiave nella specifica degenerazione dei neuroni dopaminergici caratteristici della malattia di Parkinson. I dati ottenuti sembrano dare indicazioni sulla differente attivazione di pathway di morte cellulare indotta dalla dopamina e dalle sue forme ossidate con un differente ruolo nella produzione dell'anione superossido. Poichè lo stress ossidativo è considerate uno dei meccanismi che collegano il Parkinson sporadico a quello genetico e la dopamina, nella sua chimica ossidativa, porta alla produzione di specie radicaliche, riuscire a capire quale pathway di morte cellulare è attivato e in quale misura, è cruciale per lo sviluppo di terapie per impedire l'inizio e la progressione della malattia. I dati derivanti dall'over espressione delle superossido dismutasi dimostrano che composti che impediscono la formazione dell'anione superossido (come SOD-mimetici che sono attualmente usati per altre patologie) potrebbero avere un ruolo protettivo contro lo stress ossidativo e la conseguente morte cellulare indotta. Inoltre, anche composti in grado ci bloccare la cascata apoptotica indotta da dopamina e chinoni potrebbe proteggere dalla morte le cellule affette in questa malattia neurodegenerativa.

1. Introduction

Parkinson's disease is a widespread neurodegenerative disease that was first described by James Parkinson (1755-1824) in his monograph "*An Essay of the Shaking Palsy*" (Parkinson 1817).

The author described the pathology as an "*involuntary tremolous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a runnin pace: the senses and intellect being uninjured*". (Parkinson 1817)

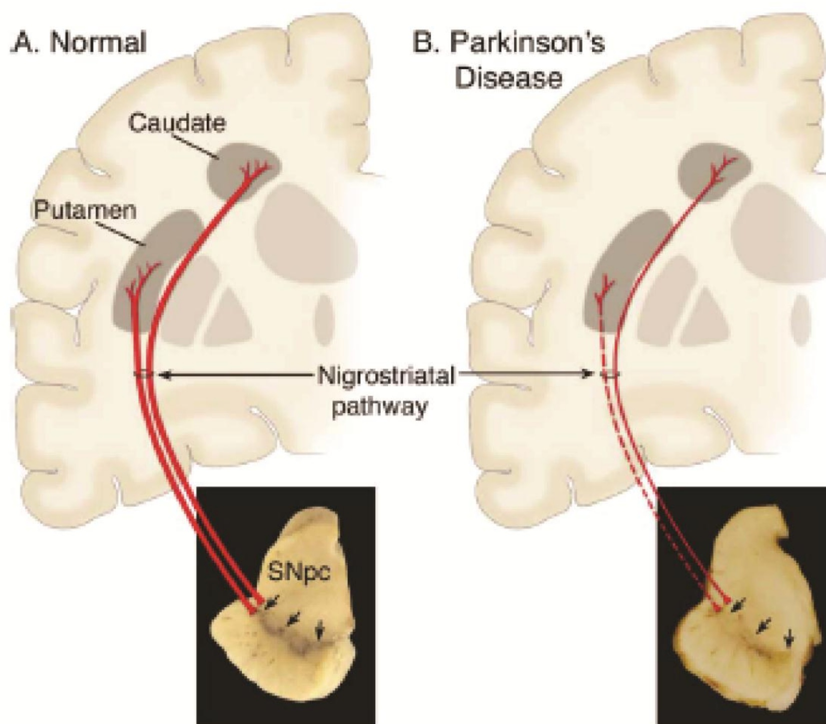
Jean Martin Charcot, one of the most important neurologist, proposed to name the disease as Parkinson's disease (Lees 2009). From its description, a great effort was put on understanding the starting point and the progression of the pathology, but nowadays the cause of the pathology is still an open field.

1.1 Parkinson's disease

1.1.1 Clinical and pathological hallmarks

Parkinson's disease (PD) is a widespread progressive neurodegenerative disease and it is second only to Alzheimer's disease with an increasing incidence associated with age with a peak above 60 years old. The onset of the disease is gradual and the pre-clinical manifestations go unnoticed until more than the 80% of the neurons are lost (Lees, 2009). The features of PD comprise tremor at rest, bradykinesia, rigidity, loss of postural reflexes, flexed posture, and the freezing phenomenon (Fahn, 2003). In addition to the motor symptoms, there are also non motor manifestations. These include bradyphrenia (slowness in mental function), decreased motivation and apathy, dementia, fatigue, depression, anxiety, sleep disturbances (fragmented sleep and REM sleep behavior disorder), constipation, bladder and other autonomic disturbances (sexual, gastrointestinal), and sensory

complaints. Dementia is associated with age, and has been reported to occur in over 70% of patients with PD eventually (Chaudhuri, 2006). The pathological hallmarks of the disease are the selectivity death of the dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) of the midbrain and in some cases of the presence of citoplasmatic inclusions called Lewy bodies composed by insoluble aggregates mainly composed of α-synuclein and ubiquitin (Spillantini 1997, Shults, 2006).



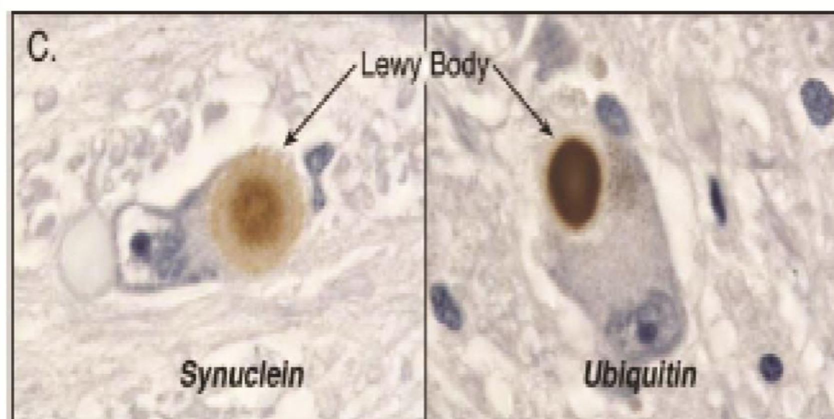


Fig. 1. Neuropathology of Parkinson's Disease. (A) Schematic representation of the normal nigrostriatal pathway (in red). dopaminergic neurons project (thick solid red lines) to the basal ganglia and synapse in the striatum (i.e., putamen and caudate nucleus). The photograph demonstrates the normal pigmentation of the SNpc, produced by neuromelanin within the dopaminergic neurons. **(B)** Schematic representation of the diseased nigrostriatal pathway (in red). In Parkinson's disease, the nigrostriatal pathway degenerates. There is a marked loss of dopaminergic neurons demonstrated by the massive depigmentation. **(C)** Immunohistochemical labeling of intraneuronal inclusions, termed Lewy bodies, in a SNpc dopaminergic neuron. On the left, immunostaining with an antibody against α-synuclein while on the right, immunostaining against ubiquitin. (Dauer and Przedborski, 2003)

In Parkinson's disease, apart from the dopaminergic neurons, other areas are affected; among them, noradrenergic neurons in the locus coeruleus and serotoninergic neurons in the dorsal raphe nucleus (Braak et al. 2004, Shen and Cookson 2004).

1.1.2 Treatments

Dopamine replacement therapy is the major medical approach in treating PD, and a variety of dopaminergic agents are available. These drugs are: dopamine agonists (such as ropinerole and pramipexole) activating pre- and post-synaptic dopamine receptors, MAO-B (selegiline) and COMT inhibitor to reduce the catabolism of dopamine. The main drug used to replenish the dopamine lost is L-DOPA: the dopamine precursor, but its use after some years leads to the appearance of other motor dysfunction such as dyskinesia (involuntary movements) or wearing-off (Schapira 2009).

Other kind of treatments include brain surgery known as deep brain stimulation (DBS) that can be appropriate for patients with advanced disease and complications from medications (Fahn 2003) and physical therapy that include exercises to maintain the joints and muscles loose and to learn techniques of better gait and balance.

1.1.3 Neuronal circuits implicated: the basal ganglia

The basal ganglia (or basal nuclei) are a group of nuclei of different origin in the brains of vertebrates that act as a cohesive functional unit. They are situated at the base of the forebrain and are strongly connected with the cerebral cortex, thalamus and other brain areas. The basal ganglia are associated with a variety of functions, including voluntary motor control, procedural learning relating to routine behaviors or "habits". (Stocco et al. 2010) The main components of the basal ganglia are the striatum (caudate nucleus and putamen), the globus pallidus (divided in internal and external segment), the substantia nigra, and the subthalamic nucleus (Fix et al. 2008).

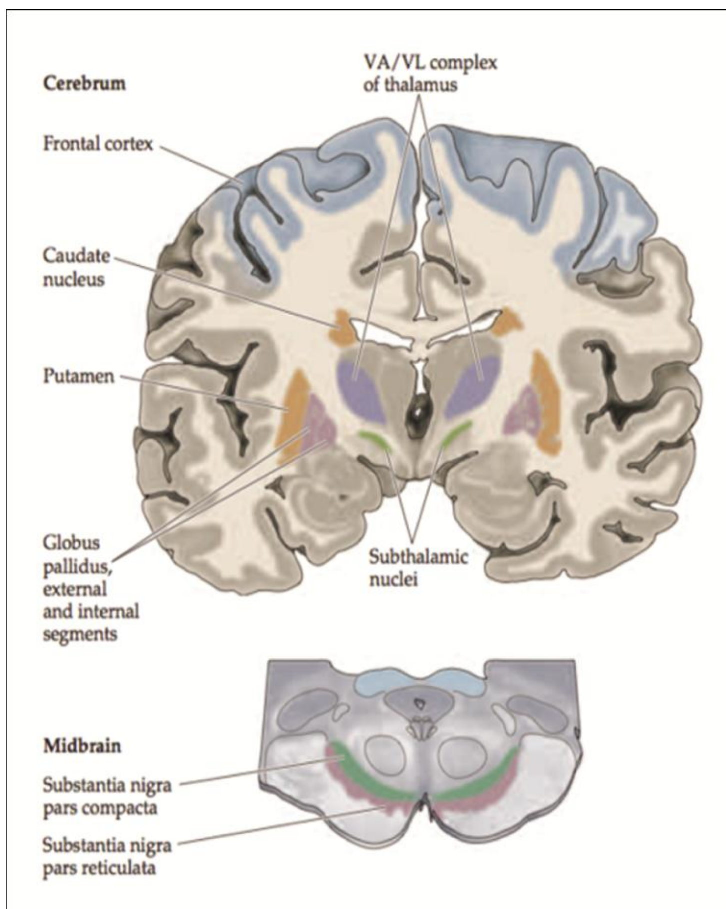


Fig. 2. Structures involved in the basal ganglia circuits. (Dale Purves 2001)

Glutaminergic neurons raising from the cerebral cortex projects to the spiny neuron of the striatum, which also receive projections from both the thalamus and

brain stem (dopaminerigc input from the midbrain and serotoninergic projections from the raphe nuclei). The striatum send its projections in the substantia nigra pars reticulate and to the globus pallidus (internal segment). Neurons of the substantia nigra pars reticulata inhibit the superior colliculus, while those of the internal segment of globus pallidus inhibit the anterior ventral nucleus and the lateral ventral nucleus (in the thalamus). The thalamus stimulates the motor cortex via glutamatergic projections. At rest, cortical neurons are inhibited while the neurons of the internal segment of globus pallidus and the susbtantia nigra pars reticulate are activated, inhibiting the thalamic neurons. On the other hand, when the cortex neurons are excited activating the striatum, they inhibit the downstream neurons, activating the thalamus leading to the activation of motor cortex neurons and facilitating movements (direct pathway). Another pathway, the indirect one, comprises spiny neurons of the striatum that projects first to the external segment of globus pallidus and subsequently to internal segment and to the subtalamic nucleus (GABAergic projections).

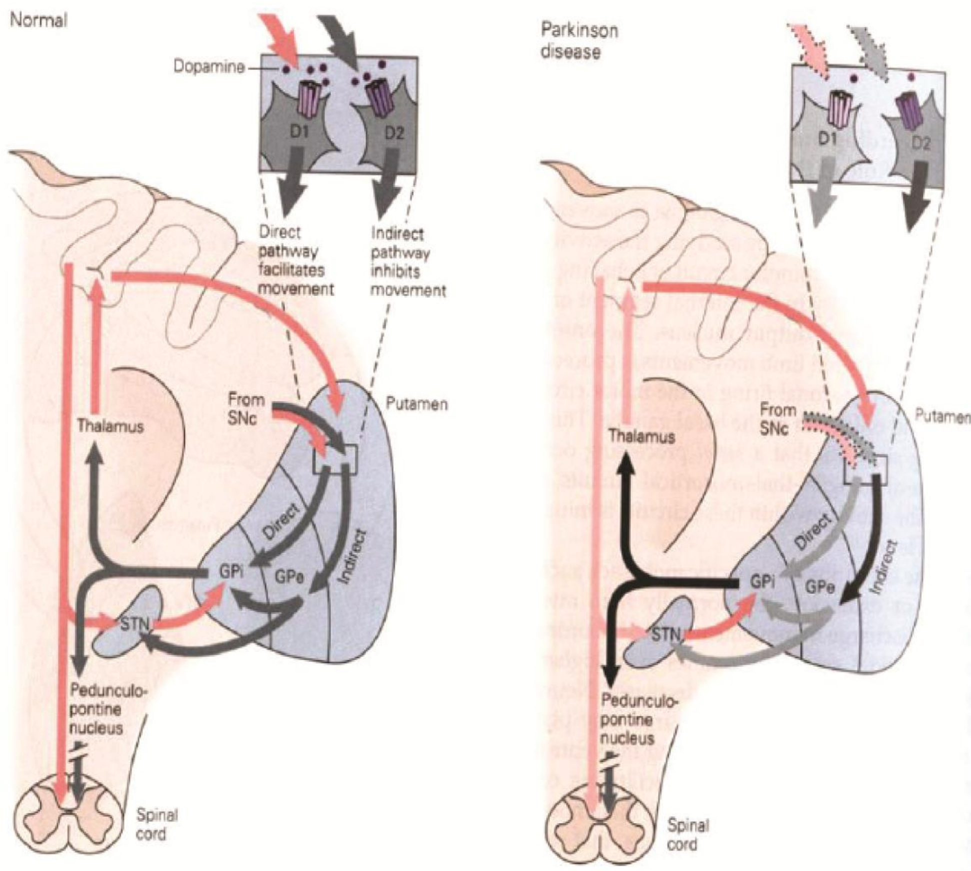


Fig. 3. The basal ganglia-thalamocortical circuit in normal conditions (left) and in parkinosn's disease (right). Excitatory connections are represented in pink, while inhibitory in grey and black arrows. Under normal conditions the indirect and the direct pathway modulate the dopaminergic neurons of the substantia nigra pars compacta. In Parkinson's disease is absent the modulation from the SNc and there's an increase in the inhibition of the thalamus leading to a difficulty to start movements. Changes in activity are represented with changes in the darkness of arrows: lighter arrows indicates a decreased activity, while the darker an increased activity. (Eric Kandel 2000).

The latter is connected to the internal segment of globus pallidus and to the substantia nigra pars reticulata via excitatory glutamatergic projections. Once the indirect pathway is activated by cortical projections, the external segment of globus pallidus is inhibited leading to the disinhibition of subtalamic nucleus stimulating the internal segment of the globus pallidus. The result is the inhibition of thalamus leading to the inhibition of movement. The direct pathway provides a positive feedback while the indirect a negative feedback on the circuit between the

basal ganglia and the thalamus. Neurons of the substantia nigra pars compacta have a role both in the direct and indirect pathway leading to an excitatory effect and inhibitory effect on the direct and indirect pathway respectively. These neurons present two different kinds of dopamine receptors: D1 type receptor in the direct and D2 type receptor in indirect pathway (Dale Purves, 2001, Eric Kandel 2000). Both type of receptors are coupled to G-proteins leading to the activation of adenylyl cyclase (D1) and its inhibition (D2) resulting in a different cellular response (Vallone et al. 2000). Dopaminergic input leads to the inhibition of the thalamocortical circuit facilitating the movement. The dopaminergic neurons degeneration that occur in Parkinson's disease, leads to the increase of inhibition in the basal ganglia. The output activity of the nuclei is increased leading to an increased inhibition of the thalamus inhibiting the activation of cortical neurons. This finally results in impaired movements (Dale Purves 2001, Eric kandell 2000)

1.2 Etiology of parkinson's disease

1.3 Genetics forms of PD

Parkinson's disease has been described as a multifactorial pathology and nowadays the etiology of the disease is still unknown. Genetic predisposition, environmental toxins, protein misfolding and aggregation, neuroinflammation, oxidative stress, mitochondrial and proteasomal dysfunction have been proposed as possible mechanisms with aging considered the major risk factor associated with the appearance of the pathology. About 5% of the cases are linked to mutation on several genes that are listed in the tab. 1. (for a review see Bekris et al. 2010)

Locus	Gene	Chromosome	Inheritance	Probable function
PARK1/4	alfa-synuclein	4q21	AD	Presynaptic protein. Lewy bodies
PARK2	Parkin	6q25.2-27	AR	Ubiquitin E3 ligase
PARK3	Unknown	2p13	AD	Unknown
PARK4	Unknown	4p14	AD	Unknown
PARK5	UCHL-1	4p14	AD	Ubiquitin C-terminal Hydrolase
PARK6	PINK1	1p35-36	AR	Mitochondrial kinase
PARK7	DJ-1	1p36	AR	Chaperone, antioxidant
PARK8	LRRK2	12p11.2	AD	Mixed lineage kinase
PARK9	ATP13A2	1p36	AR	Unknown
PARK10	Unknown	1p32	AD	Unknown
PARK11	Unknown	2q36-37	AD	Unknown
PARK12	Unknown	Xq21-q25	Unknown	Unknown
PARK13	HTRA2	2p12	Unknown	Mitochondrial serine protease

AD: autosomal dominant, AR: autosomal recessive.

Tab. 1. Gene associated with PD. (Farrer 2006, Yang 2009).

1.3.1 Genes associated with autosomal dominant PD

α -Synuclein (PARK1/4)

Alfa-synuclein was the first gene discovered and associated with autosomal dominant PD in its mutations A30P (Kruger 1998) E46K (Zarranz 2004) and A53T (Polymeropoulos 1997), duplication and triplication (Singleton 2003).

The gene product is a small protein (14,5 KDa) of 140 amino acids, natively unfolded whose physiological function is still unknown. Alfa synuclein is one of the major components of Lewy bodies (Shults 2006) and its ability to form oligomeric species and fibrils seems to account for its toxicity at the cellular level (Auluck 2010, Goedert 2001, Lotharius and Brundin 2002).

Leucine-rich repeat kinase 2 (PARK8)

Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are the most common forms of inheritable Parkinson's disease and likely play a role in sporadic disease as well. LRRK2 is a large multidomain protein with several physiological roles. The first mutation associated with the pathology was reported in 2002 (Funayama 2002), but later on other mutations were associated with the onset of the disease. The disease appears to be dominant with an incomplete penetrance and Lewy bodies are not always present (Latourelle 2008). The phenotype of LRRK2 mutation is referred to be pleomorphic since this protein is implicated in multiple signaling pathways (Zimprich 2004)

1.3.2 Genes associated with autosomal recessive PD

PARKIN (PARK2)

Mutations in Parkin, an E3 ubiquitin ligase, cause a recessive, early-onset, slowly progressive parkinsonism accounting for the majority of early-onset familial PD. More than 100 distinct mutations have been identified for this gene. In one study of patients with onset of Parkinson disease prior to age 40 (10% of all PD patients), 18% had Parkin mutations, with 5% homozygous mutations.(Poorkaj

2004). Parkin catalyzes the transfer of ubiquitin to target proteins to either mark them for degradation by the ubiquitin–proteasome system or for non-degradative signaling purposes. Recently, parkin has been implicated also in mitochondrial maintenance (Abou-Sleiman et al., 2006)

PTEN-induced putative kinase 1 (PINK1)

PTEN-induced putative kinase 1 (PINK1) is a mitochondrial serine/threonine-protein kinase encoded by the PINK1 gene. Mutations in this gene are the second most common cause of autosomal recessive early-onset familial PD with most of the mutations affecting the kinase activity of the protein (Kawajiri et al. 2011, Harowitz and Greenamyre 2010, Thomas and Cookson 2009). Loss of PINK1 function leads to a less protective effect of this protein on mitochondria maintenance and function against oxidative stress. (Akundi et al. 2011, Diedrich et al. 2011, Moore et al. 2005)

DJ-1

DJ-1 has an unknown function (Abou-Sleiman et al., 2003; Bonifati et al., 2003) and it has been linked to a rare, recessive form of parkinsonism called PARK7. (Bonifati et al. 2003). The exact function of DJ-1 is unknown, and it was first identified as a human oncogene (Nagakubo et al., 1997). Further studies demonstrated tha DJ-1 becomes more acidic in response to oxidative stressors, such as hydrogen peroxide or paraquat (Mitsumoto and Nakagawa, 2001; Mitsumoto et al., 2001). This suggests that DJ-1 might function in the oxidative stress response.

Other genes and loci associated with PD

Besides the gene listed above, there are several other gene and genetic loci that have been identified, but it is still object of debate if there is an effective connection to PD. These genes are: ubiquitin carboxy-terminal hydroxylase

(UCHL-1) (PARK5), ATP132A2 (PARK9), Omi/HtrA2 (PARK13). UCHL-1 is a protein involved in the ubiquitin-proteasome system and is implicated in the hydrolysis of polymeric ubiquitin chains. Its presence in Lewy bodies is still controversial as well as the link between its mutation I93M and the polymorphism S18Y (Leroy et al. 1998, Lincoln et al. 1999, Hutter et al. 2008, Ragland et al. 2009. ATP13A2 is a lysosomal membrane protein that belongs to the P5 subfamily of P-type transport ATPases, a transporter family of 5 proteins (ATP13A1–5) with unknown substrates (Schultheis et al., 2004). ATP13A2 was found mutated in several cases of early onset PD (Di Fonzo et al., 2007; Santoro et al., 2011). Several studies linked this protein to the network of alfa-synuclein and in the regulation of mitochondria (Gitler et al., 2009, Gusdon et al., 2012, Grunewald et al., 2012). Omi/HtrA2 is a mitochondrial serine-protease that was also found in Lewy bodies. The G399S mutation and the A141S polymorphism were identified to correlate with PD (Strauss et al. 2005) but another study performed on a larger population did not confirm these data (Ross et al. 2008). All these genes and additional genetic loci are still on validation.

1.4 Sporadic Parkinson’s disease

1.4.1 Neuroinflammation

Idiopathic Parkinson's disease (PD) represents a complex interaction between the vulnerability of the nigrostriatal dopaminergic system, a possible genetic predisposition, and exposure to environmental toxins including inflammation triggers. Mc Geer and collaborators, in 1990s, first proposed the involvement of neuroinflammation in the pathogenesis of PD, since they found activated microglia cells in the substantia nigra of PD patients. (Mc Geer et al. 1988). Other evidence, now suggest that chronic neuroinflammation is tightly associated with the pathophysiology of PD. Activation of microglia and increased levels of pro-inflammatory mediators and reactive oxygen species has been found in *post-mortem* analysis of the *substantia nigra* of PD patients and in animal models of PD. It has been suggest that there’s a vicious cycle between ROS production

and pro-inflammatory marker leading to the aggravation and progression of the pathology (Collinsa M. 2012, Glass et al. 2010).

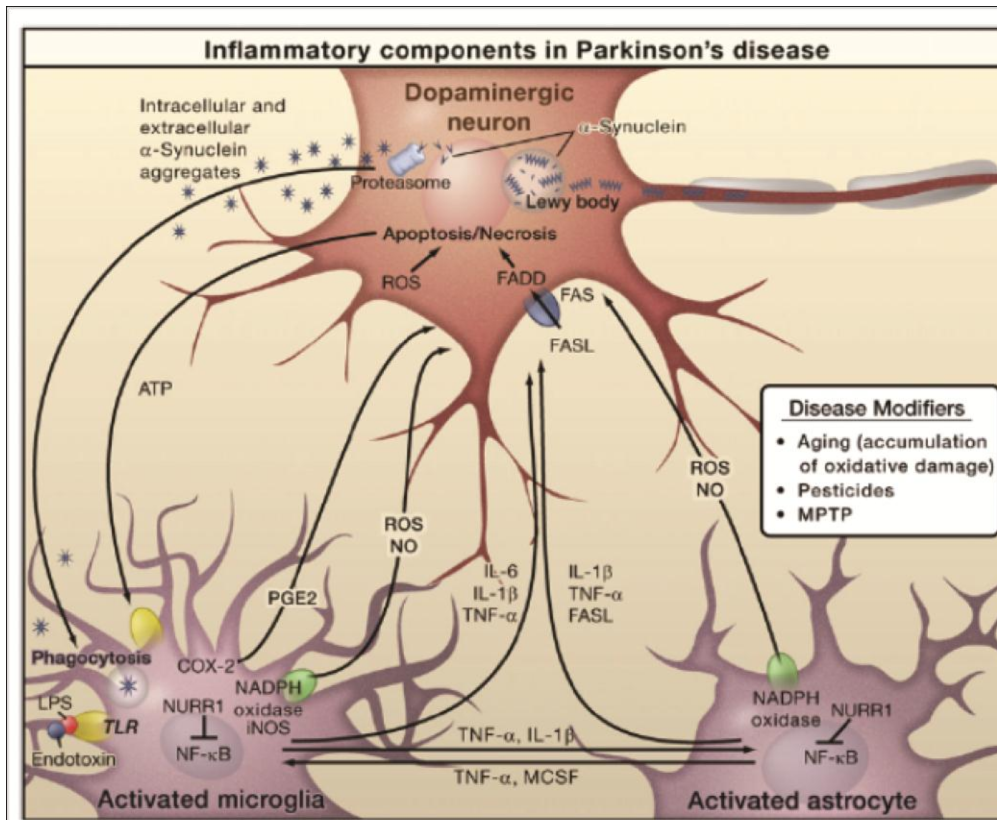


Fig. 4. Proposed mechanism of the involvement of inflammation in PD. (Glass et al. 2010)

1.4.2 Proteasome dysfunction and protein aggregation

The ubiquitin-proteasome system (UPS) is essential for the non-lysosomal degradation and clearance of dysfunctioning proteins (Sherman et al. 2001). This is mediated by a series of reactions mediated by different enzyme that identify abnormal proteins for degradation with a multiple ubiquitination tail. Ubiquitin is a key protein for the poly-ubiquitination of this kind of proteins, and it is generated by a ubiquitin-activating enzyme (E1), subsequently is transferred to ubiquitin-conjugating enzyme (E2) and ligated to lysine residues of the protein substrates in a reaction catalyzed by different ubiquitin protein ligase (E3). The complex formed is recognized and degraded by the 26S proteasome (DeMartino 1999). In sporadic PD brains, a decreased activity of the ubiquitin-proteasome

system (UPS) was found and this was linked to the abnormal accumulation of misfolded proteins (Xie 2010).

The first indication that a failure of the UPS could be a mechanism involved in the pathogenesis of PD, was suggested form the observation of the presence of Lewy bodies (Forno et al. 1996, Pollanen et al. 1993) and numerous group suggest that this inclusions were formed to prevent the possible cytotoxicity of poorly-degraded proteins but this theme is still object of debate (Robinson 2008, Dawson et al. 2003, Shin et al. 2009). Another reason that reinforced the involvement of the proteasome system dysfunction in PD was linked to the discovery of mutations in the UCHL-1 and Parkin genes. Both proteins are involved in the UPS machinery with the first responsible for the recovery of free ubiquitin protein, and the latter for its ubiquitn-ligase activity. A loss of function in these proteins could account for the accumulation of poorly degraded proteins.

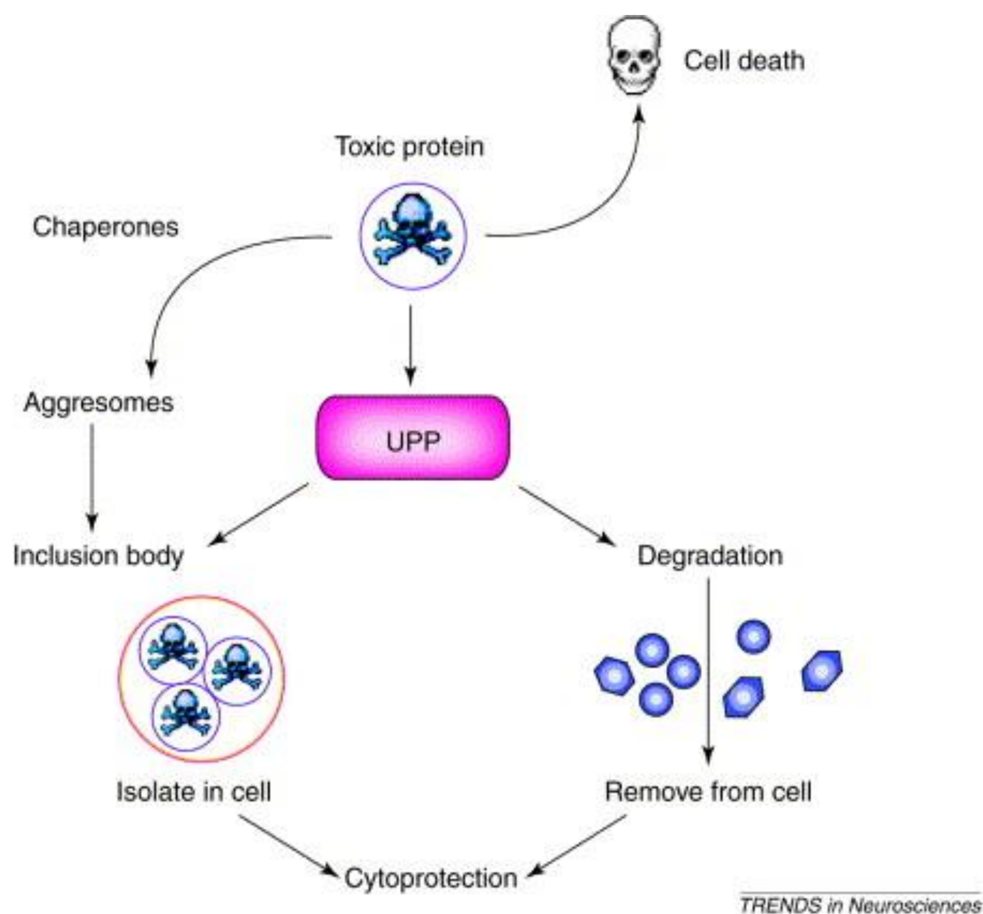


Fig. 5. A toxic or damaged protein can be detoxified via at least two pathways. The first involves the ubiquitin-proteasomal pathway (UPP) where the protein is tagged for degradation by the proteasome. If the toxic or damaged protein exceeds the capacity of the proteasome the ubiquitinated protein might serve as a nucleation center for aggregates and inclusion bodies. Aggresomes might be intermediates in the formation of inclusion bodies. Inclusion bodies and the UPP appear to work in a coordinated fashion to protect the cell from toxic or damaged proteins. The capacity of both of these systems might be overwhelmed, leading to further compromise in a feed-forward pathway that ultimately results in the demise of the neuron (Chung et al. 2001).

1.4.3 Oxidative stress

Oxidative stress is a condition in which the antioxidant defenses of the cells are not able to counteract the production of reactive oxygen and nitrogen species (ROS/RNS). The brain is more susceptible to oxidative stress for different reasons: a high consumption of molecular oxygen, high content of proteins and lipids that can be oxidized and low level of antioxidant molecules (Floyd 1999; Poon et al. 2004). Numerous studies have confirmed increased levels of several markers of oxidative damage in the SN of PD patients: DNA damage, lipid peroxidation, protein oxidation, less glutathione in its reduced form, and increased iron deposition (Jenner and Olanow 1996). The role of oxidative stress is still object of debate: is it a causative factor or simply a downstream consequence? (Andersen 2004). Oxidative stress can result from defects in several systems, such as mitochondrial dysfunction, increased calcium levels, neurotoxins and inflammatory responses and low antioxidant capacity. Moreover one potential source of ROS is dopamine (DA) itself that could provide the rational for the selectivity death of dopaminergic neurons (Graham et al. 1998).
Overall, it appears that oxidative (and nitrative) stress can be linked both to sporadic and familial PD suggesting a prominent role in initiating and amplifying effect on pathways that favor cell death in this neurodegenerative disorder.

Cellular antioxidant defenses

Oxidative stress is a condition where the over production of reactive oxygen a nitrogen species overwhelm the antioxidant capacity of a cell. Cellular levels of ROS are controlled by antioxidant enzymes and antioxidants small-molecules.

Enzymatic antioxidants

Superoxide Dismutase

As major antioxidant enzymes, superoxide dismutases (SODs), play a crucial role in scavenging O2•−. The superoxide dismutase family is responsible in the dismutation of superoxide anion radicals into molecular oxygen and hydrogen peroxide. Three distinct isoforms of SOD have been identified and characterized in mammals: mostly cytosolic copper-zinc superoxide dismutase (SOD1), mitochondrial manganese superoxide dismutase (SOD2) and extracellular superoxide dismutase (SOD3). These isoforms of SOD exhibit similar functions, but requires different co-factor and exhibit different cellular localization. (for a review see Abreu et al. 2010).

Glutathione Peroxidases

Glutathione peroxidases are a family of isozymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH) as an electron donor (H_2O_2 + 2GSH → GS-SG + $2H_2O$). (Margis et al. 2008).

Catalase

Catalase is an enzyme that is responsible for the conversion of hydrogen peroxide to water. It is localised in peroxisomes and may also be found in cytoplasm and mitochondria. (Dröge et al 2002).

Non enzymatic Antioxidants

GSH

The main antioxidant in the central nervous system, glutathione (GSH), is the most abundant antioxidant small molecule (Dringen 2003). It consists of a tripeptide of glutamate, cysteine and glycine and is particularly interesting in the context of PD because it has been found a marked depletion of this molecule in substantia nigra of PD patients. (Barker et al. 1996)

Vitamin E

The role of vitamin E in the central nervous system is not fully understood although it is a lipid soluble molecule with antioxidant function. It appears to neutralize the effect of peroxide and prevent lipid peroxidation in membranes.

1.4.4 Mitochondrial dysfunction

Mitochondrial dysfunction was proposed as one of the possible mechanisms that triggers and is part of the progression of PD. Strong evidence exists to support a role for aberrant mitochondrial form and function, as well as increased oxidative stress, in the pathogenesis of PD (Beal MF. 2007, Schapira 2008) There is complex link between mitochondria and other cellular machinery that affects cell survival, being that mitochondria have different important roles. First they have a key role in oxidative phosphorylation leading to the production of ATP, but they are also the main cellular source of free radicals, and they are involved in calcium homeostasis and in the regulation of cell-death pathways.
Furthermore, several genes involved in the genetic forms of PD are involved in the balance of mitochondrial fission and fusion, thus affecting the maintenance of these organelles. (Henchcliffe et al. 2008, Exner et al. 2012). Mitochondria are important source of energy for the cells, and an energy failure leads to an impairment of several cellular functions such as ubiquitin-proteasome system and the release of dopamine from synaptic vesicles. The first link between Parkinson’s disease and the mitochondrial dysfunction was found in 1980s, when it was

discovered that the compound MPTP, an analog of meperidine, leads to features that mimic Parkinsonian syndrome, in particular inhibiting the mitochondria respiration (Langston et al. 1983, Nicklas et al. 1985). In vivo, MPTP is converted to MPP+ by MAO-B in glial cells, then, it is transported into neurons by catecholamine transporters, and there it exert its toxicity by inhibiting complex I. This leads to an increase production of ROS and ATP depletion. MPTP, since its discovery, was used to generate several PD model aiming to mimic features of this pathology (Schober 2004). Another compound used in PD research is rotenone, an insecticide that is also a complex I inhibitor. Epidemiological studies have implicated a link between rural environment and the related exposure to herbicides and pesticides with an elevated risk of PD (Tanner et al. , 1999)

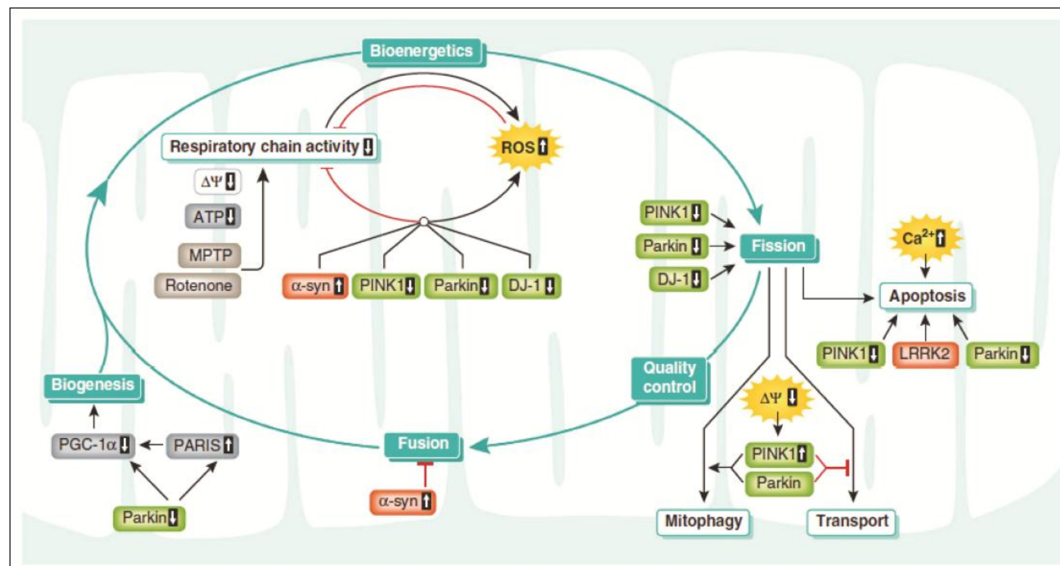


Fig. 6. Schematic representation of genes implicated in the mitochondria network. (Exner 2012).

In the following years, more precisely in 1989 a defect was first identified in the complex I of the *sustantia nigra* of PD patients (Schapira et al. 1990, Schapira et al. 1989, Mann et al. 1994) but the cause of this deficiency is not understood (Greenamyre et al. 2001. Further, mitochondria oxidative phosphorylation depends on both mitochondria and nuclear DNA-encoded proteins. Mutations in mitochondrial DNA (mtDNA) support the fact that alteration in the mitochondria

genome causes respiratory defects and phenotype typical of ageing process and age-related diseases. (Park and Larsson 2011)

1.4.5 Dopamine

Dopamine (DA) is a neurotransmitter that account for a variety of function through the body. In the central nervous system (CNS), dopamine is involved in the regulation of movements (nigrostrial pathway) and motivated behavior (mesolimbic pathway). Parkinson's disease is a neurodegenerative disorder that is characterized by the preferential degeneration of dopaminergic neurons in *substantia nigra pars compacta*. Different mechanisms have been proposed to elucidate the start and the progression of the pathology. Since all the mechanisms proposed do not explain alone the susceptibility of dopaminergic neuron loss, the main working hypothesis is that dopamine itself could present potentially toxic characteristics (Graham 1978) Beside the great attention focused on the production of reactive oxygen species (ROS) during dopamine metabolism, growing evidence suggest that this neurotransmitter itself may play a direct role. Under physiological conditions, dopamine is synthesized in the cytosol from L-tyrosine by the action of the enzyme Tyrosine hydroxylase to form L-Dihydroxyphenylalanine (L-DOPA) that is converted to dopamine by the enzyme aromatic L-amino acid decarboxylase (AADC). Upon synthesis, dopamine is transported from the cytosol into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2) where it is stabilized by the low pH. Upon the arrival of an action potential, dopamine is released in the synaptic cleft to exert its function, after that it is uptaken via the dopamine transporter and recycled in new synaptic vesicles. If the amount of cytosolic DA exceeds the physiological concentration, DA is metabolized into the non-toxic metabolite 3,4-dihydroxyphenylacetic acid and hydrogen peroxide by the action of monoamine oxidase (MAO) and aldehyde dehydrogenase, or sequestered into lysosomes where it can auto-oxidize to form neuromelanin (NM). If not buffered by these pathways, cytosolic DA can be oxidized to DA-quinone (DAQs) (spontaneously or enzymatically), a reaction that also leads to the formation of ROS.

Dopamine oxidizes producing dopamine-o-quinone (DQ), which cyclizes to leukoaminochrome. The latter oxidizes to aminochrome (AC) rearranging to 5,6-dyhydroxindole (DHI). The subsequent oxidation of DHI leads to the formation of indole-5,6-quinone (IQ) which polymerize to form neuromelanin. (Grahm 1978; Hastings 1995; Tse 1976).

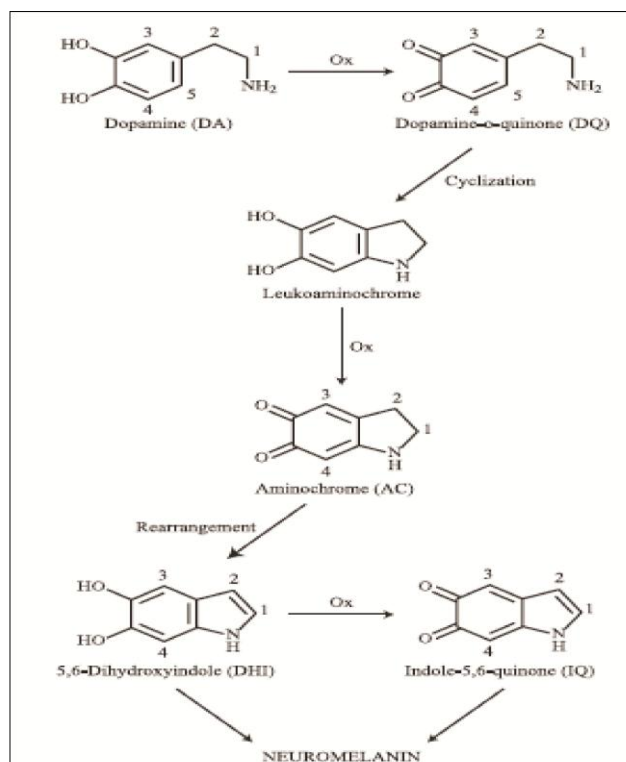


Fig. 7. The dopamine auto-oxidation pathway.

Dopamine oxidizes to dopamine-o-quinone (DQ), which cyclizes to leukoaminoscrhome. Upon oxidation leukoaminochrome is converted into aminochrome (AC), which rearranges to 5,6-dyhydroxindole (DHI). DHI can be oxidized into indole-5,6-quinone (IQ) and polymerase to neuromelanin. (Bisaglia et al. 2007)

The oxidation process of dopamine is accelerated by the presence of transition metal ions such as iron, copper and manganese (Sulzer and Zecca 2000) and by the presence ho peroxynitrite (LaVoie and Hastings 1999). Dopamine-quinones (DAQs) are electron-deficient and are able to react with cellular nucleophiles, such as cysteine residues of proteins.

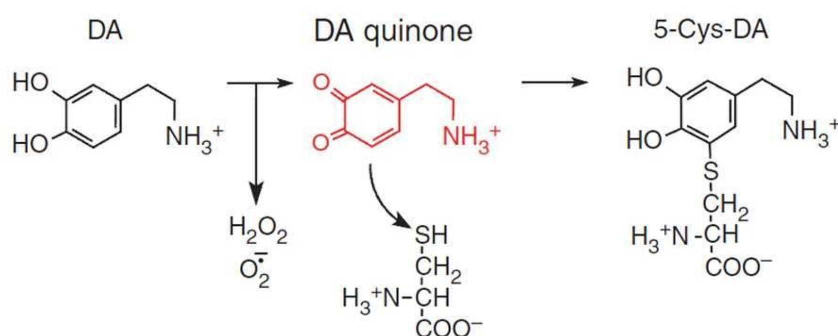


Fig. 8. Schematic representation of the oxidation of dopamine (DA) to dopamine-quinone (DQ) and its chemical reaction with cysteine residues. (Adapted for LaVoie 2005)

The covalent modification of these cysteine residues leads to the formation of 5-cysteinil-dopamine adduct leading to an impairment or a block of the proteins activities, since cysteine residues are often localized in the active site of proteins (Asanuma 2003). It has been demonstrated that different proteins are affected by the DAQs modification, such as, DJ-1, SOD2, α-synuclein, Parkin and UCH-L1 (Girotto 2012, Belluzzi 2011, Bisaglia 2007, Bisaglia 2010b, LaVoie 2005, Van Laar 2008, Van Laar 2009).

1.4.6 Apoptosis in PD

In response to excessive damage, or unfavorable intracellular or extracellular conditions, cells have evolved different mechanisms leading to cell death. Programmed cell death (PCD) is a conserved mechanism and plays a pivotal role in many physiological processes, especially during development and in maintaining cellular homeostasis. On the other hand, there is evidence that such mechanism is implied even in neurodegenerative disorders such as Parkinson's disease. Numerous assays were developed to detect and quantify apoptosis such as ultra structural analysis of cell morphology, detection of caspases activation and their substrates cleavage, DNA fragmentation and mitochondrial functionality. The involvement of apoptosis in PD, first came by from the observation that the MPTP toxin-model for PD leads to the appearance of classical features of this

kind of programmed cell death (DiPasquale et al. 1991). However, *post-mortem* studies on brains of PD patients, lead to conflicting conclusions on role for apoptosis in the neurodegeneration of dopaminergic neurons. Several laboratories found apoptotic neurons (Mochizuki et al. 1996, Anglade et al 1997, Tompkins et al. 1997) in the PD patients, but other could not replicate this findings (Banati et al. 1998, Wüllner et al. 1999) generating a debate on the matter that is still open.

The progress of the tools used to detect apoptosis with the identification of new and different markers, that combined allowed confirming the role for apoptosis in PD (Tatton et al. 2000, Tatton et al. 2003, Hartmann et al. 2000). Recently caspase-3, an effector enzyme in programmed cell death, was associated with Parkinson's disease on a post-mortem human brain study (Hartmann et al., 2000).

1.4.7 Cellular models in the context of PD

PD is a complex and multi factorial pathology that nowadays has no cure. In vitro models (establish cell line, primary cell culture or stem cells) have different advantage in understanding the molecular mechanism involved in this pathology. Besides to the limitations posed by a simple model, like the cellular models are, they have many advantages. The most prevalent PD cell models include non neuronal tumor cell lines such as pheochromocytoma (PC12) cells, neuronal tumor cell lines like human neuroblastoma (SH-SY5Y) cells and primary mesencephalic neurons. They are very useful model to mimic feature of PD since they can be used to evaluate toxic-insult, drugs screening with different compound and serves to investigate single pathogenic mechanism implications of genes or proteins.

The effect of PD-related toxins and genes were widely investigated in catecholinergic human neuroblastoma cell lines, such as SH SY5Y, SK-N-BE or BE2-M17. Further, neuron-like cellular model are the PC12, a cell line derived from a pheochromocytoma of the rat adrenal medulla and MES, a hybrid rat mesencephalic-neuroblastoma cell line.

In addition to its possible application in the cure for PD, stem cells also represent a good candidate for the PD research. The possibility to reproduce in vitro models of dopaminergic neurons comes from the use of human induced pluripotent stem cells (iPS). To date, there’s much effort in obtaining a standard protocol to reproduce iPS phenotype to obtain patient-specific stem cell lines for studying various disease mechanism (Seibler et al. 2011). Primary neuronal cultures derived from animal model can improve the investigation on PD pathogenesis at cellular level but have the disadvantage that they are not human genetic based.

SH SY5Y cell lines have been widely used as a PD model since it possess the complete dopaminergic system and have the advantage to present a human genetic background. This cell line has been used primary for reproducing the impaired dopamine homeostasis since they present a good activity of the dopamine transporter (DAT) and a low activity of the vesicular monoamine transporter (VMAT2) allowing to increase the cytoplasmatic dopamine concentration by its administration in the cell culture medium (Bossi et al 2010, Alberio, Colapinto et al. 2010). This cell line, can be differentiated using different compounds such as retinoic acid (RA), phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), brain derived neurotrophic factor (BDNF), dibutyryl cyclic AMP (dBcAMP), purine, or staurosporine (Singh 2007, Påhlman 1981, Cernaianu 2008, Kume 2008, Guarnieri 2009 and Mollereau 2007) leading to a different morphological and biochemical phenotype. The debate on the use of undifferentiated and differentiated cells is still open, but in different studies, it has been demonstrated that undifferentiated cells are more susceptible to toxins, leading to the suggestion that this kind of cells are a better cellular model to study toxicity derived from internal and external toxins. (Cheung 2009).

Aim of the thesis

In Parkinson's disease, the selectivity death of dopaminergic neurons of the *substantia nigra pars compacta* is ascribed to several mechanisms among them, oxidative stress and mitochondrial dysfunction play a pivotal role and are good candidates to interconnect both familial and sporadic forms of the disease. To explain the selectivity death of dopaminergic neurons, it has been proposed that dopamine could have a central role in the start and progression of the pathology.

Several studies suggest that an increase in cytosolic dopamine concentration (that could derive from different mechanism such as an improper vesicular storage or catabolism) leads to the oxidation of the latter to toxic dopamine-quinones (DAQs) and reactive oxygen species production. The formation of the former is a process that can occur either spontaneously or by enzymatic catalysis. Several enzymes have been proposed to be responsible for the enzyme-mediated oxidation process of dopamine, among them tyrosinase (TY). This enzyme is the rate-limiting step for the production of melanin, but several studies suggest also a role in the oxidation of dopamine into DAQs leading to cellular toxicity. The presence of the mRNA and the active protein in substantia nigra was confirmed (Greggio et al. 2005)focusing attention on the tyrosinase-mediated oxidation of dopamine as an additional mechanism in the start and progression of the disease. The aim of this project was to evaluate the toxic effect of both auto and TY-mediated oxidation processes of dopamine and their effect on cell viability in a cellular model of PD: SH SY5Y. We were interested in estimating potential differences in toxicity between these two processes and the kind of cell death activate. The identification of a possible common denominator could lead to the development of drugs for the treatment of the disease. Since oxidative stress has been implied in PD etiopathogenesis, another part of the project was focused on the oxidative damage induce by dopamine and DAQs. Among the cellular antioxidant defense against the over production of reactive oxygen species, superoxide dismutases play a central role as scavenger of superoxide anion. On the premises that dopamine oxidation chemistry leads to the production of ROS, the role of oxidative stress as a trigger and amplification mechanism for PD and the crucial role for SODs proteins, the second part of the work was focused on the possible role of superoxide dismutases 1 and 2 in protecting cells against the auto and TY-mediated processes of dopamine oxidation.

2. Materials and methods

2.1 Molecular biology

2.1.1 Cloning of SOD1 and SOD2 sequence

The Human SOD1 full length cDNA was amplified from pOTB7 (fig. 9) vector and the SOD2 full length cDNA from the pCMV-SPORT6 (fig. 10).

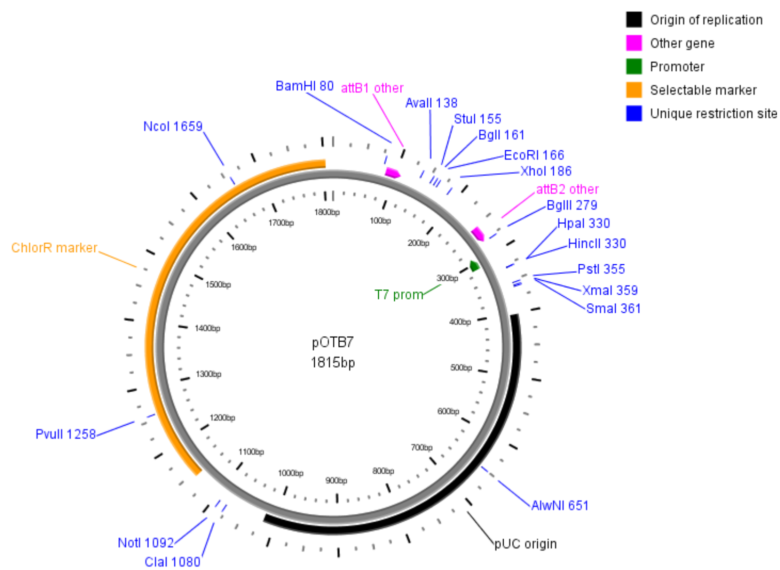


Fig. 9. pOTB7 vector map for the amplification of SOD1 full length cDNA.

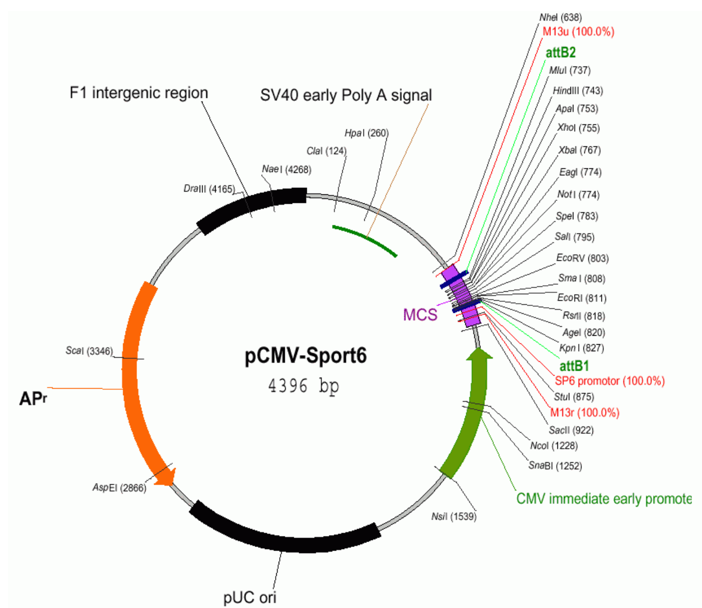


Fig. 10. pCMV-SPORT6 vector map for the amplification of SOD1 full length cDNA.

The primer used for the amplification from the origin vector were designed to carry restriction site for EcoRI and BamHI (for SOD1) and EcoRI and AgeI (for SOD2) for the subsequent cloning of the proteins in the destination vector. In table 2. are listed the primer used for the amplification, the restriction site (underlined) and the melting temperature.

primer	N. Nt	Tm °C
SOD1 Koz FOR (5'-3')	33	84.2
GGTGAATTCGTAATGGCGACGAAGGCCGTGTGC		
SOD1 BamHI (5'-3')	25	62.7
GTTTATCAGGATCCATTTCTACAGC		
SOD2 Koz FOR (5'-3')	33	84.7
GGTGAATTCAGCATGTTGAGCCGGGCAGTGTGC		
SOD2-AgeI (5'-3')	27	77.1
ACCACCGGTCTTTTTGCAAGCCATGTA		

Table 2. Primers (Sigma) used for the amplification of the SOD1 and SOD2 sequences from the origin vectors. Restriction sites for the ligation in the destination vectors are underlined. (N. Nt = number of nucleotides, Tm = melting temperature.)

2.1.2 Polymerase chain reaction (PCR)

The polymerase chain reaction is a powerful technique which allows to obtain *in vitro* millions copy of a target segments between two regions of known sequence starting from few copies. Using the primers listed in table 2.2, the full leght cDNA sequence of SOD1 and SOD2 was amplified from the origin vector and cloned in the destination vectors carrying the GFP and RFP sequence respectively. The

SOD1 sequence was inserted at the C-terminus of the GFP sequence while the SOD2 sequence was inserted at the N-terminus of the RFP sequence. Approximately 1ng of the origin vector was mixed with dNTPs (10mM each), the pfu (*Pyrococcus furiosus*) polymerase (3U/µL), pfu reaction buffer 10x and 10mM each of forward and reverse primers. The PCR program carried out by the termocycler (*My cycler thermal cycler* BIO-RAD9) was programmed to an intial denaturation at 95°C for 2', then 25 cycles of denaturation (95°C for 30''), annealing (51°C for 30'') and extension (72°C for 1'). A final extension step was performed at 72°C for 10'. PCR products were analyzed by agarose gel electrophoresis and purified. The DNA was conserved at 4°C.

2.1.3 DNA purification

DNA purification was achieved using the kit *Wizard ® SV Gel and PCR Clean-Up System* (Promega). The kit is based on the ability of the DNA to bind silica membranes of the minicolumn in the presence of choatropic salts. It consist in the addition of an equal volume of Membrane Binding Solution (guanidine isothiocyanate 4.5M, potassium acetate 0.5M, pH 5.0) to the DNA sample and the loading into the SV minicolumn. After two wash steps with Membrane Wash Solution (potassium acetate 10mM, pH 5.0, ethanol 80%, EDTA 16.7µM, pH 8.0), the DNA was eluted with H_2O mQ pH 8.4.

2.1.4 DNA and vector enzymatic restriction

Enzymes used for the DNA and vectors enzymatic restriction are summarized in the table 3.

EcoRI	BamHI	AgeI
G*AATTC	G*GATCC	A*CCGGT
CTTAA*G	CCTAG*G	TGGCC*A

Tab. 3. restriction enzymes and respective recognition sites used for the cloning.

The experimental protocols for DNA insert and vector digestion are summarized below:

DNA insert (ng)	Vector 2μg
Buffer 10x	Buffer 10x
BSA 10X	BSA 10X
EcoRI 1U	EcoRI 1U
BamHI 1U (for SOD1) and AgeI (for SOD2)	BamHI 1U (for GFP vector) and AgeI (for RFP) vector
Sterile H_2O mQ to final volume (50uL)	Sterile H_2O mQ to final volume (50uL)

All the reaction were kept at 37°C for 1-2 hours.

To verify the result of digestion, small aliquots of restricted DNA and vector were loaded on agarose gel electrophoresis, and restricted DNA was purified to eliminate endonucleases.

2.1.5 Vector dephosphorylation

During ligation, DNA ligase will catalyze the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5'-phosphate group and the other a 3'-hydroxyl group. Recircularization of plasmid DNA can therefore be minimized by removing the 5' phosphates from both ends of the linear DNA with calf intestinal phosphatase. As a result, neither strand of the duplex can form a phosphodiester bond. However, a foreign DNA segment with

5'-terminal phosphates can be ligated efficiently to the dephosphorylated plasmid DNA to give an open circular molecule containing two nicks. Because circular DNA (even nicked circular DNA) transforms much more efficiently than linear plasmid DNA, most of the transformants will contain recombinant plasmids. Up to 1μg of each vector was incubated at 37°C for 15 minutes and then at 74°C for 15 minutes in a mixture containing 27 units of TSAP (Thermosensitive Alkaline Phosphatase), reaction buffer 10x and sterile H_2O mQ to final volume 50 μL.

2.1.6 DNA ligation

To obtain the insertion of the SOD1 and SOD2 sequence in the destination vectors was performed DNA ligation. To estimate the amount of vector and insert to use, the following formula was used:

$$\text{ng insert} = \frac{\text{50ng vector x bp of insert x 5}}{2abp\ vector}$$

50ng of each destination vectors were incubated at room temperature for 2 h in a mix containing the amount (ng) of each insert (calculated before), T4 DNA ligase (from 0.1 up to 1 unit), ligase reaction buffer 10x and sterile H_2O for a final volume of 15 μL. A reaction of auto ligation was carried out (as negative control) in the presence of the vector without the insert to estimate the yield of the vector self-ligation. Afterwards, *E. coli* DH5α were transformed with both the ligation products using kanamycin antibiotic (25μg/mL). The destination vector maps are listed below (fig. 11 and 12) and in the red boxes are highlighted the restriction sites for the cloning procedure. For the properly folding of SOD1, the fluorescent tag was cloned upstream to the SOD1 sequence, while for SOD2, the cDNA sequence was cloned at the C-terminal sequence of the fluorescent tag to preserve the correct translation of the mitochondrial tergeting sequence of the gene.

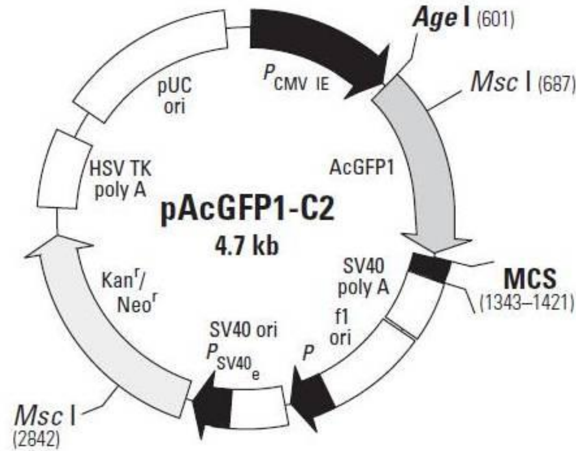
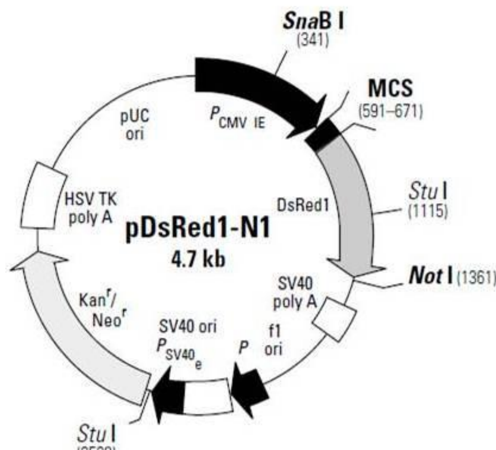


Fig. 11. Destination vectors for the expression in a mammalian cell line of pACGFP1-C2 for SOD1 cloning. In red boxes are highlighted the restriction site for the cloning procedure.



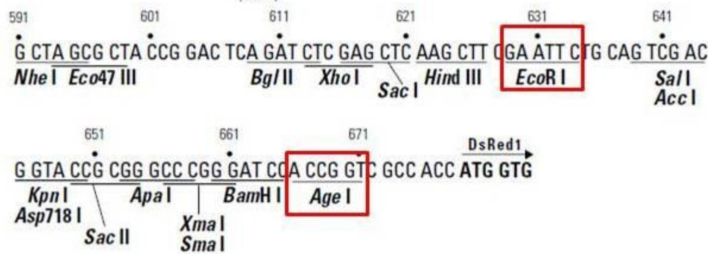


Fig. 12. Destination vectors for the expression in a mammalian cell line of pDsRed1-N1 for SOD2 cloning. In red boxes are highlighted the restriction site for the cloning procedure.

2.1.7 Bacterial transformation

For bacterial transformation an aliquot of 100 µL of frozen *E. coli* DH5α competent cells were kept in ice for 10'. Afterwards 1 µL of ligation was used to transform the cells. After the addition of DNA, the cells were left in ice for 20'. The aliquot was incubated for 90'' at 42°C (thermal shock) and then placed in ice for 2'. A 900 µL of Luria Bertani (LB) medium was added at the aliquot and incubated at 37°C for 1 hour. Subsequently the aliquot was centrifugated at 3000rpm for 2'. The supernatant was discarded and bacterial resuspended in 100 µL were plated in a LB agar plate containing kanamycin (25 µg/mL) and left overnight at 37°C.

2.1.8 Bacterial colonies screening

After the transformation the colonies were screened to select those that have incorporated the insert. For this purpose, the colonies were picked up, and used as template to perform a PCR reaction. Each colony was added (as template) in a mix containing reaction buffer 10x, dNTPs 10mm (each), forward and reverse primers (10mM each), $MgCl_2$ (25mM), Go Taq polymerase (5 unit/µL) and sterile H_2O mQ to final volume of 15 uL. The thermocycler program used was the following:

	T = °C	time	
Denaturation	95°C	2'	
Denaturation	95°C	30''	For 25 cycle
Annealing	51°C	30''	For 25 cycle
Extension	72°C	1'	
Extension	72°C	10'	

PCR product were loaded on an agarose gel to evaluate which colonies were positive. Those colonies that have incorporated the insert were inoculate in LB medium containing kanamycin

antibiotic and left overnight at 37°C for small-scale purifications of plasmid DNA (minipreps).

2.1.9 Plasmid DNA extraction

After overnight shaking at 37 °C, cells were centrifuged at 3000 g for 5 minutes. The LB broth was discarded and the cells were gently resuspended in 250 µl of Cell Resuspension Solution, an isotonic buffer (see below). Cell Lysis Solution (250 µl), containing SDS to disrupt the cell and NaOH to denaturate DNA, was added. At interval of 5 min each, the following solutions (composition described below) were added: 10µl of Alkaline Protease Solution to inactivate endonucleases; 350µl of Neutralization Solution to neutralize the pH. The lysate was centrifugated at 14000 g for 10 minutes to separate the DNA in the supernatant from the lipids, proteins and genomic DNA of the cells. The supernatant, was loaded into the spin column , a chromatographic anion exchange column (Promega Wizard® Plus SV *Minipreps* DNA Purification System kit). The DNA binds to the column and after two step of wash with Column Wash Solution, the DNA was eluted in sterile H2O mQ pH 8.4.

Buffer composition:
Cell Resuspension Solution: Tris(hydroxymethyl)aminomethane (Tris-HCl) (pH 7.5), 50mM Ethylenediaminetetraacetic acid (EDTA) 10mM, RNase A 100µg/ml.
Cell Lysis Solution: sodium hydroxide 0.2M and sodium dodecyl sulphate (SDS) 1%.
Neutralization Solution: potassium acetate (pH 4.8) 1.32M.
Column Wash Solution: potassium acetate 80mM, Tris-HCl (pH 7.5) 8.3mM, EDTA 40µM and ethanol 55%.

For high-scale DNA purification, midi or maxipreps were performed according to the manufacturer instructions.

2.1.10 DNA quantification

DNA quantification is necessary to ascertain the approximate quantity of DNA obtained for further analysis. Two methods were used: spectrophotometric analysis and gel electrophoresis. The first one consists in measuring the absorbance of DNA solution at 260nm, where DNA shows an absorption maxima. Since an absorbance of 1 corresponds to a concentration of 50 ng of doublestranded DNA in solution, the concentration of DNA in solution can be easily calculated using the following formula:

ng/μl of dsDNA = (A260nm× c) × *dilution factor*

Moreover, the purity of the sample, in terms of proteins contamination, can be calculated from the ratio of OD260/OD280. A pure DNA preparation has a value around 1,8. The second method with gel electrophoresis (see 2.1.7) consists in the comparison on the transilluminator of the sample with standard DNA (plasmids or PCRs, depending of the type of the sample) previous quantified. This method is very sensitive, since it allows to quantify down to few nanograms of DNA.

2.1.11 Agarose gel electrophoresis

Agarose (Applichem) was dissolved in the suitable electrophoresis buffer
TAE 1X. Agarose concentrations were 1% for DNA plasmids of 5-6 kbp and 2% to analyze DNA fragments of 0,1-1 kbp. The mixture was heated in a microwave oven until completely melted, then was poured into a casting tray containing a sample comb. Once the gel has cooled at room temperature, the comb was removed and the gel placed into an electrophoresis cell, filled with TAE 1X buffer. Before loading the DNA samples into the wells with a micropipette, it was necessary mix the samples with loading dye (Promega), a colored loading buffer containing glycerol and DNA intercalant, which enables to track the running of the gel and to visualize the bands under UV illumination. Beside to DNA samples, an appropriate molecular weight standard, the 100bp or 1kbp DNA ladder (fig. 2.3), was loaded in order to identify the size of DNA samples by comparison with the fragments of known size. At this point, a current field, between 50-100 volts

depending on the gel size, was applied to gel until the bromophenol blue front of the loading dye was migrated about 70-80% toward the positive pole. At the end of the running, the gel was placed on a UV transilluminator to visualize the DNA due to the presence of the DNA intercalator.

Buffer composition:
TAE 1X: Tris-Acetate 40mM, EDTA 1mM, pH 8.3.
Loading dye 6X (Promega): orange G 0.4%, bromophenol blue 0.03%, xylene cyanol FF 0.03%, Ficoll® 400 15%, Tris-HCl (pH 7.5) 10mM and EDTA 50mM (pH 8.0).

2.1.12 DNA sequencing

The PAcGFP-C2 vector containing the SOD1 insert and the pDsRed1-N1 vector containing the SOD2 insert were dried at 65°C and sequenced by BMR Genomics (spin-off of University of Padova) to verify the exact sequence and the right frame of the two fusion protein.

2.1.13 RNA extraction

Cells were lysed directly in a culture dish by adding 1 mL of TRIZOL Reagent to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette. The amount of TRIZOL Reagent added is based on the area of the culture dish (1 mL per 10 cm2). The sample was homogenized for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. 0.2 mL of chloroform were added per 1 mL of TRIZOL Reagent. Tubes were shaked vigorously by hand for 15 seconds and incubate at 15 to 30°C for 2 to 3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture presented a lower red, phenol-chloroform phase, an inter phase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube and precipitated from the aqueous phase by mixing 0.5 mL of isopropyl alcohol per 1 mL of

TRIZOL Reagent used. Samples were incubated at 15 to 30°C for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 2 to 8°C. The supernate was removed and the RNA pellet was washed once with 75% ethanol, adding at least 1 mL of 75% ethanol per 1 mL of TRIZOL Reagent used. The sample was mixed by vortexing and centrifuged at 7,500 x g for 5 minutes at 2 to 8°C. At the end of the procedure, the RNA pellet was air-dried for few minutes and dissolved in RNase-free water and incubated for 10 minutes at 55 to 60°C.
The integrity of the RNA was assessed by agarose gel electrophoresis, and the concentration was calculated by UV spectroscopy.

2.1.14 mRNA retrotranscription

for the amplification of dopaminergic markers, mRNA was retrotranscribed to obtain cDNA.Up to 1µg of RNA was mixed with 10mM of random primers (mix I). The mix was incubated for 5’ at 70°C and then chilled for 5’ at 4°C. afterwards in the first mix were added the reaction buffer 5x, 40mM $MgCl_2$, dNTPs 10mM each, RNasin® Ribonuclease Inhibitor (24 units), RT polymerase (18 units) and H_2O to final volume of 15µL. The final mix was placed in the thermocycler and the program used is listed below

25°C for 5’

42°C for 60’

70°C for 15’

Preserve at -20°C

2.1.15 Amplification of dopaminergic markers

primers used for the amplification of dopaminergic markers are listed in the table below (tab. 4.)

Oligo name	Sequence (5'-3')
TH for	CACAGGCCAAGGGCTTC
TH rev	TCCTCGATGAGGCTCTGC
AADC for	GAAGCCCTGGAGAGAGACAA
AADC rev	CCTTGTTGCAGATAGGACCG
DAT for	CACCTGCTGCCGAGTACTTT
DAT rev	TAGAGCAGCACGATGACCAG
VMAT2 for	CCGACTGTCCCAGTGAAGAC
VMAT2 rev	CGCAAATATGGGAATTGGAT
DBH for	GCCTTCATCCTCACTGGCTA
DBH rev	TTCTCCCAGTCAGGTGTGTG
GADPH for	AATGAAGGGGTCATTGATGG
GADPH rev	AAGGTGAAGGTCGGAGTCAA
SOD1 for	GCATCATCAATTTCGAGCAG
SOD1 rev	GACCTGCACTGGTACAGCCT
SOD2 for	CACCGAGGAGAAGTACCAGG
SOD2 rev	TAGGGCTGAGGTTTGTCCAG

Tab. 4. Primer sequences for the amplification of dopaminergic markers.

The mixture for the amplification of dopaminergic markers comprises: Nuclease free water (to the desired volume), 10x thermophilic polymerase reaction buffer, 2mM MgCl2, 0.2mM Nucleotide mix, 1μM upstream and downstream primers

each and 5 units/µL of *Taq* DNA polymerarse. The thermocycler program is reported below

Denaturation 94°C for 2’

Denaturation 94°C for 1’ } For 35 cycles

Annealing 55°C for 45’’

Extension 72°C for 2’

Finale extension 72°C for 7’

Hold 4°C

2.2 biochemistry

2.2.1 SDS-PAGE and western blotting

Briefly, cells were trypsinized, centrifuged and resuspended in 100 uL lysis buffer (20mM Tris pH 8.0, 150mM NaCl 1mM EDTA, 1% Triton and protease inhibitor cocktail) to prepare whole-cell lysates and maintained in ice for 30’. Subsequently lysates were centrifugated at 13.000g for 30’ to remove cell debris and the surnatant was collected. Protein concentration was determined by the BCA method (BCA protein assay kit, Thermo Scientific). Total protein cell lysate were separated in a 4-20% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membranes were probed with different primary antibodies (1:1000) for 1 hour or overnight where needed. Mouse anti-β tubulin (1:2000) was used as internal control. The incubation with primary antibody was followed by horseradish peroxidase conjugated secondary antibody (1:2000), and developed with the ECL system (Ge Healthcare).

2.3 Cellular Biology

2.3.1 Splitting adherent cell lines by trypsinisation

Cells which grow in monolayer culture are usually split (= passaged) to provide new stock flasks when they are covering the whole of the culture surface (= confluent). Failing to split cells can result in the cells either overgrowing and/or cell death. The media from the flask containing cells was removed and a volume of trypsin (enough volume to cover the layer) was added and left 1-2' at 37°C to let the enzyme to detach cells. Afterwards, trypsin was inactivated adding enough volume of serum-containing medium and the cells suspension was pipetted up and down to avoid the formation of cell clumping. The desired volume of cells were placed in a new flask to allow them to replicate.

2.3.2 Poly-lysine coating tissue culture vessels

The poly-lysine coating allows to attach cells to coverslips and perform fluorescence microscopy and immucytochemistry techniques. Coverslips were sterilized sinking them into ethanol 70% and subsequently placing them into a 12-well plate under UV illumination. After the sterilization passage, poly-lysine was added in a volume enough to cover the coverslips and the plate was incubated for 30' in the incubator at 37°C. After the incubation, poly-lysine was removed and the coversplips were washed twice with mmQ water. The coversplips were left to dry in sterile conditions and subsequently, $3x10^5$ were seeded and left to reach the desired confluency (approximately 60%).

2.3.3 Transient transfection (lipofectamine)

For transient transfection, $3x10^5$ cells were seeded in a 6-well plate on coversplips and left to reach approximately 60% of confluence. The day of the experiment, the medium of the cells were replaced with Optimem. In separate tubes, 4uL of

lipofectamine were incubated in 100uL of optimem for 5’ at room temperature. At the same time, 2ug of DNA (vectors for the transfection) were added to 100uL of optimem. After the incubation, the DNA and the lipofectamine were mixed to let the formation of DNA-lipofectamine complexes and incubate at room temperature for 30’. Subsequently the mix was added to the cells and left for 3h in the incubator. Afterwards the medium (optimem + DNA-lipofectamine mix) was replaced with fresh cell culture medium. All the treatments for the experiments were performed 36h after the transfection.

2.3.4 Paraformaldehyde recipe

50mL of distilled water were heat till 50°C, and subsequently 4g of paraformaldehyde were added and stirred. (do not heat above 70°C). Several drops of 1M NaOH were added until the solution was transparent. The solution was left to chill. 10mL of PSB 10X were added and the pH was checked (around 7.2-7.4) and corrected where necessary. Distilled water was added to reach a final vlume of 100mL. Aliquots were preserved at -20°C and thawed at room temperature.

2.3.5 Hoechst Staining for Nuclear Fragmentation

Hoechst 22432 is a cell permeable DNA binding dye that gives a blue fluorescence when excited by UV light. Identifying cells undergoing apoptosis (chromatin condensation and fragmentation) is relatively easy once stained with Hoechst. This can also be used as a nuclear counterstain for immunofluorescence. The cells were left to grown on coverslips till 60% of confluence, and subsequently treated with different conditions. The day after, cells were fixed with paraformaldheyde 4% for 20’ at room temperature. Then they were stained with 1ug/uL Hoechst in PBS, for 5 minutes at room temperature. Finally they were mounted over a glass slide and examined under UV illumination.

2.3.6 Mitosox probe

For the evaluation of mitochondrial superoxide anion production, we used mitosoxRed. The probe was resuspended in DMSO to obtain a 5mM stock. For the experimental condition the probe was used at 5uM concentration and incubated for 30' at 37°C in the incubator prior the treatments.

2.3.7 Time-lapse imaging of superoxide anion production

For the time-lapse imaging, $1x10^6$ were seeded in a 35mm plate and left to reach 80% confluency. The day of the experiment, 5uM of MitosoxRed was added to the medium and left to incubate at 37°C for 30' (in the incubator). After the incubation of the probe, the treatment was added in the cell medium. The well was left in a thermostatic chamber as in the growth condition and the microscope (Leica Microsystems DM4000) was programmed to take photos in the bright field (BF) and in the red channel (N21) every 10 minutes for 15h. the microscope parameters are summarized in the table below (tab. 5)

Channel	objective	gain	exposure
BF (brighfield)	40X	2	25
N21 (red)	40X	4.2	910

Tab. 5 microscope parameters.

2.3.8 Flow cytometry

Flow cytometry is a laser based, biophysical technology employed in cell counting, sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. It

allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. Several information can be extrapolated from the analysis, such as morphological parameters and fluorescence properties.

2.3.9 Cell detachment method evaluation

Since Flow cytometry was conceived for the analysis of cells grown in suspension, and the detachment of adherent cells leads to stress and false positive data, we evaluate the method of cell detachment that give rise to the low background in our control samples (both for ANNEXIN+PI and Mitosox). The day before the experiment, $1x10^6$ were seeded in a 6-well plate to reach approximately 80% of confluence. The next day, floating cells were collected and centrifuged at 1000g for 5'. The adherent cells were detached from the plate with four different methods that are listed in the table below (tab 6)

	Cell detachment method	Time	inactivation
mechanical	Pipetting using PBS	5'	
chemical	EDTA(0.05mM) in PBS	5'	
enzymatic	Trypsin (0.25%)	5'	DMEM containing serum
enzymatic	Papain (12U/mL)	5'	DMEM containing serum

Tab. 6 cell detachment methods.

The adherent cells were added to the floating ones and centrifuged for 5' at 1000g.

2.3.10 Fluorophore staining

Mitosox labeling: the day before the experiment, cells were incubated for 30’ with the mitosox probe and then treated with the different conditions overnight. The day after cells were analyzed with FACS (BD FACSCanto II).

Annexin-PI labeling: cells were resuspended in HBSS solution with 1uL/500uL of ANNEXIN-V-FLUOS and with 1uL/500uL of PI and incubate at 37°C for 15’ in the dark. Afterwards cells were transferred in FACS tube and analyzed.

2.3.11 Treatments

Nuclear fragmentation

For the experiments on nuclear fragmentation, both naïve cells and cells over expressing SODs and the respective negative control, were treated with increasing concentration of dopamine: from 0μM to 150 μM. In the case of DAQs, cells were treated with the same concentration of dopamine and the units of tyrosinase to use were calculated in respect to the dopamine concentration to obtain the complete oxidation of dopamine in 30 minutes. In the case of treatment with the DAT inhibitor (on naïve cells), cells were pre-treated for 30’ with 100nM of GBR 12909.

Time-lapse imaging

For the experiments of time-lapse imaging cells were treated with dopamine 100 μM, 15Units of tyrosinase, GBR 12909 100nM, and antimycin A 100 μM.

Flow cytometry

Annexin-PI: $1x10^6$ cells were seeded in a 6-well plate and let to reach approximately 62% of confluence. The day before the experiment, cells were treated with 100μM and 200 μM dopamine, 15Units of tyrosinase (only in the case of dopamine 100 μM), GBR 12909 100nM, staurosporine 500nM and 1 μM.

Mitosox: $1x10^6$ cells were seeded in a 6-well plate and let to reach approximately 62% of confluence. The day before the experiment, cells were pre-treated with 5 µM of MitosoxRed probe, afterwards they were treated with 100µM and 200 µM dopamine, 15Units of tyrosinase (only in the case of dopamine 100 µM), GBR 12909 100nM, antimycin A 100 µM and 200 µM.

Western blot analysis

$1x10^6$ cells were seeded in a 6-well plate and let to reach approximately 60% of confluence. The day before the experiment, cells were treated with dopamine 100 µM and 200 µM, 15Units of tyrosinase (only in the case of dopamine 100 µM), GBR 12909 100nM, staurosporine 500nM and antimycin A 100 µM

2.4 Statistical analysis

Statistical analysis was performed with the GraphPad Prism software (version 5.0). Values in the histograms represents media and ± SEM. T-student test was applied when two groups were compared, whereas two-way ANOVA followed by Bonferroni post-hoc test in the case of multiple comparisons. P values were considered as described below:

P value	Wording	Summary
< 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
>0.05	Not significant	n.s.

3. Results

3.1 evaluation of the cellular model

With the purpose to dissect the molecular pathway of cellular toxicity induced by dopamine and its oxidized forms and to evaluate the role of superoxide dismutases 1 and 2 in protecting cells, we used a cellular model: SH SY5Y. This cell line is widely used in PD model since it expresses all the dopaminergic machinery, and have the advantage that is genome human-based. Before starting any experiment, we were interested in testing our cellular model for the presence of all the protein involved in the dopaminergic system machinery. For this purpose, the RNA was extracted, mRNA was retrotranscribed to obtain cDNA and the dopaminergic markers were amplified with specific primers (fig. 7). Although this kind of analysis permits to obtain only information on the expression of the mRNA of the different proteins, from the data obtained we concluded that our cellular model has the entire dopaminergic system that allow us to proceed to the next experiments.

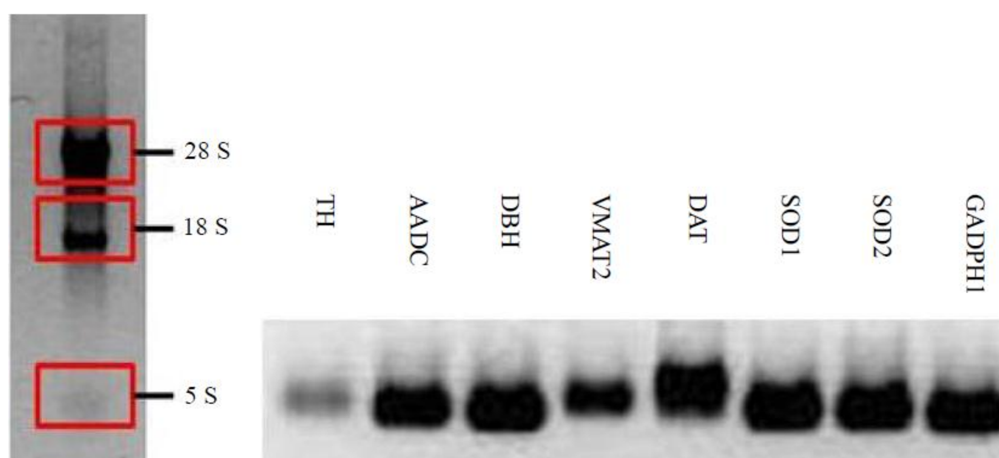


Fig. 7. SH SY5Y possesses all the dopaminergic system machinery. agarose gel of RNA extracted from SHSY5Y cell line and amplification products of dopaminergic markers. Tyrosine hydroxilase (TH), L-amino acid decarboxylase (AADC), Dopamine β-hydroxylase, vescicular monoamine oxydase (VMAT2), dopamine transporter (DAT), superoxide dismutase 1,2 (SOD). Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as positive control.

3.2 Evaluation of dopamine and dopamine-quinones citotoxicity

3.2.1. Evaluatation of apoptotic nuclei by Hoechst staining

To evaluate the citotoxicity effect of dopamine and its oxidized forms, dopamine-quinones (DAQs), we used different techniques. Since in literature is reported that the loss of dopaminergic neurons in Parkinson's disease is caused by the activation of the apoptotic pathway, we evaluate several markers of apoptosis. At first we were interested in confirming the activation of apoptosis in dopamine and DAQs induced cell death and therefore we evaluate one of the last sign of apoptosis: apoptotic nuclei. Using the DNA-binding dye Hoechst staining, we performed a fluorescence microscopy experiment to evaluate the morphology of nuclei after dopamine and DAQs treatment. In the first experiment we evaluate the toxicity of dopamine in inducing apoptotic cell death. The physiological concentration of dopamine inside cells has been reported to be in the nM range, reaching a mM concentration inside the synaptic vesicles. (Eisenhofer et al. 2004, Kopin et al. 1993, Santiago et al. 1996, West et al. 2003). These studies suggest that a slight increase in intracellular dopamine concentration could have important consequences on cell viability. For this reason we decided to treat cells with non-letal concentration of dopamine. SH SY5Y were treated with different concentration of dopamine (for a representative image see fig. 8) and the percentage of apoptotic nuclei was calculated. Dopamine induced cell death in a dose-dependent manner (fig. 9)

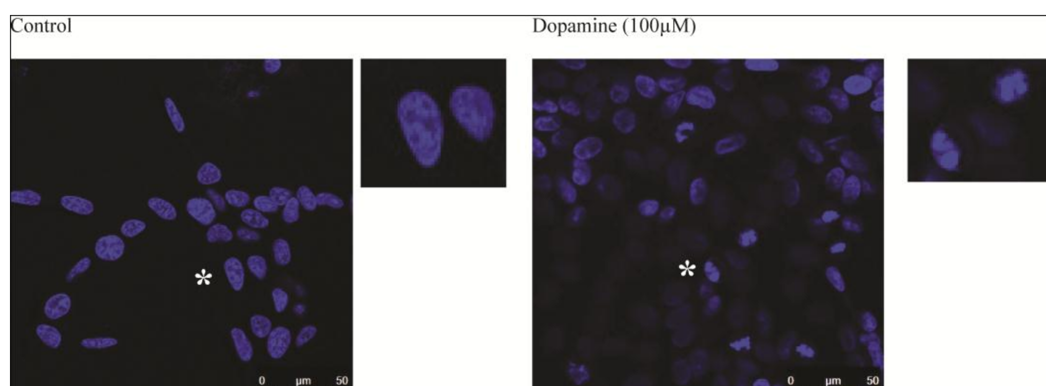


Fig. 8. Dopamine induce apoptotic cell death. Representative image of nuclei morphology in control cells and in cells treated with dopamine (100µµM). It is possible to note the changes in the morphology of the nuclei that undergo a fragmentation in small bodies confirming the apoptotic cell death. In the right small boxes there is a zoom of the nuclei.

In the graph below is represented the statistical analysis if nuclear fragmentation after dopamine challenge (Fig. 9).

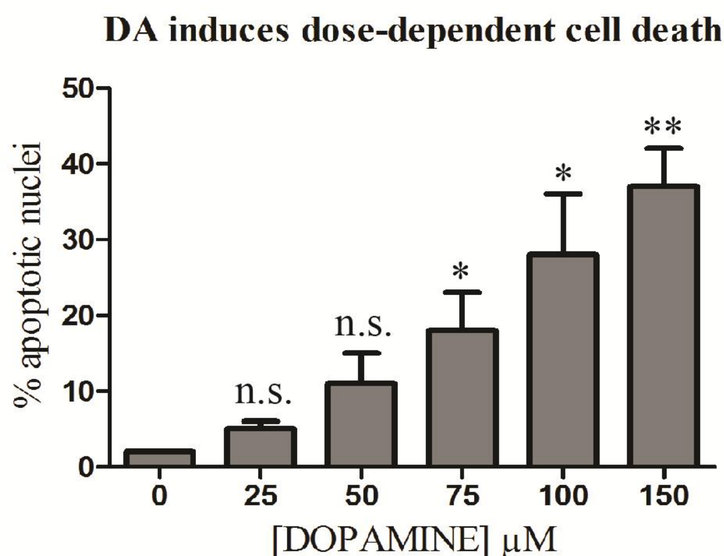


Fig. 9 Dopamine induces apoptotic nuclei appearance in a dose-dependent manner. Percentage of apoptotic nuclei counted in cells treated with increasing concentration of dopamine. (N = 3, t-test for the evaluation of statistical significance in respect to control, * $p<0.05$)

Under physiological conditions, dopamine is stored in the synaptic vesicles by the action of vesicular monoamine transporter 2 (VMAT2) and upon the arrival of an action potential, dopamine is released in the synaptic cleft. Once it has exert its function is then recycled by the activity of the dopamine transporter and re-sequestered into the synaptic vesicles. A study of Manáková and colleagues (Manáková et al 2004) demonstrated a prominent role for the DAT in protecting cells against 6-OHDA. Other studies on dopamine toxicity led to controversial results with some showing no effect on inhibition of the dopamine transporter (Jiang et al. 2008) and others the contrary (Fazeli et al. 2011). Since in literature is still on debate the role of the DAT, and since we treated cells adding dopamine in the cell media (extracellular environment), we were interested in evaluating the role of the dopamine transporter (DAT) in mediating the dopamine-induced cell death in our PD cellular model. The GBR 12909 compound have been reported to have high affinity and selectivity for the dopamine transporter, and a slow dissociation leading to a long-lasting effect (Rothman 1991, Choi et al 1991) and in the study of Manáková concerning about the toxicity of 6-OHDA, the pre-treatment of cells with 100nM of the dopamine transporter (DAT) inhibitor GBR 12909 rescued cells against 6-OHDA toxicity (Manáková et al 2004) . We pre-

treated cells with 100nM GBR and then we added dopamine. In the (fig. 10) is visible that the DAT inhibitor had a rescue effect against dopamine toxicity, we therefore concluded that dopamine needs to enter cells to exert its toxicity.

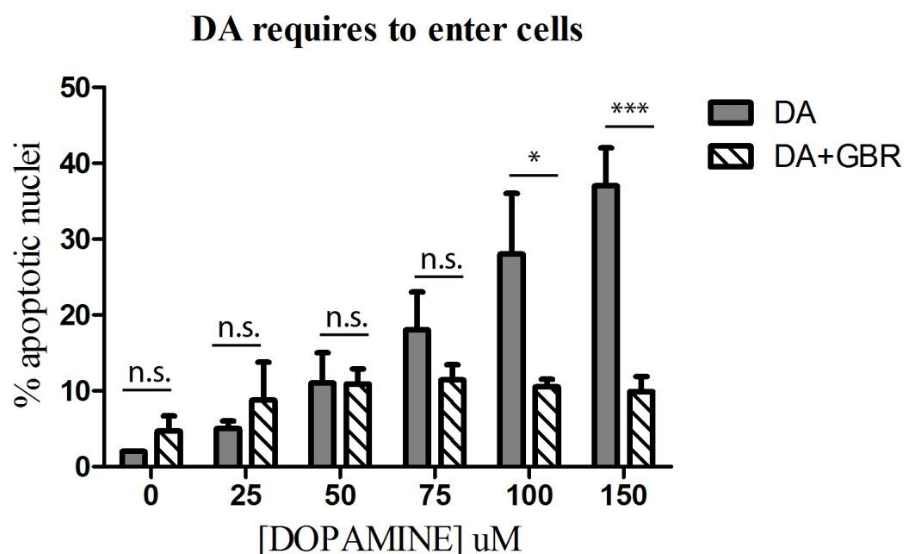


Fig. 10 DAT inhibitor (GBR 12909) have a protective role against dopamine toxicity. Percentage of apoptotic nuclei counted in cells treated with dopamine and dopamine plus the DAT inhibitor GBR 12909. (N = 3, * p<0.05)

Once we demonstrated that extracellular dopamine exert its toxicity only in the case of its internalization in the cells, we tested if the enzyme-mediated oxidation of dopamine also leads to apoptotic cell death. Cytosolic dopamine cad undergo two different pathway of oxidation: by an auto-oxidation mechanism or a oxidation mediated by the action of enzymes. Several enzymes are responsible for the oxidation of dopamine. Monoamine oxidase A and Catechol-O-methyl transferase are responsible for the degradation of dopamine and dopamine that is not broken down by enzymes is repackaged into vesicles for reuse by VMAT2. It has been demonstrated that other enzyme are responsible for dopamine oxidation, among them tyrosinase, prostaglandin H synthase, xanthine oxidase (Sulzer and Zecca 2000; Asanuma et al. 2003). Tyrosinase has been implied as one of the enzyme responsible for the oxidation of dopamine to DAQs (Miranda and Botti 1983; Costa et al. 1992). In 1996 Stokes and colleagues observed that the formation of dopamine quinone is accelerate by the presence of tyrosinase in an in vitro model (Stokes et al. 1996). In another study Xu and collaborators showed that tyrosinase can enhance the concentration of dopamine quinone thus exacerbating the covalent binding and inactivation of TH via adduct formation (Xu et al.

1998). Tyrosinase may then function as a quinone generator within the dopaminergic neurons, but it was also reported to have a protective role. This enzyme could also contribute to NM formation, the synthesis of which is thought be driven by excess cytosolic dopamine not accumulated in synaptic vesicles (Sulzer et al. 2000). These considerations show that tyrosinase has a potential dual role: to damage neurons by producing dopamine-quinones or protect cells by enhancing NM synthesis. The expression of tyrosinase mRNA in the brain is a data supported by different studies (Xu et al. 1997; Tief et al. 1998), and the presence of a functional protein in this area was confirmed (Greggio et al. 2005). On these premise, we wanted to evaluate the different mechanism of dopamine oxidation, therefore we treated cells with dopamine plus tyrosinase to oxidize dopamine in DAQs in a enzymatic way and we evaluate if there was a difference in the toxicity between the auto-oxidation and the enzyme-mediated process.The decision to use tyrosinase to oxidize dopamine was considered to compare the kinetics of production of DAQs species. As a enzyme-mediated reaction, the dopamine oxidation process by the action of tyrosinase was faster than the autoxidation one. It is possible to follow the kinetic of dopamine oxidation mediated by the tyrosinase enzyme using UV spectroscopy. The peak absorption at λ = 280 nm represent dopamine and it decreases through time indicating the disappearance of dopamine and immediately, an absorption maximum appears at λ = 395 nm, corresponding to the yellow dopamine-o-quinone chromophore (DQ), which is progressively replaced by the orange aminochrome (AC). The kinetic of the tyrosinase-mediated oxidation of dopamine led to the complete stabilization of the AC within 30 minutes. Dopamine and DAQs treatment didn't suggest a difference in the rate of toxicity (fig 11)

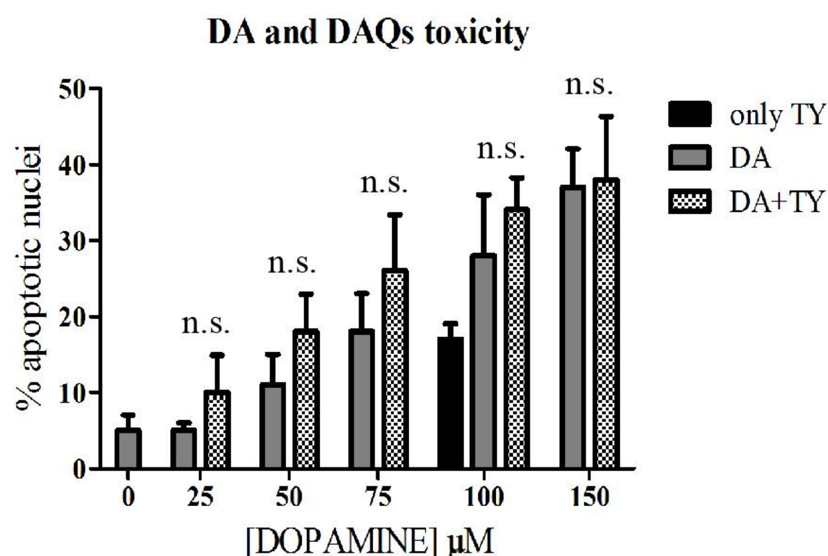


Fig. 11 Dopamine and DAQs do not display different toxicity in cells. Percentage of apoptotic nuclei counted in cells treated with dopamine and dopamine plus tyrosinase. (N = 3, n.s. = p > 0.05)

The positive control with the treatment of cells only with tyrosinase (in respect to dopamine 100µM concentration) showed also a toxic effect since in the cell medium is present L-tyrosine. Tyrosinase catalyze the idroxylation of L-tyrosine directly to dopamine quinones or indirectly to form before L-DOPA and after dopamine quinones. Anyway, this data do not influence our results, since the ratio between tyrosinase units and different dopamine concentration is the same, and the increase in dopamine concentration led to a consequent increased in percentage of nuclear fragmentation in a linear manner. Our previous result demonstrate that dopamine requires to enter cells to exert its toxicity, since the pre-treatment with the DAT inhibitor had a protective effect on cell viability. We therefore asked if dopamine quinones toxicity dependes on the DAT internalization.The treatment with GBR did not show a difference in the toxicity between the DAT not inhibited and inhibited leading to the conclusion that dopamine quinones toxicity is not mediated by the dopamine transporter and presumably they permeate the cell membrane (fig 12).

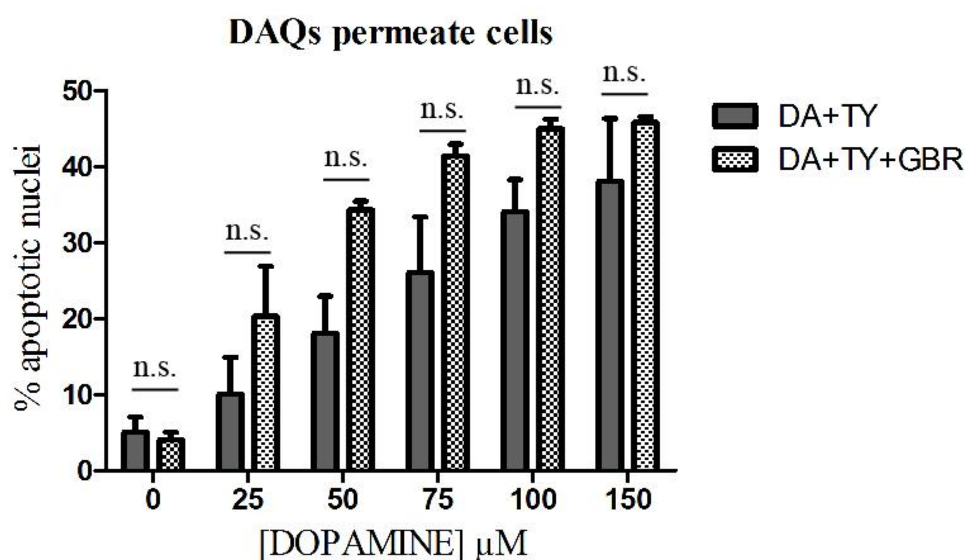


Fig. 12 DAQs toxicity is not mediated by the DAT internalization activity. Percentage of apoptotic nuclei counted in cells treated with dopamine and dopamine plus tyrosinase and the DAT inhibitor (N = 3, n.s. = p > 0.05).

The images of all the treatments and the relative morphology of the nuclei are summarized in figure 13

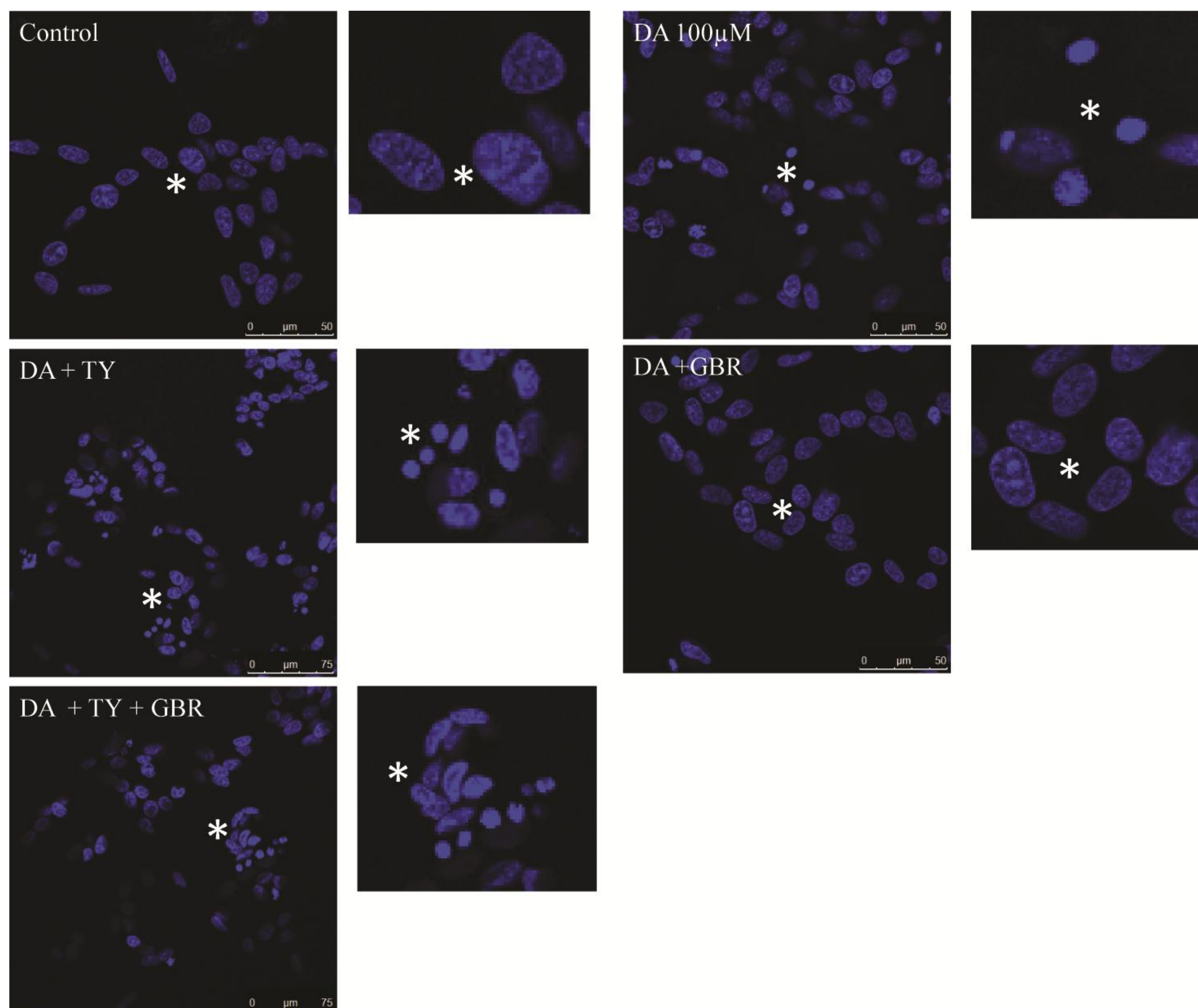


FIG. 13. Both dopamine and DAQs lead to nuclear fragmentation. Representative images of different treatments. It is possible to note that both dopamine and DAQs induce nuclear fragmentation and the treatment with GBR (dopamine transporter inhibitor) have a rescue effect only in the case of dopamine. In small boxer are zoom of the respective images.

To confirm the activation of apoptotic pathway mediated by dopamine auto-oxidation and oxidation mediated by enzymes to generate dopamine-quinones, we evaluate another apoptotic marker with flow activated cell sorter: the externalization of phosphatidil-serine. (Vermesa 2000)

3.2.2 Flow cytometry

For our experiment in the evaluation of apoptosis we performed a double labeling experiment using two different fluorophores: ANNEXIN-V -FLUOS for the detection of phosphatidil-serine externalization (an early marker of apoptosis) and propidium iodide (a marker for necrosis). Flow cytometry was conceived to analyze cells growing in suspension. The detachment of the adherent cells, required to use FACS, give rise to stress. To quantify the potential damage to adherent cells, we first evaluate which method of detachment was the best choice to minimize the level of background signal only due to the procedure. We evaluate four different methods: one mechanical using only PBS, one chemical using EDTA in PBS (0.05mM) and two enzymatic using tripsin (0.1mM) and Papain (12 U/mL).

We choose this four methods because they represent the classical methods used to detach adherent cell and to test the sensitivity of our cells to the detachment procedure. PBS is used to detach cells in a mechanical way, but usually is not recommended for flow cytometric analysis since it give rise to high degree of damage to cell membranes. EDTA in PBS is used as a chemical method since this compound is a metal chelator and impedes to the cell adhesion molecule (CAMs), that depends on Ca^{++} presence, to exert their function. The two enzymatic methods chosen were trypsin and papain. Both are enzyme typically used to detach adherent cells. In particular trypsin was used to passage cells when they were at confluency, while papain is usually used with very sensitivity cells or neuronal culture that are more susceptible to insults. (Panchision 2007)

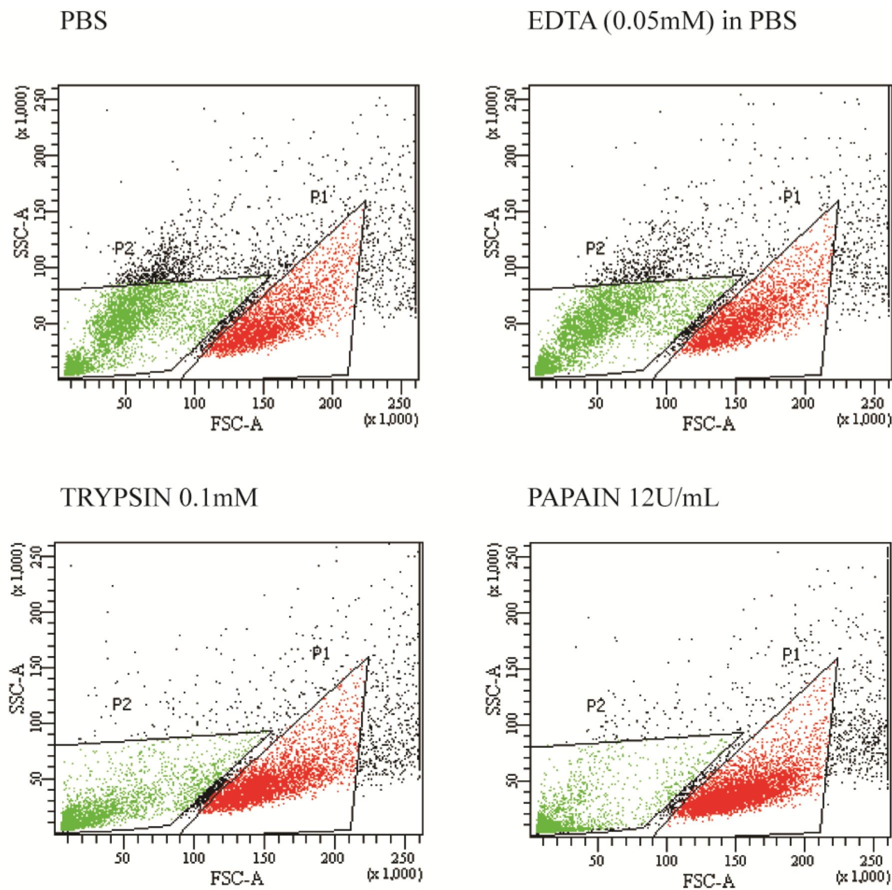


fig. 14. Papain minimize the production of debris during the cells detachment procedure. In the image are reported the dot-plots of FSC and SSC, representing the morphological parameters of cells. In the control sample it is important to have the major percentage of cells in the P1 population that represent the “healthy” population, while in the P2 population are represented death cells and debris. The procedure of cell detachment could lead to a high background of cell debris therefore it is important to use the proper experimental procedure.

The cell detachment method is crucial to proceed with the next experiments. In fig. 14 is evident that papain permits the proper detachment of cells and that the procedure maintain the morphological feature of healthy cells (P1 population), while the other methods led to a high level of cell debris (P2 population). In fig 15 is represented the peak of fluorescence intensity of the ANNEXIN-V probe that is the marker of apoptosis. In control sample this peak is centered to zero values since all the cells do not exhibit fluorescence, while upon an apoptotic stimulus, the phosphatidil-serine externalization leads to the bond of the fluorescence probe that in the histograms is represented by the shift of the peak to higher values. Again papain showed a peak centered to zero values, allowing to proceed to the next experiment.

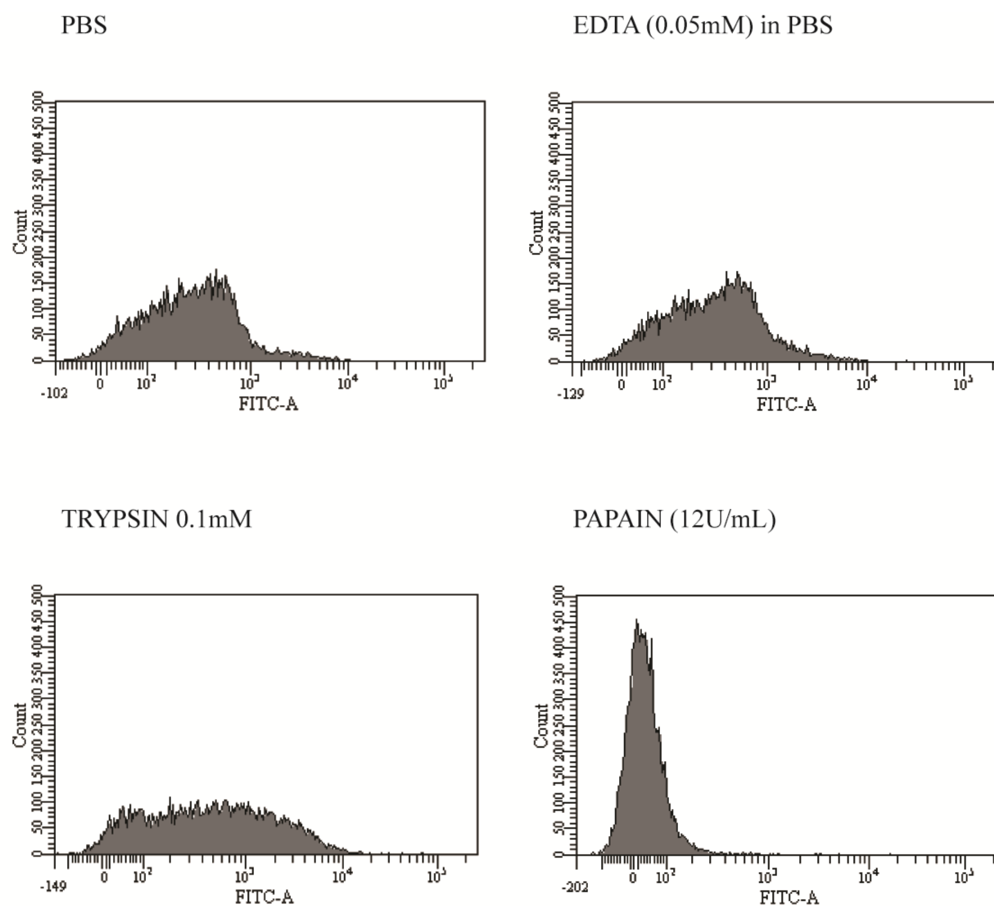


fig. 15. Papain exhibit the lower fluorescence background in control sample. in this image is reported the fluorescent signal from the ANNEXIN-V probe. In control sample the peak must be centered to zero values to appreciate its shift to higher values upon induction of apoptosis.

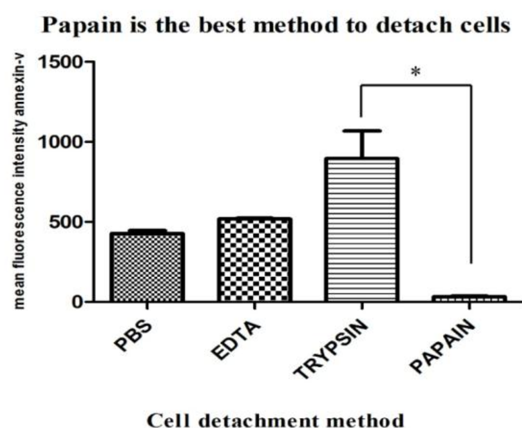


Fig. 16 Papain is the best cell detachment method. Statistical analysis was shown only in the case of a statistical difference between methods.

The use of flow cytometry is a powerful technique for the analysis of single cells allowing the analysis of millions of events each run. Lots of information can be extrapolated from the experiments, both morphological and on the fluorescence properties of the cells (for a review Givan 2011). An important principle of flow cytometry data analysis is to selectively visualize the cells of interest using a procedure called gating in which is possible to perform analysis only on a desired population excluding the other cells. For the apoptosis assay, we decided to use papain (12U/mL) for its lowest background in the control sample. After the optimization of the cell detachment method, we performed all the experiments with the different conditions as in the experiment with apoptotic nuclei. Cells were treated with dopamine (100µM), dopamine plus the DAT inhibitor GBR (100nM), dopamine plus tyrosinase, and dopamine plus tyrosinase in the presence of the DAT inhibitor GBR. In the first image (fig.17) is represented the dot-plot of FSC and SSC. This type of plot gives information about cell size. It is possible to note that in control sample, cells are mainly grouped in the "healthy" population (the red dots in the P1 population). This is because they have specific parameters of dimensions. On the other hand, in the different treatments that leads to apoptosis, cell shrinkage (a morphological sign of apoptosis) results in a decrease in forward light scatter (FSC) and a reduced side scatter (SSC) leading the cells to concentrate in the "dead" population (P2). In the second image (fig. 18) is represented the double labeling experiment, in which there are four quadrants. The low-left quadrant (Q3) represents cells, which are negative for both ANNEXIN-V -FLUOS (marker for apoptotis) and PI (marker for necrosis) and are considered healthy. The upper left and the lower-right quadrants are cells that are positive for PI (Q1) and annexin (Q4) representing respectively necrotic and apoptotic cells. The upper-right quadrant (Q2) comprises cells that are positive both for annexin and PI, representing the late-apoptotic population. As demonstrated in the previous experiment on apoptotic nuclei, both dopamine and DAQs leads to the externalization of phosphatidil-serine (PS) with no statistical difference on the rate of cell death between dopamine and DAQs. Dopamine, as demonstrated previously, needs to enter in cells to exert its toxicity, since the treatment with the DAT inhibitor had a rescue effect. DAQs exerted their cellular toxicity inducing the externalization of PS, but their toxicity do not dependent on their internalization by the action of the DAT, demonstrated by the fact that the inhibition of the DAT did not show a protective effect. From the data of FSC and SSC is possible to note that both dopamine and its oxidized products leads to changes in morphological parameters, represented by the relocation of cells from the healthy population (P1) to the dead one (P2). This data confirmed that both dopamine and DAQs are toxic for cells. In the histograms (fig. 19), relative to the shift of the fluorescence intensity of the ANNEXIN-V probe, is visible that both dopamine and DAQs leads to a reposition of the peak from lower values in the healthy population to higher values in the

apoptotic one. The increase in the ANNEXIN-V fluorescence is a parameter that confirm that both dopamine and DAQs leads to the externalization of phosphatidil-serines. In the case of pre-treatment with the DAT inhibitor in addition to dopamine, the percentage of ANNEXIN-V positive cells are comparable to the control sample confirming the dopamine DAT-dependent pathway of toxicity. In the case of DAQs, the DAT inhibitor did not show a rescue effect suggesting again that DAQs are toxic species, but do not depend on DAT internalization activity. With the use of a different technique, we can confirm that both dopamine and DAQs are toxic for cells, but dopamine needs to enter cells to exert its toxic effect, while DAQs do not. In the third image (fig. 19) is represented the peak of fluorescence of the ANNEXIN-V probe. Since in the apoptotic process there are two concomitant events: number of cells that undergoes apoptosis and number of phosphatidil-serine that are externalized, it is possible to note the shift of the fluorescence of annexin and the cell number that exhibit fluorescence. This kind of shift is also visible in the fluorescence channel for PI (fig 20) leading to the conclusion that in the toxic affect of dopamine and DAQs are implied two different mechanism: apoptosis and necrosis with the first prevalent on the latter in terms of percentages. With the data extrapolated from the FACS analysis we plotted the percentage of cells that were positive for annexin in the Q4 and Q2 population (positive for annexin representing apoptotic cells, and positive for both annexin and PI representing cells that are in late-apoptosis stage.)

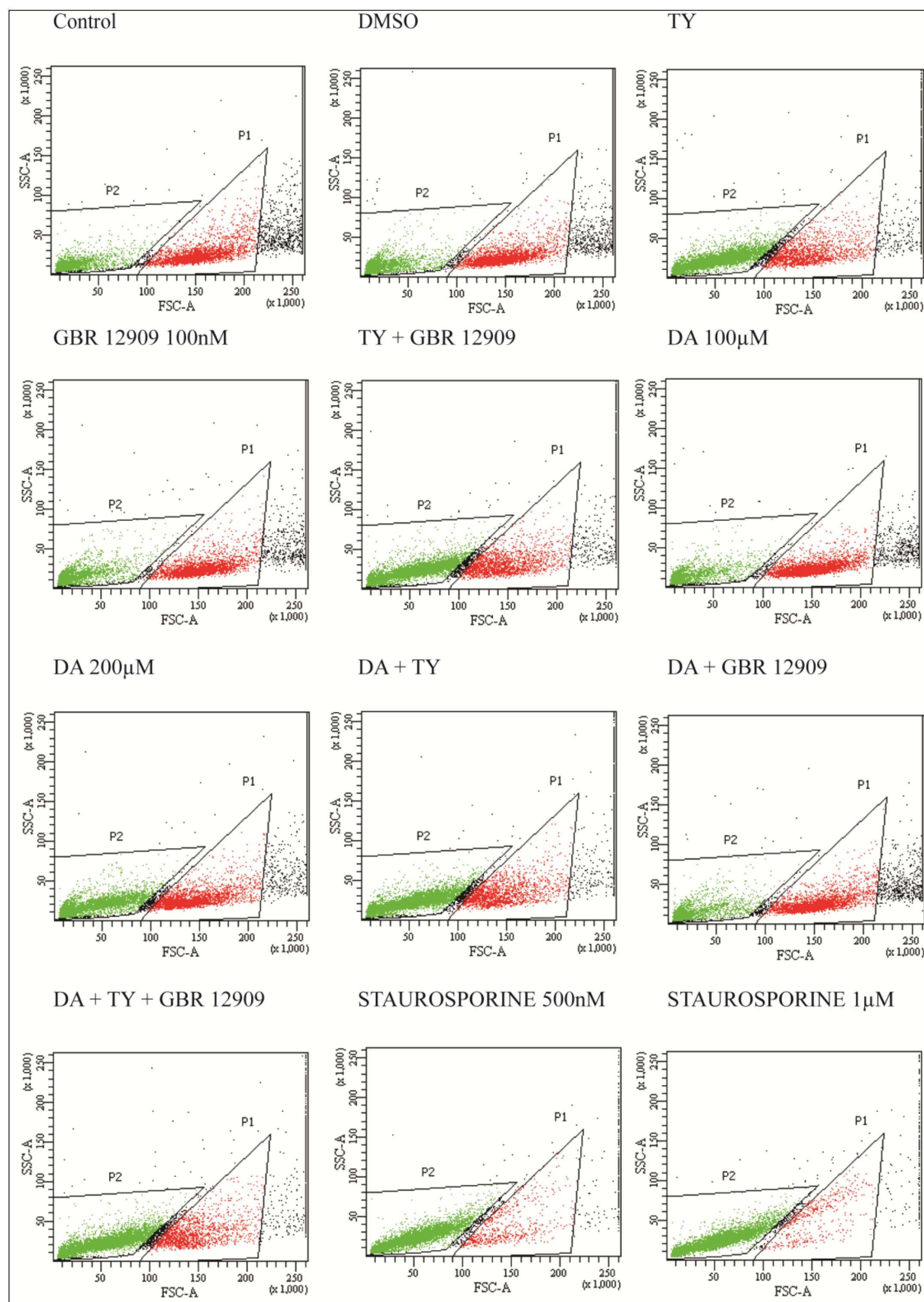


Fig. 17. dopamine and DAQs affect cell morphology. Dot-plot of FSC and SSC. Is evident the relocation of cells from the population of "healthy" cells (P1 population) to "dead" cells (P2) population.

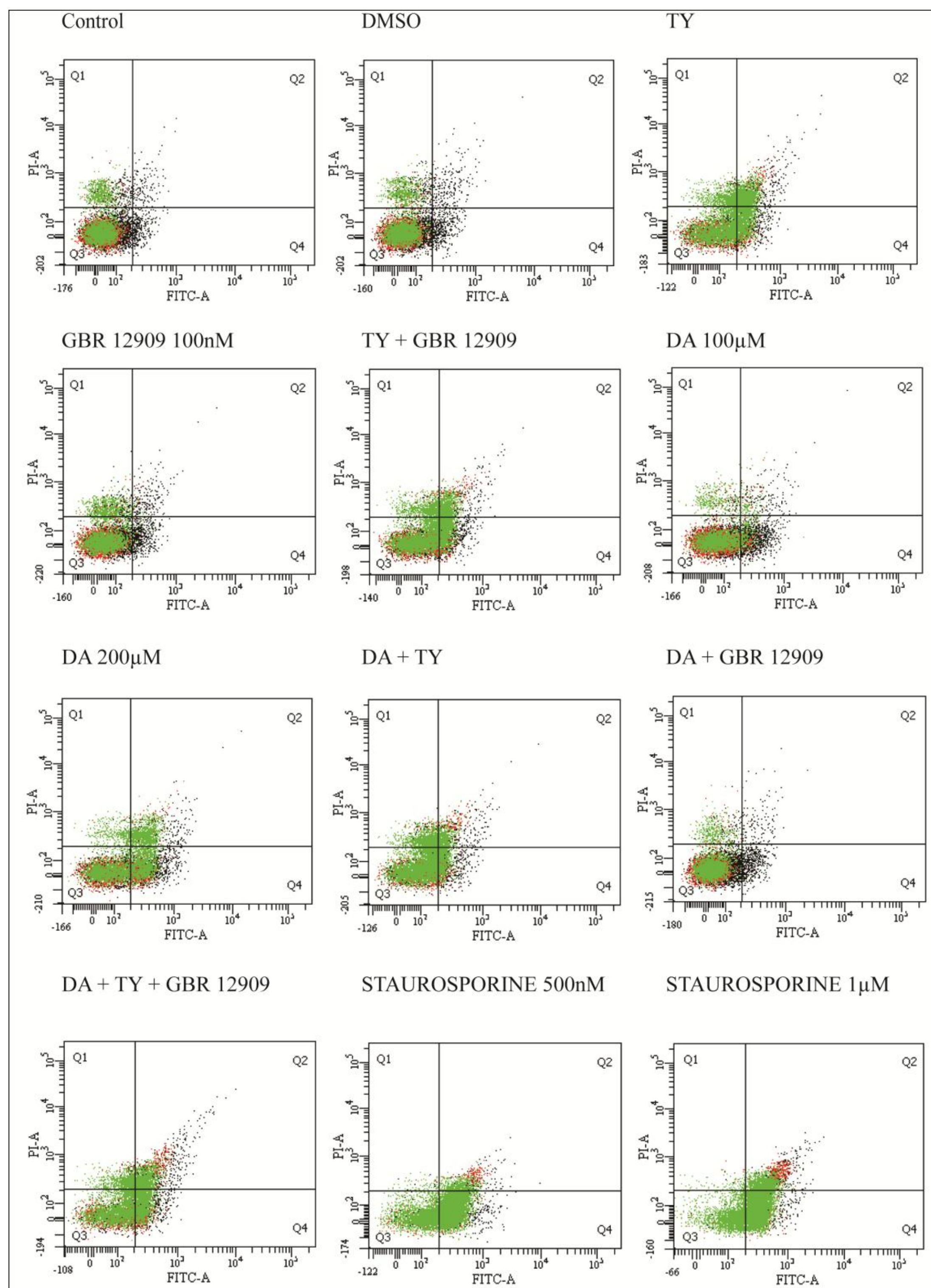


Fig. 18. dopamine and DAQs lead to the externalization of phosphatidil-serine. Dot-plot of double labeling experiment.

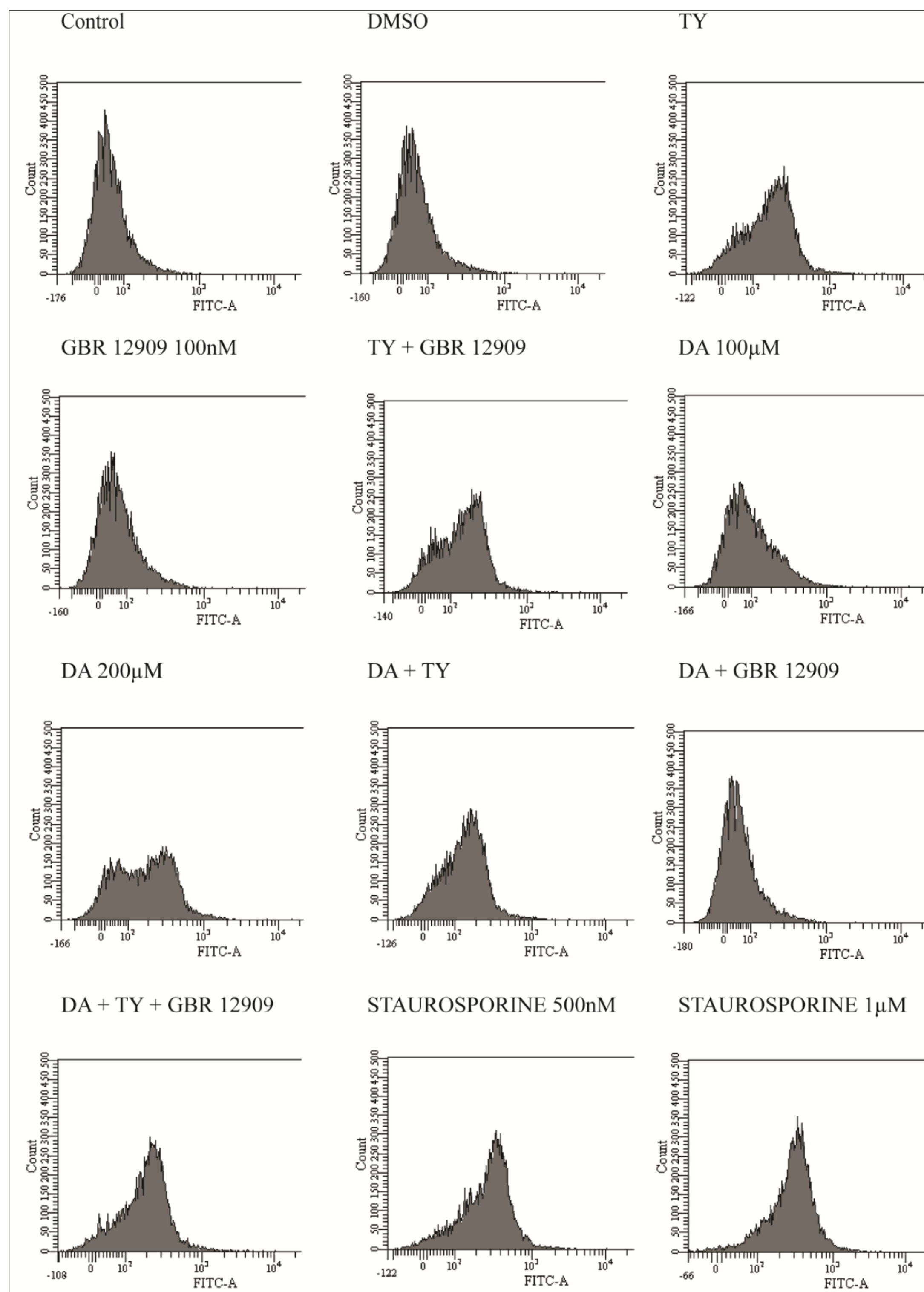


Fig 19. dopamine and DAQs induce a shift in the ANNEXIN-V signal due to externalization of phosphatidil-serine. Histogram of the fluorescence intensity of ANNEXIN-V probe.

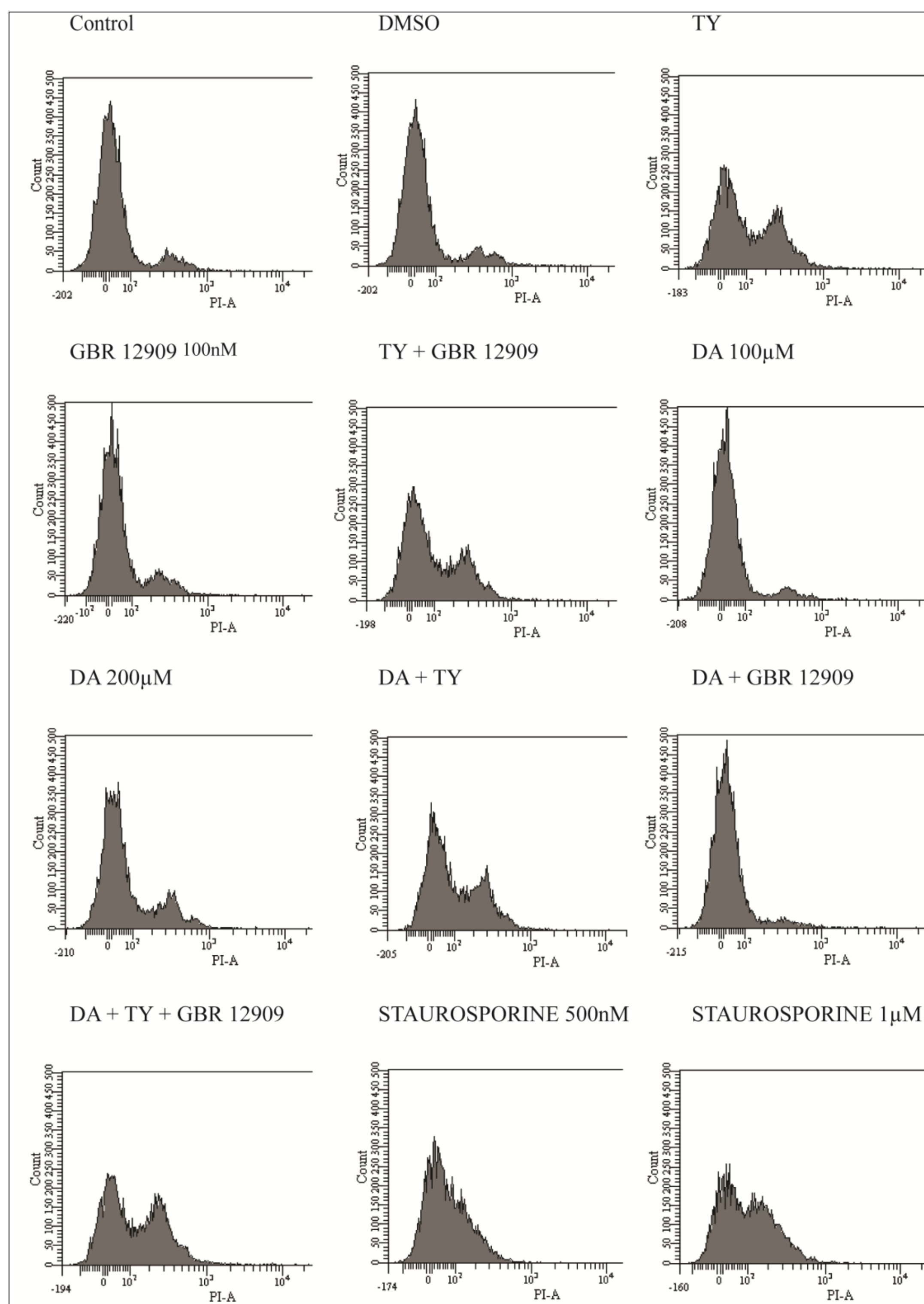


Fig. 20. dopamine and DAQs also induce a shift in the PI fluorescence, a marker of necrosis or late apoptosis. Histograms of the PI fluorescence shift.

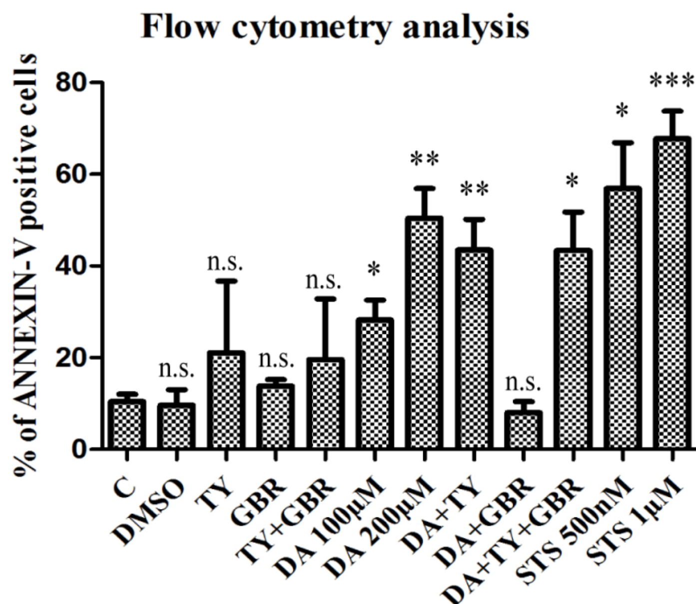


Fig. 21. Both dopamine and DAQs leads to the externalization of phosphatidil-serine. Statistical analysis of FACS data, represented by the percentage of ANNEXIN-V positive cells with a statistical difference in respect to the control sample. Staurosporine was used as positive control.

With the use of FACS technique, we evaluate one of the first marker of apoptosis: phosphatidil-serine externalization and we confirmed the previous data obtained from apoptotic nuclei. Both dopamine and DAQs led to morphological changes in cells but the pre-treatment with the DAT in the case of dopamine led to a rescue effect. On the other hand DAQs and the co-treatment with the DAT inhibitor show the same degree of morphological changes. From the double labeling experiments is possible to appreciate the rise in the percentage of cells that are both positive for ANNEXIN-V probe and PI indicating a small amount of necrotic signal and a high degree of apoptotic signal that is also visible in the fluorescence peak shift for both the probes used.

3.3 Mitochondrial superoxide production

Oxidative stress and mitochondrial dysfunction have been proposed as possible mechanism implied in the pathogenesis of Parkinson’s disease. To explain the selective death of dopaminergic neurons, dopamine has been proposed to have also toxic properties when not properly stored in synaptic vesicles. Chemistry of auto-oxidation and enzyme-mediated oxidation of dopamine leads to the production of ROS. The main site of ROS production is the mitochondria where a 1-2% of the

molecular oxygen is converted into superoxide anion: the first ROS produced. With the purpose to dissect the mechanism of auto-oxidation and enzyme-mediated oxidation of dopamine toxicity, and to evaluate if there are differences in this different mechanism to induce cell death, we evaluate the production of superoxide anion using a specific probe: MitosoxRed. This probe, specific for mitochondrial superoxide anion, is live-cell permeable and it is rapidly and selectively targeted to the mitochondria. Once inside the mitochondria, the probe is oxidized by superoxide anion, but no other reactive oxygen species, and exhibits red fluorescence. (fig. 22)

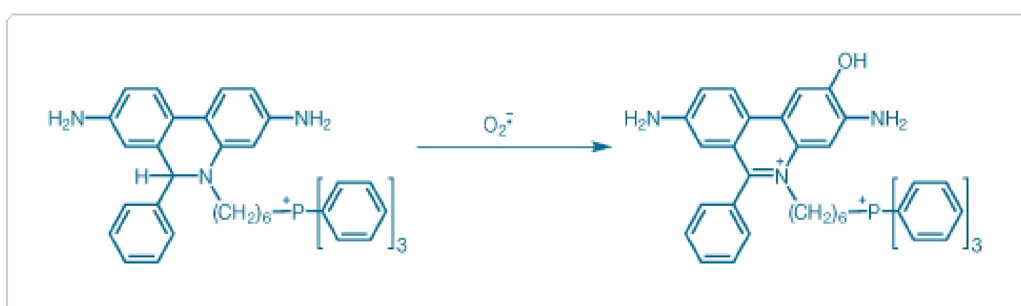


Fig 22. Oxidation of MitoSOX™ Red indicator to 2-hydroxy-5-(triphenylphosphonium)hexylethidium by superoxide (O2 –.)

The experiment was carried out with two different techniques: time-lapse imaging for the evaluation of the kinetics of superoxide anion production and with FACS for the quantification of the superoxide anion produced.

3.3.1 Time-lapse imaging

In the first experiment, we evaluate the production of superoxide anion in cells treated with dopamine. The cells were incubated with the probe and then treated with dopamine 100μM for 15 hour. In the image below (fig. 23) is evident the increasing red fluorescence that demonstrate the production of superoxide anion.

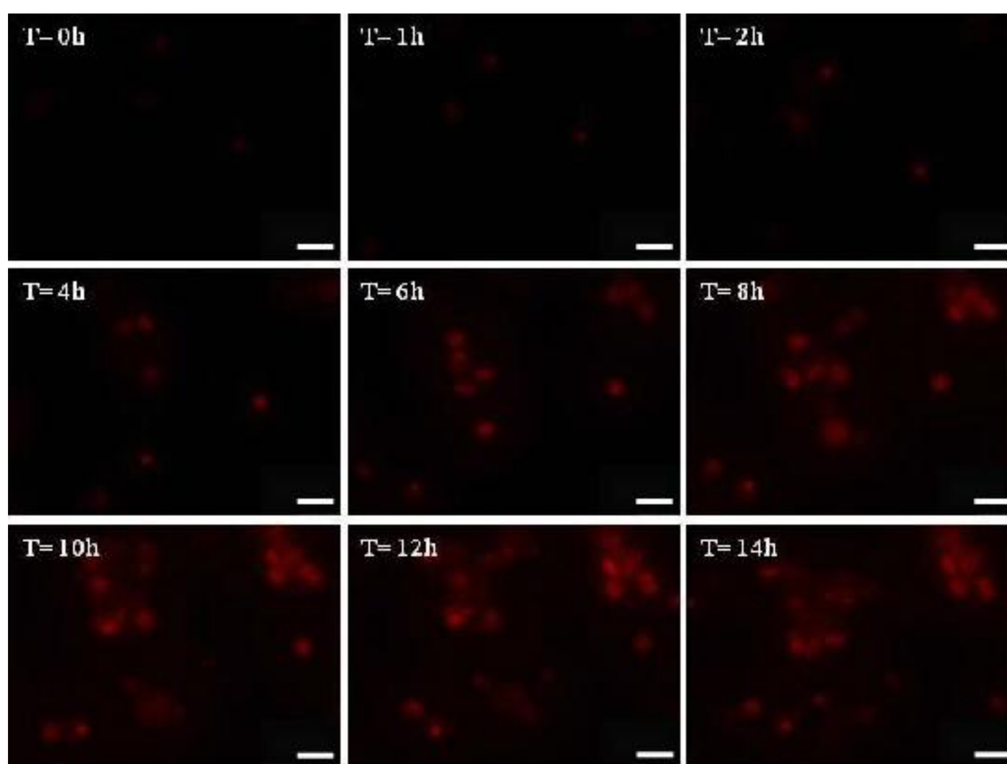


Fig 23. dopamine (100µM) produces superoxide anion through time.

After demonstrating that dopamine auto oxidation induce the production of mitochondrial superoxide anion, we treated cells with the DAT inhibitor GBR 12909 and as expected we saw a decrease in the red fluorescence intensity demonstrating again that dopamine needs to enter cells to exert it's toxicity that is mediated by the production of superoxide anion. Afterwards we evaluate both the action of DAQs and their co-treatment with GBR for the production of superoxide anion. As is possible to see from the time-lapse images (fig. 24), the pre-treatment with the DAT inhibitor lowered the signal of mitosox probe confirming that dopamine needs to enter cells to induce the production of mitochondrial superoxide anion. On the other hand, the treatment of cells with dopamine plus tyrosinase did not lead to an increase in the mitosox fluorescence and also in the case of pre-treatment with the DAT inhibitor, therefore with conclude that the tyrosinase-mediated oxidation of dopamine did not induce the production of superoxide anion. Antimycin A (Nakayama et al., 1956) was used as positive control since it inhibits the complex III of the mitochondrial electron transport chain (Alexandre and Lehninger, 1984; Campo et al., 1992; Maguire et al., 1992) leading to an increase in superoxide anion production.

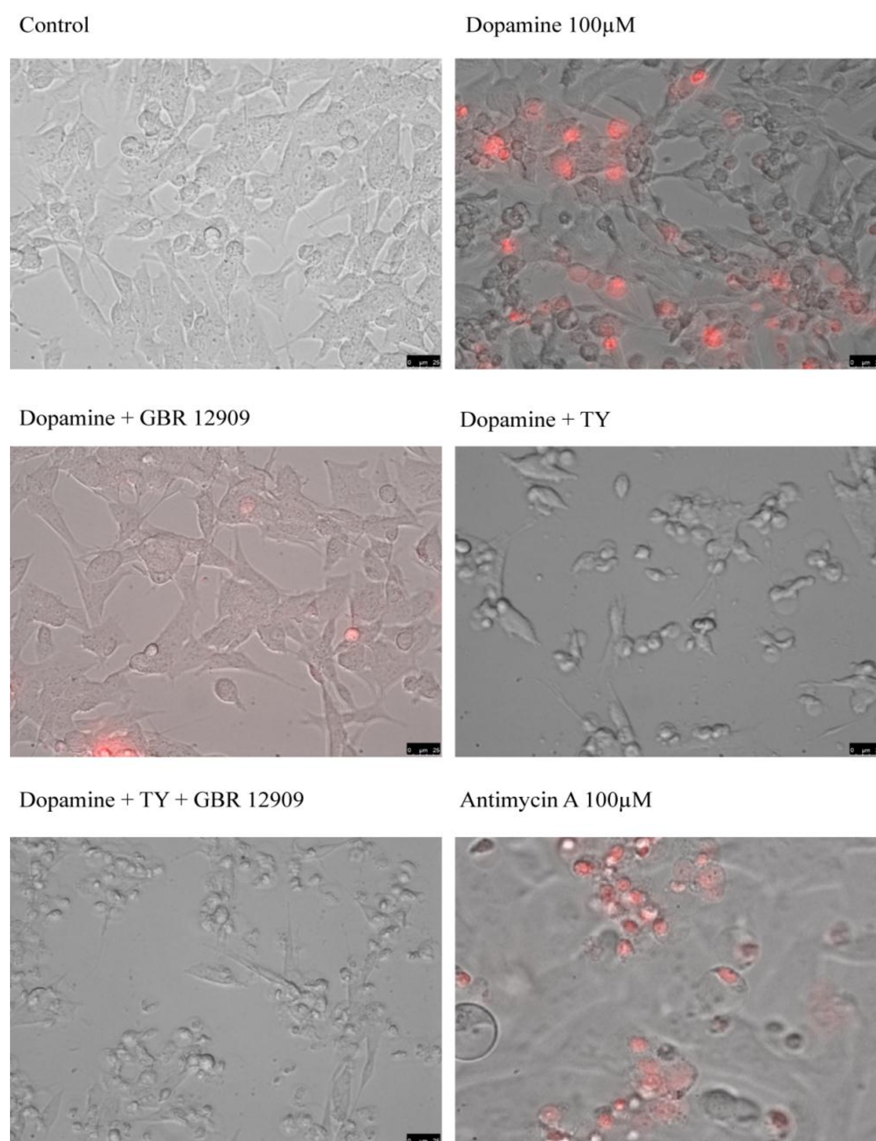


Fig 24. Only intracellular dopamine induce the production of mitochondrial superoxide anion. Antimycin A was used as a positive control since it is a complex III inhibitor.

Using ImageJ software is possible to quantify the increase of the fluorescence intensity of the probe through time.

The photos of the red channel fluorescence were analyzed from the 15h to evaluate the number of fluorescent cells and to perform a densitometric analysis. Using the ImageJ software, each cell that exhibit red fluorescence was analyzed to obtain the densitometric value of the fluorescence and the media of all values of fluorescence of the cells at a given time point to calculate obtaining the Y value the variation of red fluorescence intensity through time (fig 25).

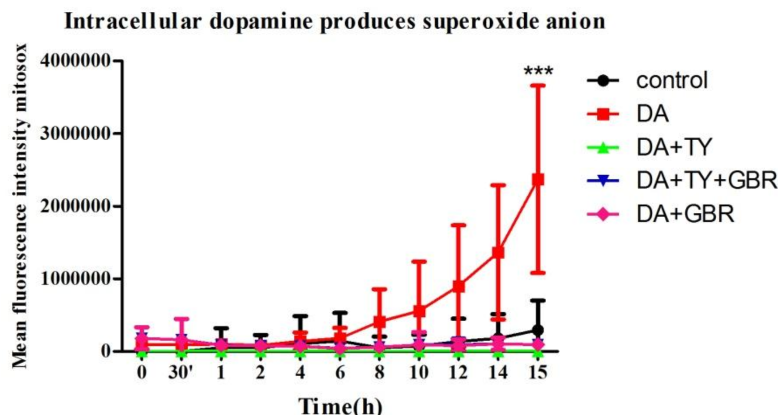


Fig 25. kinetics analysis of superoxide anion production. Photos at 15h were analyzed with imaJ software. Each cells was analyzed with the densitometric analysis to obtain the fluorescent intensity value and at every time-point was made a madia of the intensity values to obtain the Y value. The high degree of deviation is due to the high variability in the number of mitosox positive cells, and the fluorescence intensity of each cells.

As seen from the images of the time-lapse, only cytosolic dopamine was able to produce mitochondrial superoxide anion, whereas the other treatments were comparable to the basal level observed for the control. Two are the possible interpretation for this data: (i) dopamine-quinones do not affect the production of mitochondrial superoxide anion or their concentration in not enough to increase its level, or (ii) DAQs produce mitochondrial superoxide anion in low quantity and the antioxidant defense of cells are able to cope with this production.. Taking advantage from the images of the time-lapse we performed a kinetics analysis of the morphological changes associated to cell death under the different conditions. An elongated form characterizes our cells, but upon death, they become rounded and they detach from the flask. Analyzing the experiment and counting the number of dead cells (the “rounded” ones) is possible to note the different rate of cell death under the different treatment conditions. (fig. 26)

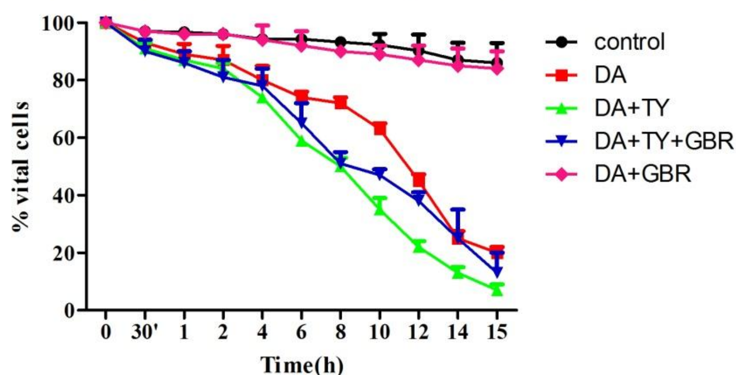


Fig 26. morphological analysis of cell death kinetics induced by the different treatments.

3.3.2 Flow cytometry

To confirm and to quantify with a different technique, the production of superoxide anion induced by our experimental condition, we performed the same experiment as in the time-lapse imaging described above, using flow cytometry. Also in this case, we evaluate which cell detachment method was the most suitable to minimize background in the control sample. We used the same method used for annexin experiments, confirming the use of papain as the best method.

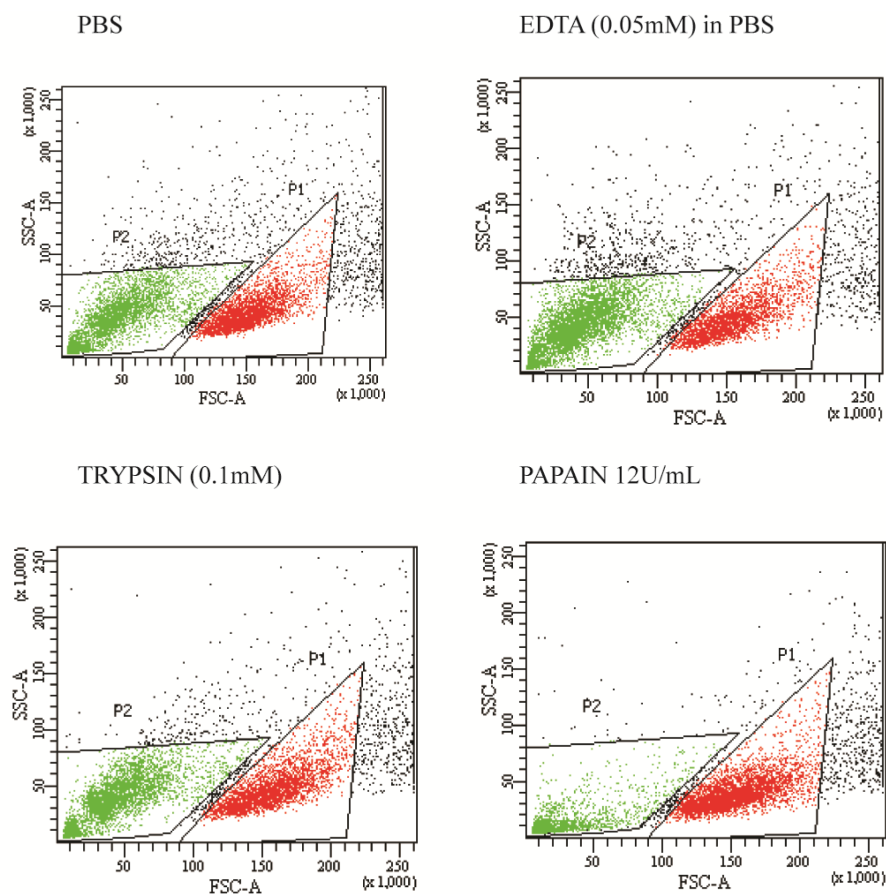


FIG. 27. Papain preserves the morphological features of healthy cells (in P2 population).

In figure 27 are represented dot-plots of FSC and SSC of FACS analysis. Since the procedure of cell detachment is a fundamental requisite for the preparation of sample for FACS analysis, it is important to preserve cell morphological features in control sample to be able to appreciate the changes in these parameters upon induction of dead stimuli. As in the case for ANNEXIN-V, papain was choose as the proper method since cells retain their morphological features as is represented in the P1 population (healthy cells). On the other hand, other methods leads to a high degree of cell debris.

In the figure below (fig. 28) are represented histograms of the fluorescence peak of mitosox probe. Again it is important to have the lower background signal, and papain was reconfirmed as the proper choice due to its peak position on zero values.

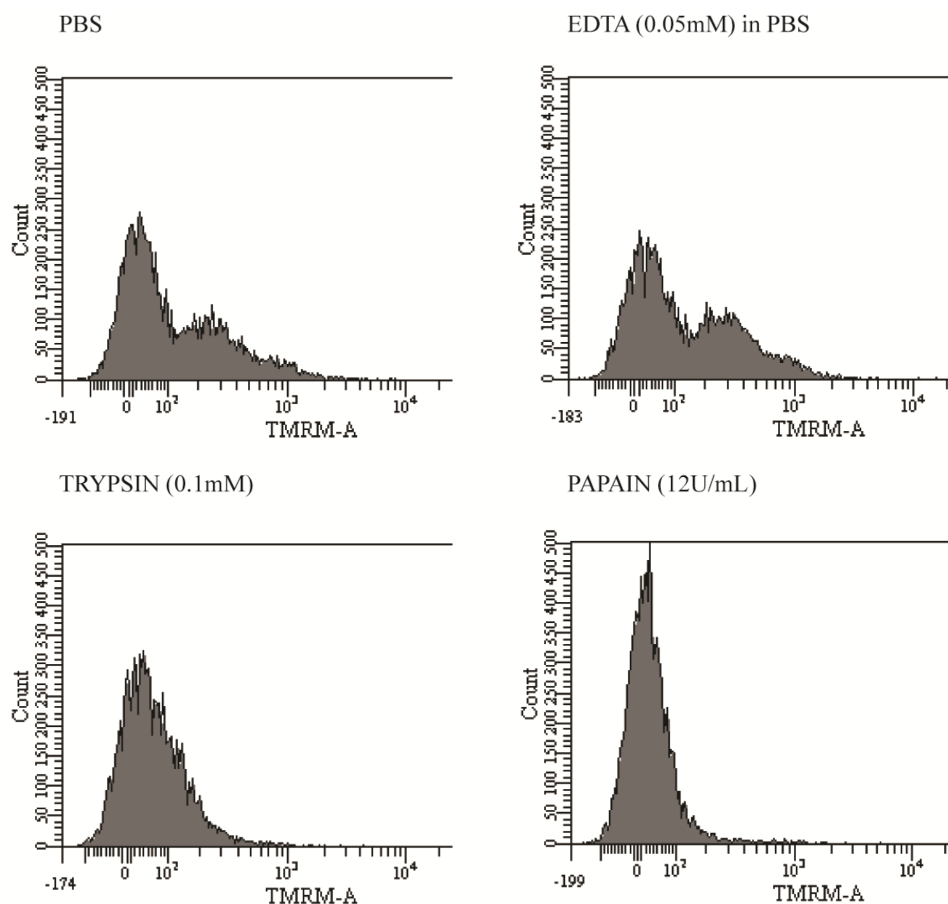


FIG. 28. Papain leads to the lower background in control sample.

After the evaluation of cell detachment method, we performed the experiments treating cells under different condition. In the first image (fig.29), it's possible to observe the morphological changes of the cells after the different treatments. Both dopamine and DAQs leads to a relocation of cells from the "healthy population (P1) to the dead one (P2). In the second image (fig. 30), in P3 gate are represented cells that exhibit the fluorescence of mitosox, and this population is reported even in the third image (fig.31) where it can be appreciate the fluorescence shifts from lower values (associated to the absence of superoxide anion production) to higher values. Again this technique confirmed that only cytosolic dopamine was responsible for the increasing in mitochondrial superoxide anion production represented in the P3 gate in the second figure and in the fluorescence shift of the mitosox fluorescence in the third one. Antimycin A was used as a positive control since it is a complex III inhibitor leading to the production of superoxide anion.

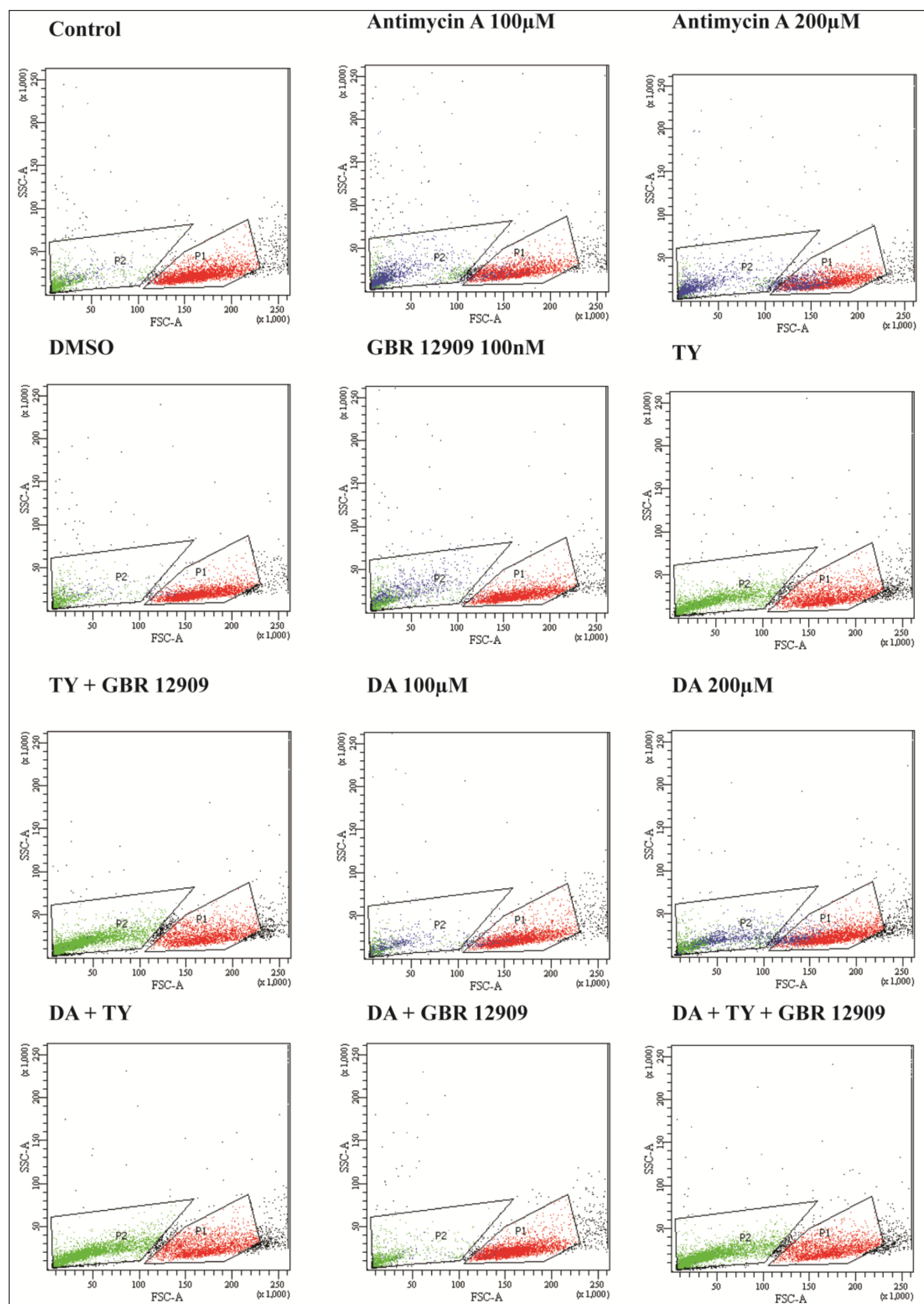


Fig 29. Flow cytometry analysis of mitosox red in cells treated with the different treatments. The dot-plot shows changes in cells morphology: P1 population (in red) represents healthy cells while P2 population (in green) represent death cells.

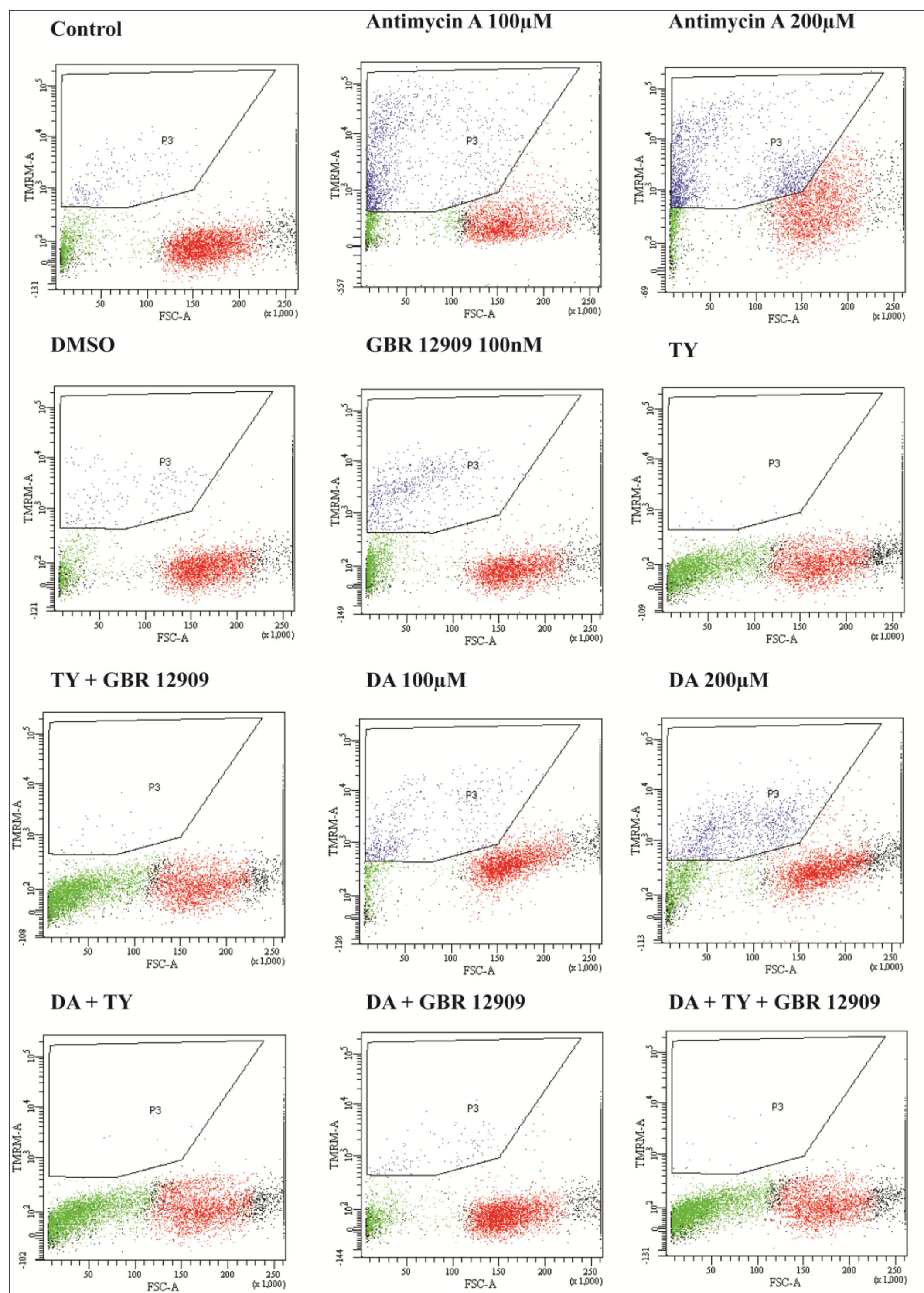


FIG. 30. Dopamine induces the mitochondrial superoxide production while DAQs do not. In the P3 population are represented cells that exhibit the mitosox fluorescence.

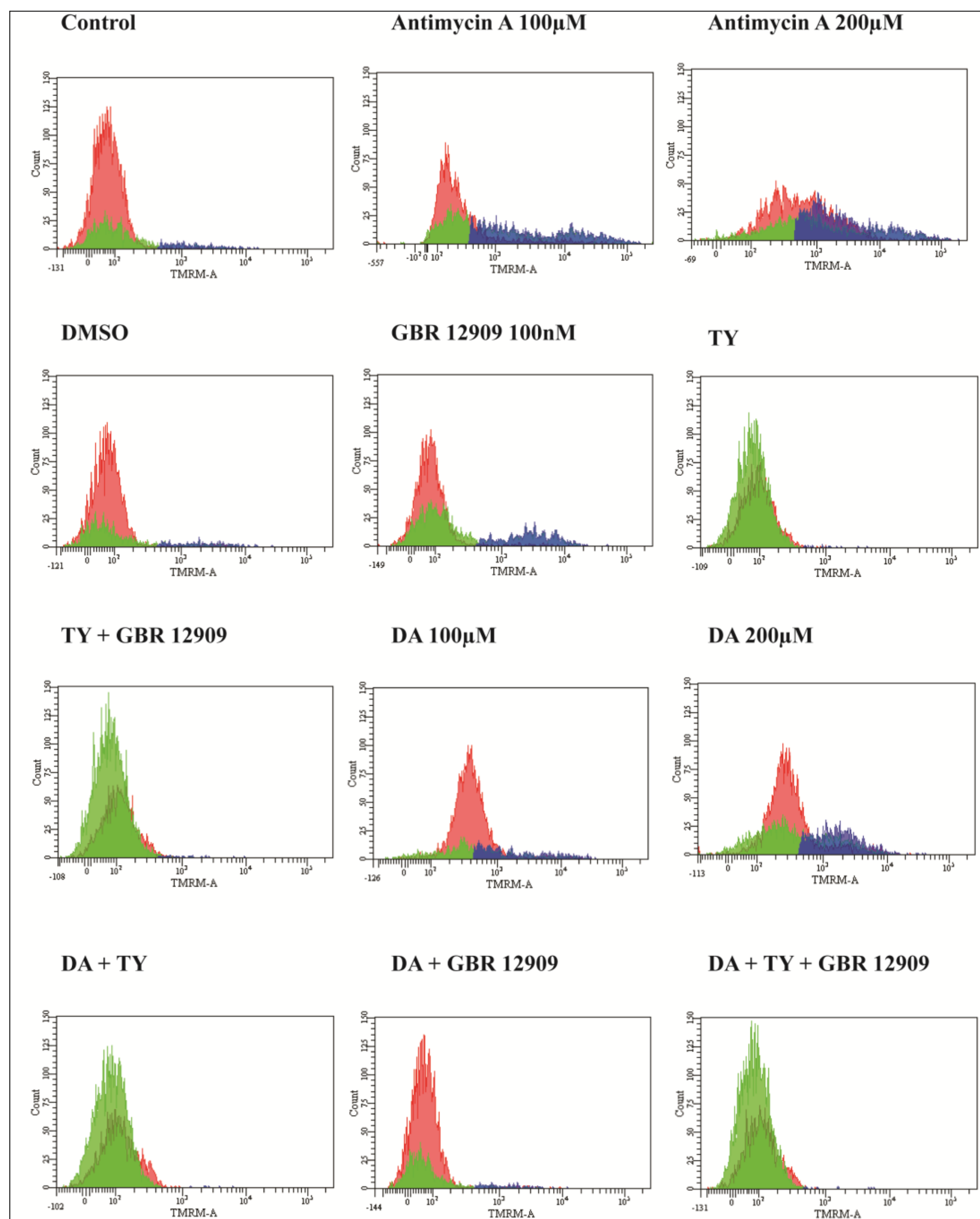


FIG. 31. Dopamine induces the shift of the fluorescence peak of mitosox. In this histograms is possible to note the fluorescence peak of mitosox and its shift only in the case of dopamine.

With the use of flow cytometry we confirmed the previous results obtained in the time-lapse imaging experiments. Only cytosolic dopamine give rise to the production of mitochondrial superoxide anion together with changes in morphological features. On the other hand, DAQs led to the changes in morphological parameters, but no production of mitosox fluorescence was detected leading to the conclusion that DAQs toxicity do not depend on the production of this ROS specie.

3.4 Activation of apoptotic cascade

Apoptosis is a tightly regulated mechanism that can be activated by different stimuli and is mediated mainly by two different mechanisms: the extrinsic (death-receptor mediated) and intrinsic pathway (mitochondrial pathway) that ultimately converges to apoptosis. From previous data, we demonstrated that both dopamine and DAQs leads to the activation of apoptosis with typical features of this programmed cell death process such as phosphatidil-serines externalization and nuclear fragmentation. The data regarding the role of ROS production led to different results between dopamine and DAQs. Dopamine led to the production of mitochondrial superoxide anion only in the case of its internalization since DAT inhibitor treatment lowered this production to control level. On the other hand, DAQs both in the presence and absence of the DAT inhibitor did not lead to the production of superoxide anion. We asked if dopamine and DAQs activate the same pathway but in different ways, one superoxide anion-dependent and the other one superoxide anion-independent, or if they activates different apoptotic ways evaluating other markers for apoptosis. By western blot analysis using antibody against caspase 3 and Poly (ADP-ribose) polymerase (PARP) we evaluate the apoptotic cascade.

3.4.1 Caspase 3 cleavage

The progression of apoptotis is regulated by different proteins among them caspases have a pivotal role. The caspase-cascade is one of the several way by which the apoptotic signal is transduced and amplified. Caspases are aspartate-specific cysteine proteases and exist all in a inactivated forms as pro-caspases. Upon apoptotic signal, they undergo an internal cleavage (performed by other proteins or other caspases) to become activated. Caspase 3 is one of the executioner caspases and its activation can be induced by different stimuli that are connected both with the extrinsic or intrinsic pathway. Once activated, caspase 3 can activates other caspases or affect other proteins that are involved in cell shrinkage, membrane blebbing and DNA rapair (for a review Fan 2005). Upon induction of apoptosis, caspases play a important role in triggering and amplifying the death signal. Different class of caspases are present in mammals, mainly grouped on the level at which they exert their role. Caspase-3 is one of the caspases activated in the execution phase of apoptotic cascade, therefore we evaluate its cleavage (and subsequent activation) in our experimental conditions. The cells challenged with the different treatments were subjected to SDS-PAGE and probed for an antibody against total caspase 3. This antibody recognize both full length and cleaved form of

caspase-3. From preliminary data is possible to notice that DAQs led to a high degree of caspase-3 cleavage and therefore to its activation, while dopamine led only to a slight increase in its cleavage.(fig.32)

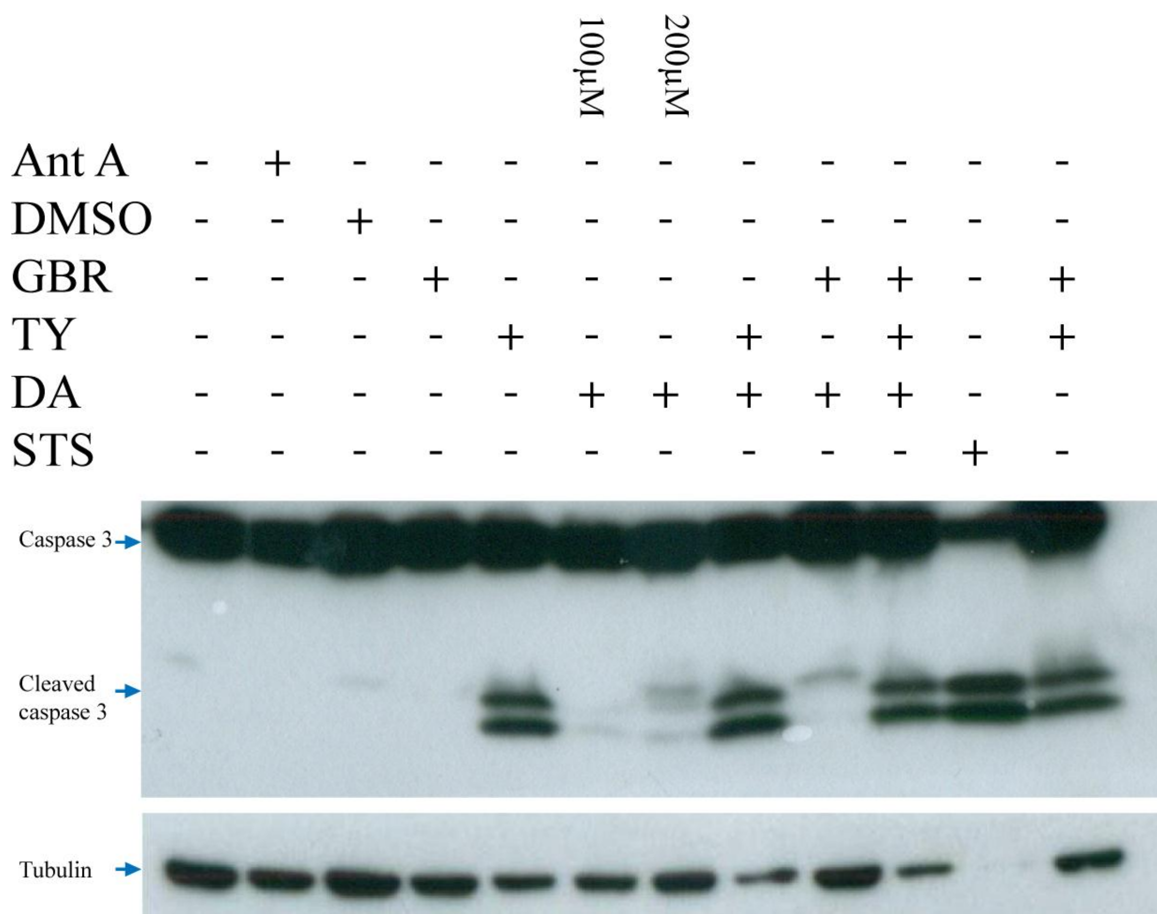


Fig 32 DAQs leads to the cleavage and subsequent activation of caspase 3. Representative image of one of the two western blot analysis of lysates of cells treated with the different conditions.

In the figure below (fig. 33.) is represented the densitometric analysis of the cleaved form of caspase-3. Since the experiment was performed only in duplicate (only one data shown), it was not possible to perform a statistical analysis, but the data of these experiment led to the same result.

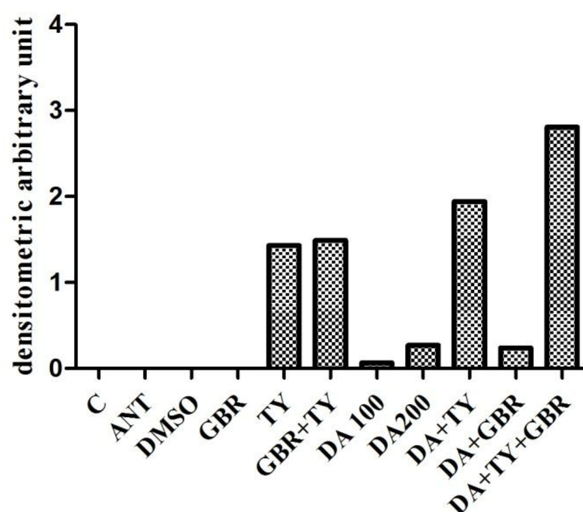


Fig. 33. DAQs leads to a high degree of caspase-3 cleavage. Densitometric analysis of two experiments on caspase-3 cleavage. (N = 2). Values are means of the two experiments. For the lack of further experiments, we were not able to perform a statistical analysis.

3.4.2.Poly (ADP) ribose polymerase (PARP) cleavage

Poly (ADP) ribose polymerase (PARP) is a nuclear protein involved in several cellular processes including mainly DNA repair functions. Upon the induction of a stress stimulus, PARP is responsible to detect signals of DNA damage and activation of the enzymatic machinery for the DNA repair. If the stimulus leads to an extensive DNA damage, cells undergo cell death rather than spending energy for repairing internal damage. The inactivation of PARP through its internal cleavage is achieved by different inducers among them capsase-3. (For a review see Herceg 2001)

We previous demonstrate that only DAQs were able to activate caspase 3, so we expect to obtain the same results in the cleavage of PARP. Control cells and cells treated with dopamine and DAQs (the complete analysis is missing) led to observation of a high degree of PARP cleavage only in the case of DAQs (fig 34). Despite the lack of the entire experiment and internal controls, this data (performed in triplicate) is in agreement with the data obtained from the western blot analysis of cleaved caspase 3 leading to the hypothesis that dopamine and DAQs follow different apoptotic pathways.

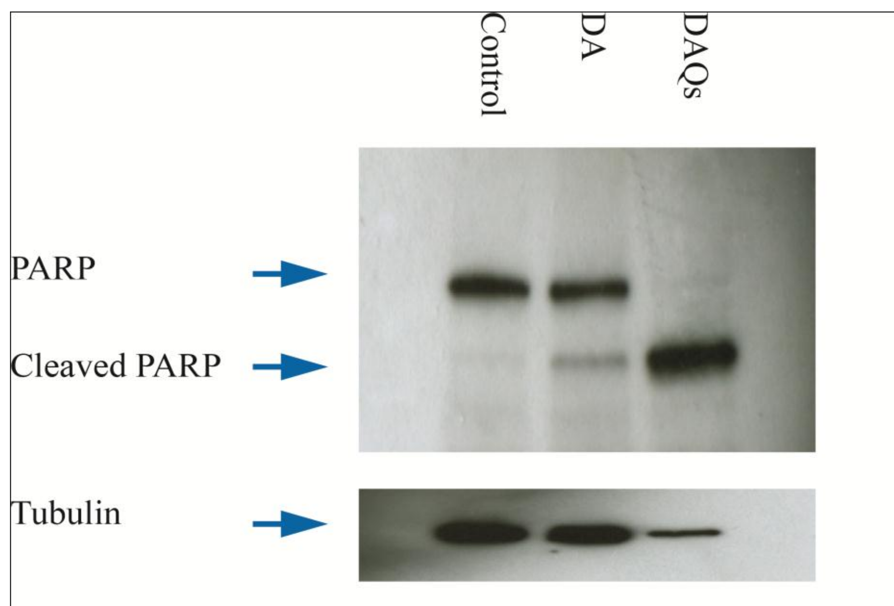


FIG. 34. DAQs leads to an extensive PARP cleavage

In the fig 35. below is reported the densitometric analysis of PARP cleavage.

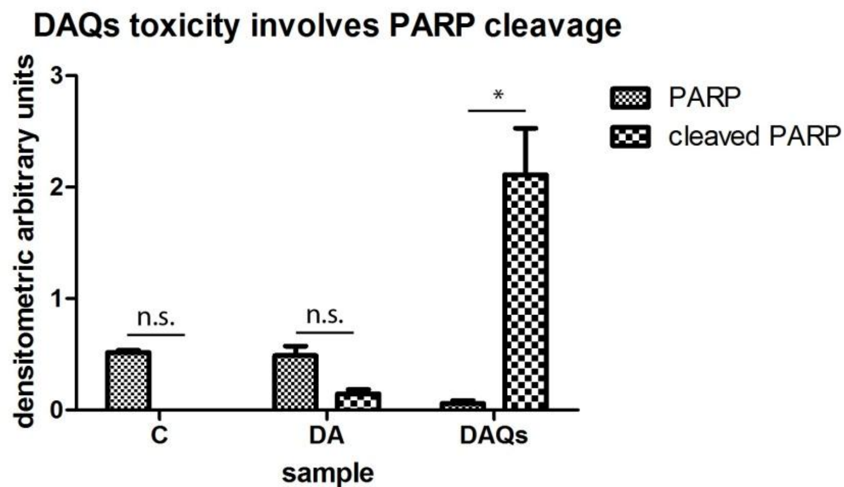


Fig. 35. Densitometric analysis of PARP cleavage.

3.5 Superoxide dismutase 1 and 2 over expression

Oxidative stress along with mitochondrial dysfunction has been implied in the pathogenesis of Parkinson's disease. This condition is a possible link between sporadic and familial form of the

disease being that several genes implicated in the familial forms are linked to the maintenance of mitochondria morphology, integrity and function. The data presented in the previous sections on the toxicity exerted by dopamine and its oxidation products (DAQs) revealed that both are cytotoxic leading to apoptotic cell death, but only cytosolic dopamine led to the production of mitochondrial superoxide anion. In this second part of the project we were interested in evaluating the potential protective role of superoxide dismutase 1 and 2. Superoxide dismutases are a class of enzymes that are responsible for the dismutation of superoxide anion into molecular oxygen and hydrogen peroxide and are referred as the first line of defense against the production of ROS. In mammals, different superoxide dismutases have been found, which differ for their metal co-factor and cellular localization. Superoxide dismutase 3 is a copper-zinc anzyme (SOD3) and is found in the extracellular space, superoxide dismutase 1 as well as SOD3, is a copper-zinc enzyme (SOD1) mainly found in the cytosol, while superoxide dismutase 2 is a manganese enzyme (SOD2) that is found in the mitochondria. (for a review Abreu 2010). Nowadays there is no genetic mutation on the superoxide dismutases genes that has been linked to Parkinson's disease, but several studies implied the role for SOD1 and 2 activities in the progression of the disease. Two different studies by the same group, based on proteomic analyses of isolated rat brain mitochondria following exposure to DAQs, suggested a direct interaction with SOD2 in brain's mitochondria. In the first one, by using a combination of fluorescent probes directed against cysteine or lysine residues, the investigators demonstrated that DA oxidation results in the loss of mitochondrial proteins, among which SOD2 (Van Laar 2008). In the second study, SOD2 was identified as one of the proteins modified by 14C-DAQs in rat brain mitochondria (Van Laar 2009). From a study of Iglesias-González and colleagues it has been demonstrated that pre-treatment with superoxide dismutase had a protective effect against 6-OHDA induced cell death (Iglesias-González et al. 2012). Since mitochondria dysfunction and oxidative stress is implied in the pathogenesis of PD and considering the importance of SOD1 and SOD2 in protecting cells against superoxide anion production, we evaluate the role for this two enzymes in protecting cells against dopamine and DAQs toxicity. For this purpose the cDNA of superoxide dismutase 1 and 2 were cloned in vectors carrying a fluorescence tag (GFP for SOD1 and RFP for SOD2) to monitor at the single cell level the effect of the two proteins over expression.

3.5.1 Cloning of SOD1 and SOD2

The human full length cDNA of superoxide dismutase 1 was a kind gift of Dott. Marco Bisaglia in the pOTB7 Vector while the human full-length cDNA of superoxide dismutase 2 of Dott. Elisa

Belluzzi in the pCMV-SPORT6 Vector. Since these vectors are not suitable for the expression in mammalian cells, the sequence of SOD1 and SOD2 were amplified from the origin vector (fig. 36) using two primers carrying the EcoRI and BamHI restriction sites and EcoRI and AgeI restriction sites (for SOD1 and SOD2 respectively) and cloned into the pAcGFP1-C2 vector and pDsRed1-N1vector for SOD1 and SOD2 respectively. (fig. 37 and fig. 38).

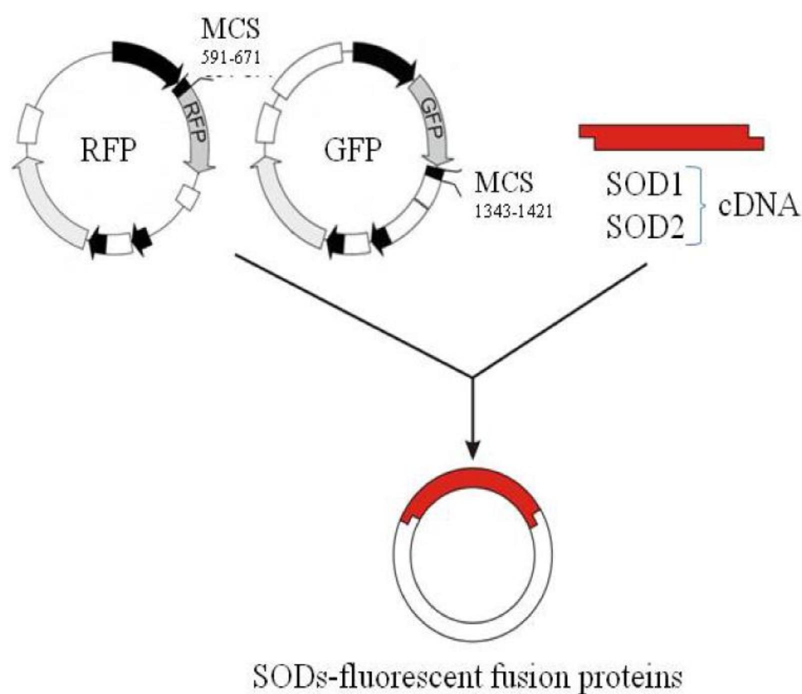
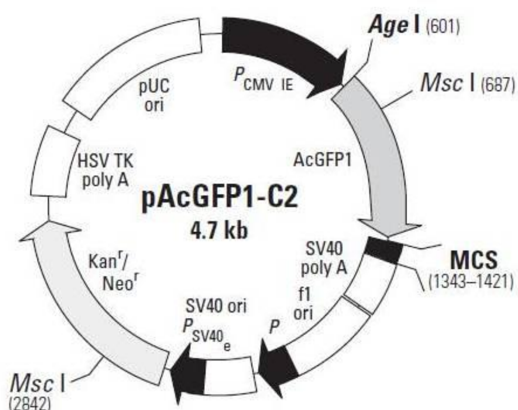


Fig 36 . Schematic representation of cloning strategies.

The enzymes used to clone the SOD1 figx and SOD2 figx sequence are summarized in the red boxes .



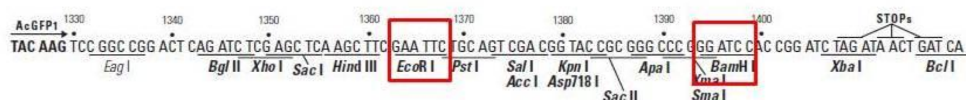


Fig. 37. pAcGFP1-C2 vector map. In the red boxes are highlighted the restriction sites used to clone the SOD1 sequence in the destination vector

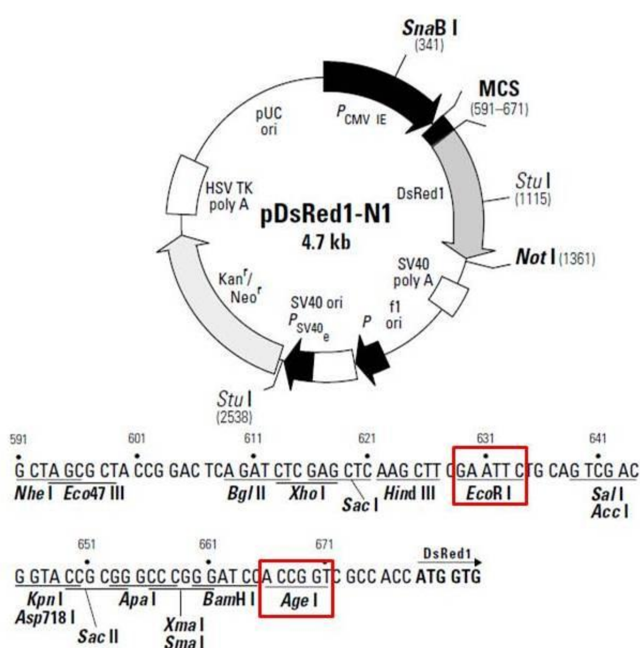


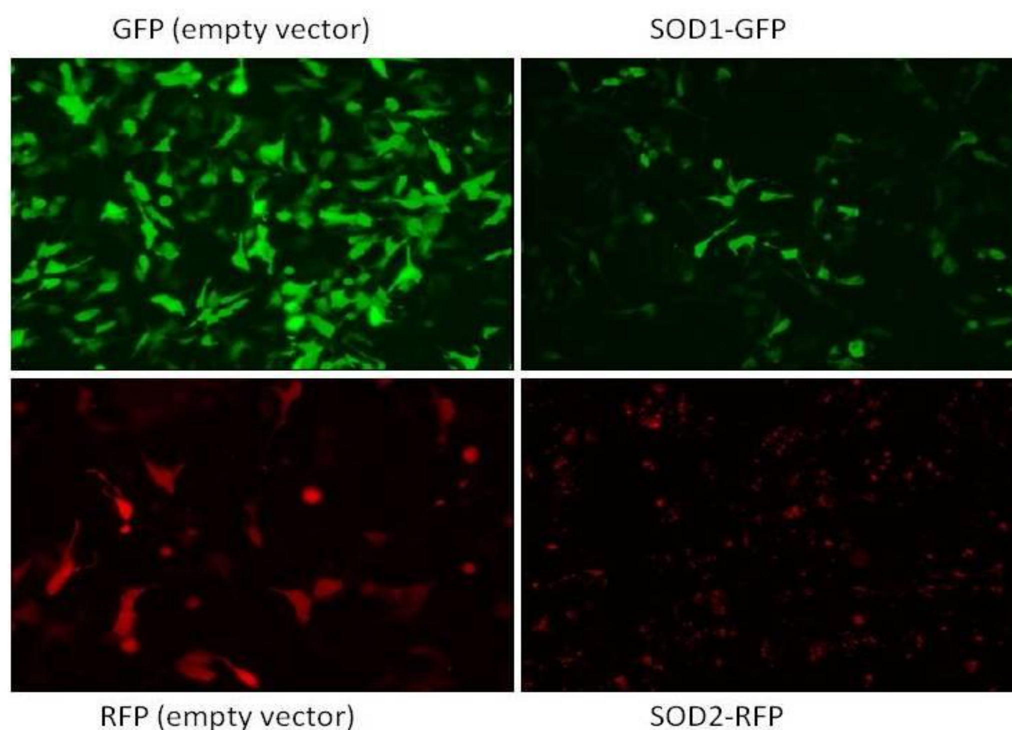
Fig. 38. pDsRed1-N1 vector map. In the red boxes are highlighted the restriction sites used to clone the SOD2 sequence in the destination vector.

For the properly folding of SOD1, the fluorescent tag was cloned upstream to the SOD1 sequence. For SOD2, the cDNA sequence was cloned at the C-terminal sequence of the fluorescent tag to preserve the correct translation of the mitochondrial tergeting sequence of the gene. The sequence

of the SOD1 and SOD2 and the correct frame of the protein in respect to the N-terminal GFP tag (for SOD1) and the C-terminal RFP tag (for SOD2) were verified by sequencing at the BMR genomics, proving that both proteins were in frame to the respective fluorescent tag.

3.5.2 Transient transfection and cellular localization

Cells were transfected with both the empty vectors and the SODs construct to evaluate the correct localization of the two protein inside the cells (fig. 3.9).



(fig. 39). Transient transfection of both empty vectors (on the left)m and SODs-fusion proteins (on the right). SOD1-GFP shared a cytosolic localization, while SOD2-RFP shared a spotted pattern suggesting a precise localization insede cells.

SOD1 conjugated to the GFP protein was expected to share a cytosolic localization, while the SOD2 fusion protein was expected to localized into mitochondria. From fluorescence microscopy experiment, we confirmed that our construct were in the correct localization. SOD1 localized mainly in the cytosol. To address the correct localization of SOD2 into the mitochondria, we transfected cells with an ampty vector carrying the sequence of yellow-fluorescent protein targeted

to the mitochondria. The correct localization of the SOD2-RFP protein was assessed by confocal microscope evaluating the merge of YFP florescence of YFP-construct and the SOD2-RFP vector. In the the merge visualized, confirmed us that the protein is correct localized in the mitochondria. (fig. 40)

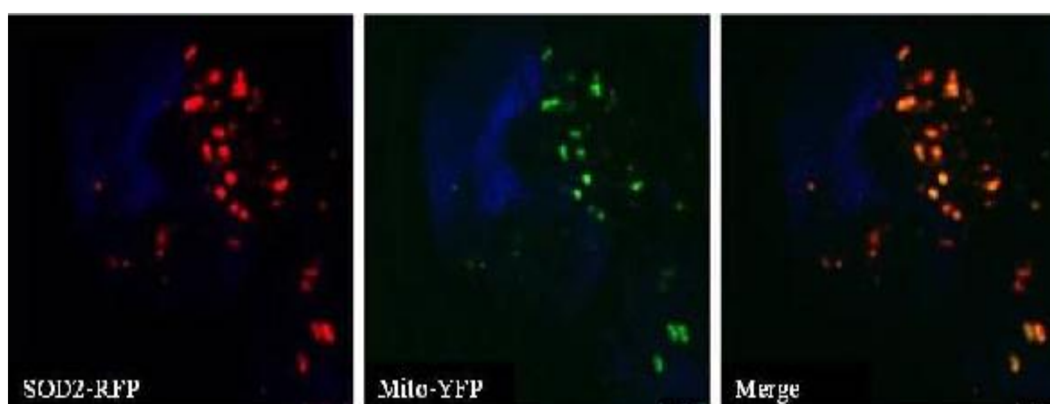


Fig. 40. SOD-RFP construct correctly localized into mitochondria. Confocal microscopy images confirm the correct localization of the SOD2-RFP fusion protein inside mitochondria.

3.5.3 western blot analysis of SODs over expression

To evaluate the over expression of the two SODs fusion proteins in respect to the transfection efficiency and for tubulin, cells transfected with the empty vectors and the SODs vector were subjected to SDS-PAGE (fig. 41) and quantified by densitometric analysis (fig 42)

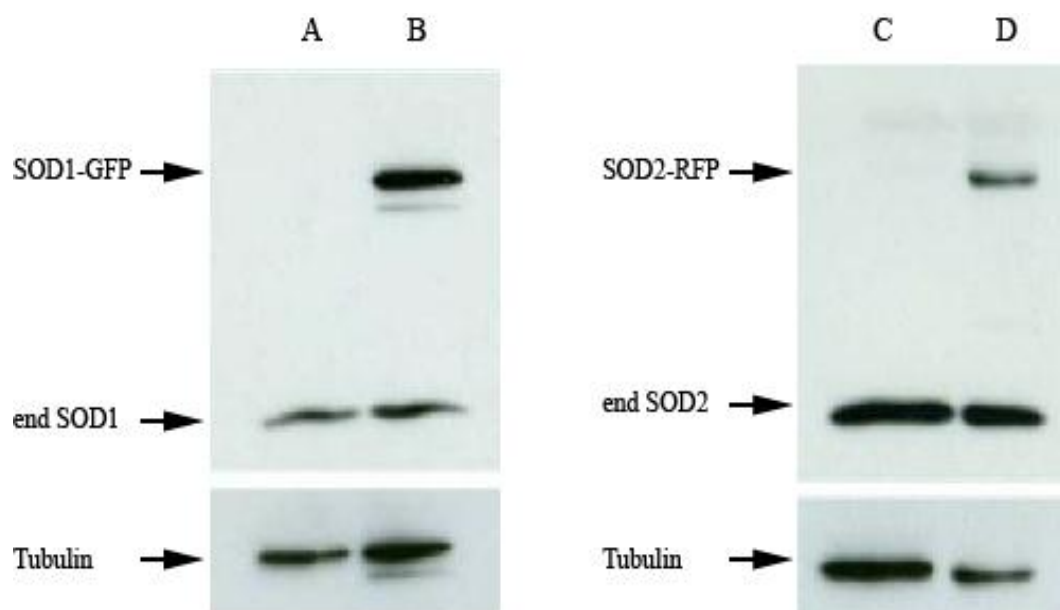


Fig. 41. western blot analysis of cell transfected with the pAcGFP vector and the vector carryng the SOD1 sequence (A and B respectively) and the pDsRed vector and the vector carrying the SOD2 sequence (C and D respectively).

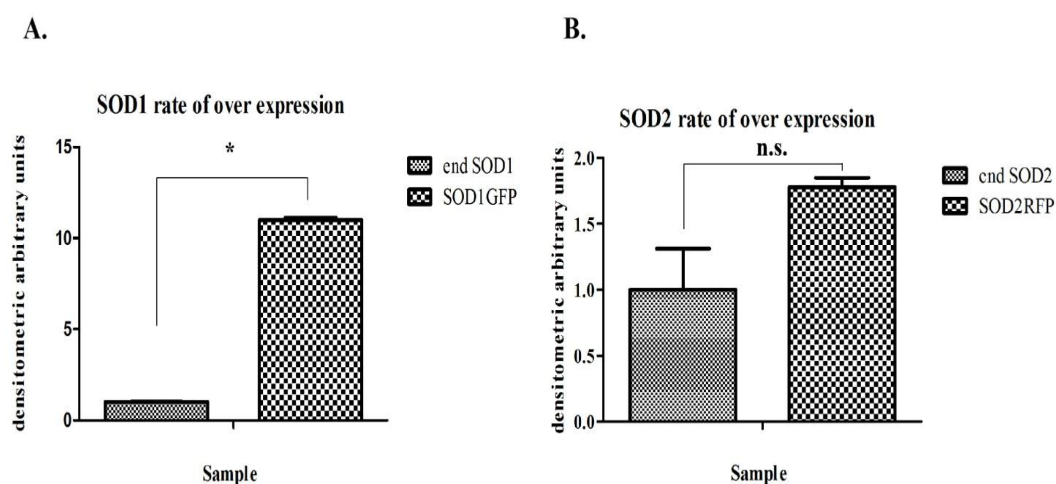


Fig. 42. Densitometric analysis of the over expression of SOD1 (A) and SOD2 (B) in respect to transfection efficiency and normalized for tubulin.

Once evaluated the cellular model over expressing the two SODs conjugated with the fluorescence tag, we evaluate their role against dopamine and DAQs induced toxicity.

3.5.4 SODs effect against dopamine toxicity

Transfected cells were treated with increasing concentration of dopamine (like in the experiment in the previous section) and the percentage of apoptotic nuclei was calculated for both SOD1 (fig. 43) and SOD2 (fig. 44)

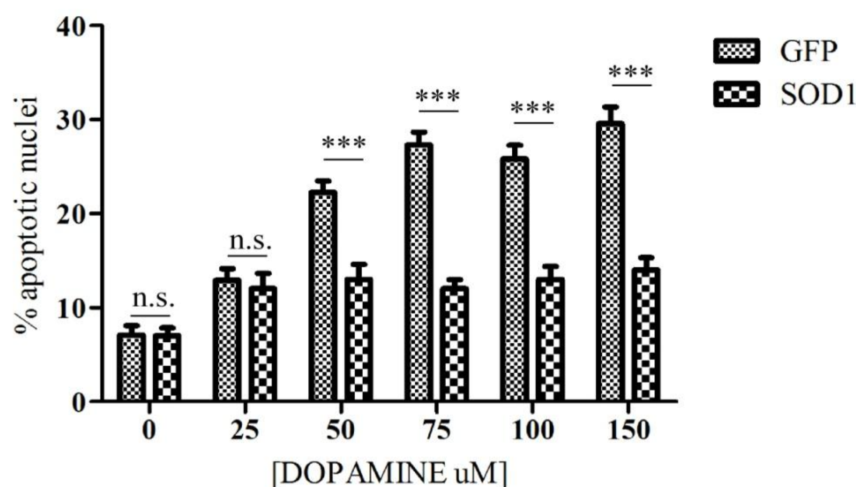


Fig. 43. SOD1 over expression has a rescue effect against dopamine toxicity. Percentage of apoptotic nuclei in cells over expressing the empty vector (GFP) and the SOD1-GFP (SOD1). (N = 3, n.s. = p > 0.05, *** = p < 0.01

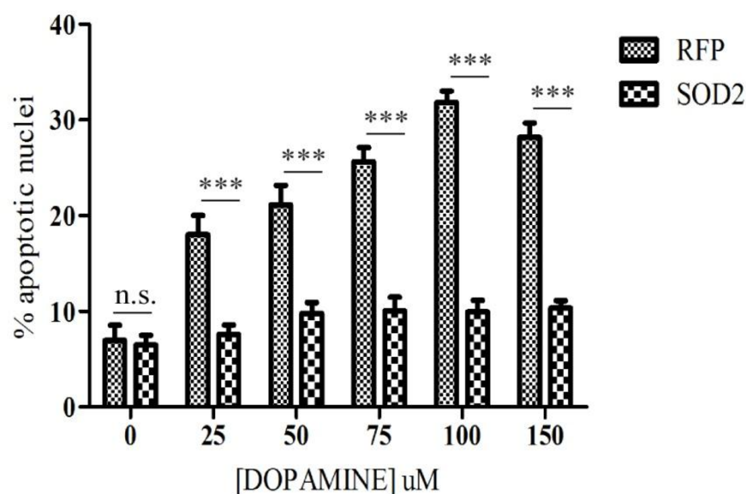


Fig. 44. SOD2 over expression has a rescue effect against dopamine toxicity. Percentage of apoptotic nuclei in cells transfected with the empty vector (RFP) and SOD2-RFP (SOD2).

As expected, both superoxide dismutases 1 and 2 exert their rescue effect on cell viability by lowering the level of superoxide anion induced by dopamine accumulation and blocking the activation of apoptotic cascade induce by dopamine auto-oxidation process (since we did not detect apoptotic nuclei) with no differences in rescue effect exerted by SOD1 or SOD2.

3.5.5 SODs effect against dopamine-quinones toxicity

The next experiment was carried out to evaluate the effect of the over expression of SODs in protecting cells against DAQs. Cells were tranfected with the empty vectors and the vectors carrying the sequence of SOD1 and SOD2 and were treated with increasing concentration of dopamine plus tyrosinase. For each condition, apoptotic nuclei were counted and the percentage of apoptotic nuclei at the different condition were calculated for SOD1 (fig. 45). and SOD2 (fig.46)

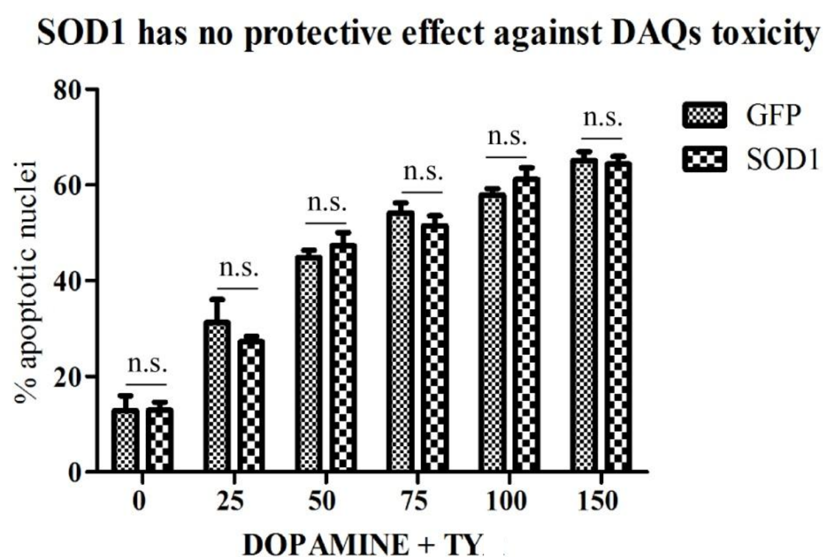


Fig. 45. SOD1 has no protective effect against DAQs toxicity. Percentage of apoptotic nuclei in cells over expressing the empty vector (GFP) and SOD1.

The same experiment was carried out for SOD2 using the empty vector (RFP) as a negative control. (fig. 46)

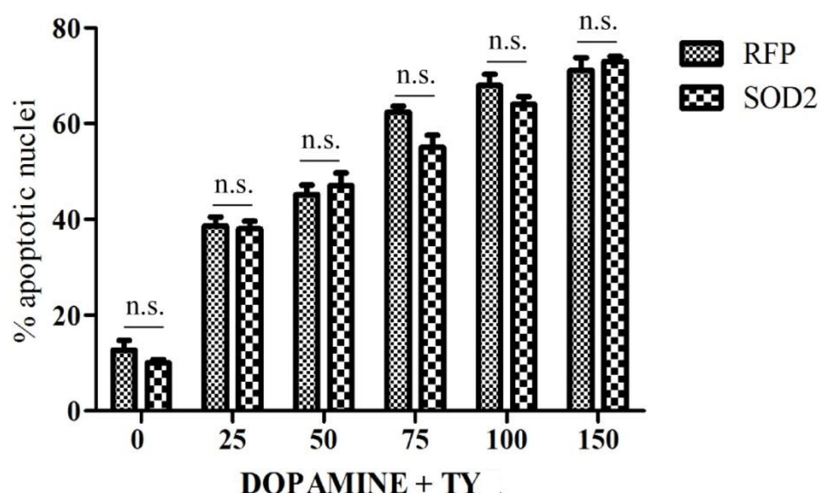


Fig. 46. SOD2 has no protective role against DAQs toxicity. Percentage of apoptotic nuclei in cells transfected with the empty vector (RFP) and SOD2-RFP (SOD2).

Previous data demonstrated that tyrosinase-mediated oxidation of dopamine induced toxicity in a superoxide-independent manner since there was no production of mitosox fluorescence, however there was the induction of cell death by activation of apoptosis. The evaluation of the over expression of both superoxide dismutases 1 and 2 did not show a rescue effect as in the case of dopamine treatment supporting the different role of superoxide anion production in the auto and enzyme-mediated process of dopamine oxidation and subsequent cell toxicity.

4. Discussion and conclusion

Parkinson's' disease is a widespread neurodegenerative disorder that still nowadays has no cure. The clinical and pathological hallmarks of the disease are related to motor dysfunction and to the selective death of dopaminergic neurons in the *substantia nigra pars compacta* of the midbrain. Less than 5% of the cases are linked to mutations in several genes but in the vast majority of PD patients the cause is still object of debate. Among the several mechanism that have been proposed to be triggers and amplifiers of the disease, mitochondrial dysfunction and oxidative stress play an important role (Beal MF. 2007, Schapira 2008). *Post mortem* studies provided evidences of both, decreased mitochondrial complex I activity (Schapira et al. 1990, Schapira et al. 1989, Mann et al. 1994) as well as the presence of oxidative stress markers (Jenner and Olanow 1996). Although mitochondrial dysfunction and oxidative stress have been associated to both the beginning and the progression of this disease, these processes cannot explain the selective death of the dopaminergic neurons of the *substantia nigra pars compacta*. These neurons are characterized by the production of dopamine whose improper oxidation chemistry was proposed to lead to toxic products (Graham et al. 1978). Under physiological condition dopamine is produced in the cystosol and after synthesis it is stored in the synaptic vesicles by the action of the vesicular monoamine transporter 2 (VMAT2) where dopamine is stabilized by the low pH. Upon the arrival of an action potential, dopamine is released in the synaptic cleft where it can exert its function of neurotransmitter. Afterward it is recycled by the action of the dopamine transporter (DAT), which placed on the outer membrane transports back dopamine to the cytoplasm. If the amount of cytosolic dopamine increases, it is degraded into non-toxic species by the action of monoamine oxidase (MAO) and catecol-o-methyl transferase (COMT) or it is sequestered into lysosomes to form neuromelanin (NM). If dopamine is not cleared in these catabolic pathways, it undergoes a process of autoxidation or enzyme-mediate oxidation leading to the production of toxic dopamine-quinones (DAQs) and reactive oxygen species (ROS) The former have been demonstrate to interact with cysteinyl residues of proteins (Belluzzi 2011, Bisaglia 2007, Girotto 2012,

Bisaglia 2010b, LaVoie 2005, Van Laar 2008, Van Laar 2009) and to cause toxicity by diminishing or blocking proteins activity (Asunama 2003), the latter can damage cellular components such as proteins, lipids and DNA leading eventually to cell death (Jenner P. 2007). Several enzymes are good candidates for the enzymatic oxidation of dopamine, among them tyrosinase has been implied as one of the enzyme responsible for the oxidation of dopamine to DAQs (Miranda and Botti 1983; Costa et al. 1992). Several studies demonstrated that DAQs production in the tyrosinase-mediated oxidation of dopamine are responsible for the cellular toxicity (Stokes 1996, Xu 1998, Greggio et al. 2005) but others suggest a protective role for tyrosinase by the induction of the production of neuromelanin (Sulzer et al. 2000).

The aim of this project was to understand the mechanism of cell toxicity induced by the auto and the tyrosinase-mediated oxidation of dopamine and to evaluate the role for superoxide dismutases 1 and 2 in dopamine and DAQs intoxication in a cellular model for PD: SH SY5Y.

The first part of the project focused on dopamine auto and tyrosinase-mediated oxidation and the effect on cell viability. Initially, we carried out an experiment to test our cellular model for the presence of all the dopaminergic machinery that comprises enzymes responsible for the synthesis, storage,and uptake of dopamine. To this purpose RNA was extracted from cells, mRNA was retro transcribed to obtain cDNA and the proteins of interest were amplified with specific primers. These experiments gave us information regarding only the expression level of the mRNA of the proteins involved in the dopamine system, but were sufficient to confirm the presence of the dopaminergic markers of interest.

For the first aim of the project concerning about the toxicity of dopamine and DAQs we were interested in revealing which type of cell death mechanism was induced. In the literature it has been proposed that dopamine induces cell death by activating apoptosis (Ziv et al. 1996, Mochizuki et al 1996, Anglade et al. 1997) although there are conflicting results (Kosel et al. 1997, Burke et al. 1998, Burke et al. 1998).Using different techniques, we investigated the role of apoptosis in cell death induced by dopamine and its oxidation products. Using a DNA binding dye and fluorescence microscopy, we checked for one of the later stages of

apoptosis: nuclear fragmentation. This kind of experiment permits to evaluate the morphology of nuclei, which in control sample are rounded, but in the case of induced apoptosis became small and fragmented (apoptotic bodies). The physiological concentration of dopamine inside cells is not clearly defined, although has been reported to be in the nM range, reaching a mM concentration inside the synaptic vesicles. (Eisenhofer et al. 2004, Kopin et al. 1993, Santiago et al. 1996, West et al. 2003). These studies suggest that a slight increase in intracellular dopamine concentration could have important consequences on cell viability. For this reason we decided to treat cells with non-letal concentration of dopamine. Cells treated with increasing concentration of dopamine (from 0μM to 150μM) showed a rise in the percentage of apoptotic nuclei in an overnight treatment leading to the conclusion that apoptosis was activated. Cells were treated with extracellular dopamine, therefore we asked if its toxicity was mediated by its internalization in cells by the action of the dopamine transporter (DAT). Cell were pre-treated with the specific DAT inhibitor, GBR 12909, leading to a rescue effect against dopamine toxicity. This led us to the conclusion that dopamine toxicity depends on its internalization by the activity of DAT. Cytosolic dopamine can undergo an auto-oxidation process and one enzyme-mediated. Several studies implied the role of tyrosinase in the oxidation process of dopamine and the presence of the enzyme in substantia nigra. (Stokes 1996, Xu 1998, Greggio et al. 2005). On these premise, to compare dopamine auto oxidation process and its enzymatic oxidation, we investigate the role of tyrosinase-mediate oxidation of dopamine to speed up the production of dopamine quinones and to assess if there was a different rate of cellular toxicity between these species generated by different mechanism. Dopamine auto oxidation and the enzyme-mediated process did not show a difference in cellular toxicity. The positive control with the treatment of cells only with tyrosinase (when compared to dopamine 100μM sample) showed also a toxic effect due to the presence in the cell medium of L-tyrosine. Tyrosinase poorly catalyze the hydroxylation of L-tyrosine to DOPA and after the oxidation to dopaminequinones. Anyway, this data did not influenced our results, being the ratio between tyrosinase units and dopamine is the same in all the concentration tested, and the increase in dopamine concentration led to a consequent increased in percentage of nuclear

fragmentation in a linear manner. Since we previously demonstrated that dopamine toxicity is mediated by the DAT activity, we asked the same question for DAQs . Cell were pre-treated with the DAT inhibitor and then treated with dopamine plus tyrosinase. We did not see any difference in the toxicity between DAT inhibitor treated and untreated experiments leading to the conclusion that DAQs are toxic species but their toxicity do not depends on a DAT mediated internalization. In this first part of experiments we confirmed that both dopamine and DAQs are toxic species since they induce cell death. To confirm that the death pathway activated by dopamine and DAQs was apoptosis, we tested our cells for another marker of apoptosis: the externalization of phosphatidil-serines (PS). During apoptosis there is the loss of membrane asymmetry and this process can be detected using Annexin-V-FLUOS, a Ca^{2+}-dependent phospholipid-binding protein with a high affinity for PS. From flow cytometry experiment we confirmed the previous data of nuclei fragmentation. Both dopamine and DAQs were toxic for cells, as demonstrated by the increasing percentage of cells positive for the annexin-v probe, and confirming the reliance on the internalization process mediated by the dopamine transporter only in the case of dopamine treatment. In the literature it has been proposed that the oxidation of dopamine contribute to the production of reactive oxygen species and mitochondrial dysfunction and oxidative stress have been long implicated in Parkinson's disease. To comprehend more deeply the toxicity mechanism induced by dopamine and its oxidation products, we estimate the production of mitochondrial superoxide anion (using a specific probe: MitosoxRed) in relation to exposure to these stressors. Data from time-lapse fluorescence microscopy and flow cytometry demonstrated that only the auto oxidation process of dopamine induces the production of this reactive oxygen species and, once more, this production depends on the internalization process of dopamine. DAQs did not induce the production of mitochondrial superoxide anion but in any case, activated apoptosis. This data suggested a difference in the induction of apoptosis between DA and Ty generated DAQs, and convinced us to investigate if there were different apoptotic pathways activated or just a different role of superoxide anion- in induced cell death a different oxidative dependence induction. Apoptosis is a very complex and tightly regulated process that involves several proteins and interaction, but there are mainly two activation

pathways that are responsible for the cell demise. One is the death-receptor mediated pathway while the other is the mitochondrial one. We performed western blot analysis testing our cells challenged with dopamine and DAQs for caspase 3 activation (represented by its internal cleavage) and consequent poly (ADP) ribose polymerase inactivation (proved by its cleavage). These two proteins can be recruited both in the death-receptor pathway and in the mitochondrial one, leading to cell death. Data from preliminary results on the cleavage of caspase showed a marked degree of caspase3 cleavage impling its activation after treatment of cells with DAQs. On the other hand, dopamine did not show suche activation. Other experiment are required to perform a statistical analysis. Since poly (ADP) ribose polymerase (PARP) is a target of caspase 3 activation, we evaluate the cleavage of PARP in consequence of dopamine and DAQs treatment. Beside the lack of the complete experiment, from preliminary western blot analysis, we found a high degree of PARP cleavage after DAQs treatment. Even in this case we have to complete the result to reach a robust statistical analysis. This preliminary data highlighted a marked difference in dopamine and DAQs mediated toxicity convincing us to proceed in the investigation on apoptosis process. We are now trying to update the analysis of apoptotic pathway to understand the toxicity mechanism induced by dopamine and DAQs following backward the apoptotic cascade to identify any differences in proteins and interactions activated by the two oxidation processes and to reveal at which level it is possible to block the progression of apoptotic cascade. This work could reveal common denominators that could highlights possible targets involved in the dopamine and DAQs induction of toxicity and for the design of new drugs aim at ameliorate and slow down the progression of this disease.

The second part of the project was focused on the role of superoxide dismutases 1 and 2 in protecting cells against dopamine and DAQs toxicity. These two enzymes play a crucial role and are referred as the first line of defense against reactive oxygen species, because they catalyze the dismutation of superoxide anion into molecular oxygen and hydrogen peroxide. Considering the crucial functions of these two enzymes and the importance of dopamine oxidation chemistry in cell death pathway, we designed a cellular model for the over expression of both superoxide dismutase 1 and 2. The full length cDNA of both

proteins was cloned in two vectors carrying the sequence of green fluorescence proteins (GFP) and red fluorescence protein (RFP) for SOD1 and SOD2 respectively. For the proper SOD1 folding, the protein was cloned at the C-terminus of the fluorescent tag. while the SOD2 sequence was cloned at the N-terminus of the fluorescent tag to preserve the mitochondrial targeting sequence. Both SOD1 and SOD2 were first tested for their correct localization inside cells. SOD1 shared mainly a cytosolic localization, while SOD2 mitochondrial localization was tested in a co-transfection experiment with another fluorescent protein (mito-YFP) targeted to the mitochondria. The co localization of the latter two proteins was assessed by confocal microscopy. Once demonstrated that our proteins were correctly localized inside cells, we evaluate their level of over expression in comparison with the endogenous ones. For SOD1 we reached about eleven fold of over expression with respect to the endogenous one, while for SOD2 it was about twofold. To test the effect of both SOD1 and SOD2 in dopamine induced cell death, we transfected cells both with empty vectors (as negative control) and the SODs vectors. We treated cells with increasing concentration of dopamine and the nuclear fragmentation was evaluated with fluorescence microscopy experiments. We previous demonstrated that dopamine causes an increase in mitochondrial superoxide anion, and we expected to find a protective role. Indeed, the experimental data demonstrated that over expression of both SOD1 and SOD2 had a rescue effect against dopamine induced cell death, lowering the production of superoxide anion. In the same way, we tested SODs effectiveness in protecting cells against DAQs induced toxicity. From this data we confirmed the superoxide anion-independent toxicity for DAQs, since the over expression both of SOD1 and SOD2 did not rescued cells. These data reported a different potential for superoxide dismutases against dopamine and DAQs toxicity confirming the reliance on superoxide anion production for the former but not in the case of latter. Further experiments are necessary to ameliorate the knowledge on dopamine toxicity and the differences of the auto oxidation and enzyme-mediated process. The cell model used has the characteristic features of a high DAT and a low VMAT2 activity that permits to easily obtain an increase in the cytosolic dopamine concentration providing a useful model to study the toxicity of exogenous compounds that are used for PD research (Alberio, Bossi et al 2010,

Alberio, Colapinto et al. 2010). The more prominent result of this research is the great importance of a proper storage or catabolism of dopamine since a slight increase in cytosolic dopamine led to the activation of apoptotic cascade and the role for dopamine transporter in mediating extracellular dopamine toxicity. Both auto oxidation and enzyme-mediated processes involved in the dopamine chemistry are toxic for cells and a more comprehensive knowledge on these processes could help to find new strategies to counteract the beginning and the progression of the pathology both detoxifying ROS produced by dopamine and DAQs, and blocking the apoptotic cascade activated by these two different processes.

5. References

Abou-Sleiman P.M., Healy D.G., Quinn N, Lees AJ, and Wood NW. (2003). The role of pathogenic DJ-1 mutations in Parkinson's disease. Annals of Neurology 54: 283-6.

Abou-Sleiman, P.M., Muqit M.M., and Wood, N.W. (2006). Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nature Reviews Neuroscience 7: 207-219.

Abreu IA, Cabelli DE. (2010). Superoxide dismutases-a review of the metal-associated mechanistic variations. Biochimica et Biophysica Acta 1804: 263-274.

Akundi R. S., Zhi L., and Bueler H. (2011). PINK1 enhances insulin-like growth factor-1-dependent Akt signaling and protection against apoptotis. Neurobiology of disease 45: 469-478.

Alberio T, Bossi AM, Milli A, Parma E, Gariboldi MB, Tosi G, Lopiano L, Fasano M. (2010). Proteomic analysis of dopamine and α-synuclein interplay in a cellular model of Parkinson's disease pathogenesis. FEBS Journal 277: 4909-4919.

Alberio T, Colapinto M, Natale M, Ravizza R, Gariboldi MB, Bucci EM, Lopiano L, Fasano M. (2010). Changes in the two-dimensional electrophoresis pattern of the Parkinson's disease related protein DJ-1 in human SH-SY5Y neuroblastoma cells after dopamine treatment. International Union of Biochemistry and Molecular Biology Life 62: 688-692.

Alexandre A, Lehninger AL. Bypasses of the antimycin a block of mitochondrial electron transport in relation to ubisemiquinone function. Biochim Biophys Acta. 1984;767:120–129.

Andersen J. K. (2004). Oxidative stress in neurodegeneration: cause or consequence? Nature Medicine 10 Suppl: S18-25.

Andersen P. H. (1989). The dopamine uptake inhibitor GBR 12909: selectivity and molecular mechanism of action. European journal of pharmacology, 166; 3, 493–504.

Anglade P., Vyas S., Javoy-Agid F., et al. (1997). Apoptosis and autophagy in nigra neurons of

patients with Parkinson's disease. Histol histophatol 12: 25-31
Asanuma M, Miyazaki I, Ogawa N. (2003). Dopamine- or L-DOPA-induced neurotoxicity: the role of dopamine quinone formation and tyrosinase in a model of Parkinson's disease. Neurotoxicity Research 5: 165-176

Auluck P.K., Caraveo G., and Lindquist S. (2010). Alpha-Synuclein: membrane interactions and toxicity in Parkinson's disease. Annual Review of Cell and Developmental Biology 26: 211- 233.

Banati RB, Daniel SE, Blunt SB. (1998). Glial pathology but absence of apoptotic nigral neurons in long-standing Parkinson's disease. Movement Disorders 13: 221-227.

Barker JE, Heales SJ, Cassidy A, Bolaños JP, Land JM, Clark JB. (1996). Depletion of brain glutathione results in a decrease of glutathione reductase activity; an enzyme susceptible to oxidative damage. Brain research 716: 118-22.
Beal M.F. (2007). Mitochondria and neurodegeneration. Novartis Foundation Symposia 287:183-92.

Beal, M.F. (2005) Mitochondria take center stage in aging and neurodegeneration. Ann. Neurol., 58, 495–505.

Bekris LM, Mata IF, Zabetian CP. (2010) The Genetics of Parkinson Disease. Journal of Geriatric Psychiatry and Neurology 23: 228–242.

Belluzzi E., Bisaglia M., Lazzarini E., Tabares L.C., Beltramini M., and Bubacco L. (2012). Human SOD2 modification by dopamine quinones affects enzymatic activity by promoting its aggregation: possible implications for Parkinson's disease. PLoS One 7:e38026.

Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, Jaros E, Hersheson JS, Betts J, Klopstock T, Taylor RW, Turnbull DM. (2006). High levels of mitochondrial DNA deletions in substantia nigra. Nature Genetics 38: 515-517.

Bender, A., Krishnan, K.J., Morris, C.M., Taylor, G.A., Reeve, A.K.,
Bisaglia M, Mammi S, Bubacco L. (2007). Kinetic and structural analysis of the early oxidation products of dopamine: analysis of the interactions with alpha-synuclein. The Journal of Biological

Chemistry 282: 15597-15605.

Bisaglia M, Tosatto L, Munari F, Tessari I, de Laureto PP, Mammi S, Bubacco L. (2010). Dopamine quinones interact with alpha-synuclein to form unstructured adducts. Biochemical and Biophysical Research Communications 394: 424-428.

Bonifati V., Rizzu P., Squitieri F., Krieger E., Vanacore N., van Swieten J.C., Brice A., van Duijn C.M., Oostra B., Meco G., and Heutink P. (2003). DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. Neurological Sciences 24: 159-60.

Braak H., Ghebremedhin E., Rub U., Bratzke H., and Del Tredici, K. (2004). Stages in the development of Parkinson's disease-related pathology. Cell and Tissue Research 318: 121-134.

Campo ML, Kinnally KW, Tedeschi H. The effect of antimycin A on mouse liver inner mitochondrial membrane channel activity. J Biol Chem. 1992;267:8123–8127
Cell. 1992 Aug 21;70(4):539-51.
A cDNA that suppresses MPP+ toxicity encodes a vesicular amine transporter.
Liu Y, Peter D, Roghani A, Schuldiner S, Privé GG, Eisenberg D, Brecha N, Edwards RH.

Cernaianu G., Brandmaier P., Scholz G., Ackermann O.P., Alt R., Rothe K., et al. (2008). All-trans retinoic acid arrests neuroblastoma cells in a dormant state. Subsequent nerve growth factor/brain derived neurotrophic factor treatment adds modest benefit. Journal of Pediatric Surgery 43: 1284-1294.

Chaudhuri K.R., Healy D.G., and Schapira A.H. (2006). Non-motor symptoms of Parkinson's disease: diagnosis and management. The Lancet Neurology 5: 235-245.

Cheung Y.T., Lau W.K., Yu M.S., Lai C.S., Yeung S.C., So K.F., and Chang R.C. (2009). Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology 30: 127–135.

Choi SW, Elmaleh DR, Hanson RN, Fischman AJ. (2000). Novel 3-aminomethyl- and 4-

aminopiperidine analogues of 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazines: synthesis and evaluation as dopamine transporter ligands. Journal of Medicinal Chemistry 43: 205-13.

Chung K., Dawson V. L. and Dawson T. M. (2001). The role of the ubiquitin-proteasomal pathway in Parkinson's disease and other neurodegenerative disorders. Trends in neurosciences 24: S7–S14.

Collinsa L.M., Toulouse A., Connor T.J., and Nolan Y.M. (2012). Contributions of central and systemic inflammation to the pathophysiology of Parkinson's disease. Neuropharmacology 62: 2154–2168.

Costa C., Bertazzo A., Allegri G., Toffano G., Curcuruto O. and Traldi P. (1992) Melanin biosynthesis from dopamine. II. A mass spectrometric and collisional spectroscopic investigation. Pigment Cell Research 5: 122-131.

Dale Purves G.J.A., Fitzpatrick D., Lawrence C Katz, Anthony-Samuel LaMantia, James O McNamara, and S Mark Williams. (2001). Neuroscience (Sinauer Associates).

Dauer W, Przedborski S. (2003). Parkinson's disease: mechanisms and models. Neuron 39: 889-909.

Dawson T.M., and Dawson V.L. (2003). Molecular pathways of neurodegeneration in Parkinson's disease. Science 302: 819-822.

DeMartino G.N., and Slaughter C.A. (1999). The proteasome, a novel protease regulated by multiple mechanisms. The Journal of Biological Chemistry 274: 22123-6.

DeMartino GN, Slaughter CA. (1999). The proteasome, a novel protease regulated by multiple mechanisms. The Journal of Biological Chemistry 274: 22123-22126.

Dexter DT, Sian J, Rose S, Hindmarsh JG, Mann VM, Cooper JM, Wells FR, Daniel SE, Lees AJ, Schapira AH, et al. (1994). Indices of oxidative stress and mitochondrial function in individuals with incidental Lewy body disease. Annals of Neurology 35: 38-44.

Di Fonzo A., Chien H.F, Socal M., Giraudo S., Tassorelli C., Iliceto G., Fabbrini G, Marconi R, Fincati E., Abbruzzese G, et al. (2007). ATP13A2 missense mutations in juvenile parkinsonism and young onset Parkinson disease. Neurology 68: 1557-1562.

Diedrich M, Kitada T, Nebrich G, Koppelstaetter A, Shen J, Zabel C, Klose J, Mao L. (2011). BBrain region specific mitophagy capacity could contribute to selective neuronal vulnerability in Parkinson's disease. Proteome science 9: 59.

Dipasquale B, Marini AM, Youle RJ. (1991). Apoptosis and DNA degradation induced by 1-methyl-4-phenylpyridinium in neurons. Biochemical and Biophysical Research Communications 181: 1442-1448.

Dringen R., and Hirrlinger J. (2003). Glutathione pathways in the brain. Biological Chemistry. 384: 505–516-

Dröge W. (2002). Free radicals in the physiological control of cell function. Physiological Reviews 82: 47–95.

Eisenhofer G, Kopin IJ, Goldstein DS. (2004). Catecholamine metabolism: a contemporary view with implications for physiology and medicine. Pharmacological Reviews 56: 331-349.

Elsworth JD, Roth RH. (1997). Dopamine synthesis, uptake, metabolism, and receptors: relevance to gene therapy of Parkinson's disease. Experimental neurology 144: 4-9.

Eric R. Kandel, J.H.S., Thomas M. Jessell (2000). Principles of Neural Science, Fourth Edition edn (The McGraw-Hill Companies, Inc).

Erickson JD, Eiden LE, Hoffman BJ. (1992). Expression cloning of a reserpine-sensitive vesicular monoamine transporter. Proceedings of the National Academy of Sciences USA 89: 10993-10997.

Exner N., Lutz A.K., Haass C., Winklhofer K.F. (2012). Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences. The EMBO Journal 31:

3038-62.

Fahn S. (2003). Description of Parkinson’s disease as a clinical syndrome. New York Academy of Sciences 991: 1–14.

Fan TJ, Han LH, Cong RS, Liang J. (2005). Caspase family proteases and apoptosis. Acta Biochimica et Biophysica Sinica 37: 719-727.

Fazeli G., Oli R.G., Schupp N., and Stopper H. (2011). The role of the dopamine transporter in dopamine-induced DNA damage. Brain Pathology 2: 237-48.

Fix James D. (2008). "Basal Ganglia and the Striatal Motor System". Neuroanatomy (Board Review Series) (4th ed.). Baltimore: Wulters Kluwer & Lippincott Wiliams & Wilkins. pp. 274–281

Floyd R.A. (1999). Antioxidants, oxidative stress, and degenerative neurological disorders. Proceedings of the Society for Experimental Biology and Medicine 222: 236–245.

Forno L.S. (1996). Neuropathology of Parkinson's disease. Journal of Neuropathology & Experimental Neurology 55: 259-72.

Fridovich I. (1995). Superoxide radical and superoxide dismutases. Annual review of biochemistry 64: 97-112.

Funayama M., Hasegawa K., Kowa H., Saito M., Tsuji S., Obata F. (2002). A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. Annals of Neurology 51: 296-301.

Gilgun-Sherki Y, Melamed E, Offen D. (2001). Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacology 40: 959-75.

Girotto S, Sturlese M, Bellanda M, Tessari I, Cappellini R, Bisaglia M, Bubacco L, Mammi S. (2012). Dopamine-derived quinones affect the structure of the redox sensor DJ-1 through modifications at Cys-106 and Cys-53. The Journal of Biological Chemistry 287: 18738-18749.

Gitler A.D., Chesi A., Geddie M.L., Strathearn K.E., Hamamichi S., Hill K.J., Caldwell K.A., Caldwell G.A., Cooper A.A., Rochet J.C., and Lindquist S. (2009). Alpha-synuclein is part of a diverse and highly conserved interaction network that includes PARK9 and manganese toxicity. Nature Genetics 41: 308-15.

Givan A.L. (2011). Flow cytometry: an introduction. Methods in Molecular Biology 699:1-29.

Glass C.K., Saijo K., Winner B., Marchetto M.C., and Gage F.H. (2010). Mechanisms underlying inflammation in neurodegeneration. Cell 140: 918-34.

Goedert M. (2001). Alpha-synuclein and neurodegenerative diseases. Nature Reviews Neuroscience 2: 492-501.

Graham D.G. (1978). Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. Molecular Pharmacology 14: 633-643.

Greenamyre JT, Sherer TB, Betarbet R, Panov AV. (2001). Complex I and Parkinson's disease. International Union of Biochemistry and Molecular Biology Life 52: 135-141.

Greggio E, Bergantino E, Carter D, Ahmad R, Costin GE, Hearing VJ, Clarimon J, Singleton A, Eerola J, Hellstr√∂m O, Tienari PJ, Miller DW, Beilina A, Bubacco L, and Cookson MR. (2005). Tyrosinase exacerbates dopamine toxicity but is not genetically associated with Parkinson's disease. Journal of neurochemistry 93: 246-256.

Grünewald A., Arns B., Seibler P., Rakovic A., Münchau A., Ramirez A., Sue C.M., and Klein C. (2012). ATP13A2 mutations impair mitochondrial function in fibroblasts from patients with Kufor-Rakeb syndrome. Neurobiology of Aging 33: 1843.e1-7.

Guarnieri S., Pilla R., Morabito C., Sacchetti S., Mancinelli R., Fanò G., et al. (2009). Extracellular guanosine and GTP promote expression of differentiation markers and induce S-phase cell-cycle arrest in human SH-SY5Y neuroblastoma cells. International Journal of Developmental Neuroscience 27: 135-147.

Gusdon A.M., Zhu J., Van Houten B., Chu C.T. (2012). ATP13A2 regulates mitochondrial bioenergetics through macroautophagy. Neurobiology of Disease 45: 962-72.

Gwinn-Hardy K. (2002). Genetics of parkinsonism. Movement Disorders 17: 645-656.

Harowitz M. P. and Greenamyre T. (2010). Gene-environment interactions in parkinson’s disease: the importance of animal modeling. Clinical pharmacology and therapeutics 88: 467-474.

Hartmann A, Hunot S, Michel PP, Muriel MP, Vyas S, Faucheux BA, Mouatt-Prigent A, Turmel H, Srinivasan A, Ruberg M, Evan GI, Agid Y, Hirsch EC. (2000). Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. Proceedings of the National Academy of Sciences USA 97: 2875-2880.

Henchcliffe C., and Beal M.F. (2008). Mitochondrial biology and oxidative stress in Parkinson disease pathogenesis.Nature Clinical Practice Neurology 4: 600-609.

Herceg Z, Wang ZQ. (2001). Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. Mutation Research 477: 97–110.

Hutter C.M., Samii A., Factor S.A., Nutt J.G., Higgins D.S., Bird T.D., Griffith A., Roberts J.W., Leis B.C., Montimurro J.S., et al. (2008). Lack of evidence for an association between UCHL1 S18Y and Parkinson's disease. European Journal of Neurology 15: 134-139.

Iglesias-González J., Sánchez-Iglesias S., Méndez-Álvarez E., Rose S., Hikima A., Jenner P., and Soto-Otero R. (2012). Differential Toxicity of 6-Hydroxydopamine in SH-SY5Y Human Neuroblastoma Cells and Rat Brain Mitochondria: Protective Role of Catalase and Superoxide Dismutase. Neurochemical Research 37: 2150–2160.

Jenner P., and Olanow C.W. (1996). Oxidative stress and the pathogenesis of Parkinson’s disease. Neurology 47: S161–S170.

Jiang Y., Pei., Li S., Wang M., and Liu F. (2008). Extracellular Dopamine Induces the Oxidative Toxicity of SH-SY5Y Cells. Synapse 62: 797–803.

Kawajiri S., Saiki S., Sato S., and Hattori N. (2011). Genetic mutations and functions of PINK1. Trends in pharmacolocial Sciences 32: 573-580.

Keeney P.M., Xie J., Capaldi R.A., and Bennett J.P. Jr. (2006). Parkinson's disease brain mitochondrial complex I has oxidatively damaged subunits and is functionally impaired and misassembled. The Journal of Neuroscience 26: 5256-64.

Khan N.L., Valente E.M., Bentivoglio A.R., et al. (2002). Clinical and subclinical dopaminergic dysfunction in PARK6-linked parkinsonism: an 18F-dopa PET study. Annals of Neurology 52: 849–853.

Kopin I.J. (1993). Neurotransmitters and disorders of the basal ganglia, in: G.J.Siegel, B.W. Agranoff, R.W. Albers, P.B. Molinoff (Eds.), BasicNeurochemistry, Raven Press, New York, , p. 903.

Kraytsberg Y., Kudryavtseva E., McKee A.C., Geula C., Kowall N.W., and Khrapko, K. (2006) Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nature Genetics 38: 518–520.

Krüger R., Kuhn W., Müller T., Woitalla D., Graeber M., Kösel S., Przuntek H., Epplen J.T., Schöls L., and Riess O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nature Genetics 18: 106-108.

Kume T., Kawato Y., Osakada F., Izumi Y., Katsuki H., Nakagawa T., et al. (2008). Dibutyryl cyclic AMP induces differentiation of human neuroblastoma SH-SY5Y cells into a noradrenergic phenotype. Neuroscience Letters 443: 199-203.

Langston J.W., Ballard P., Tetrud J.W., and Irwin I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219: 979-980.

Latourelle J.C., Sun M., Lew M.F., Suchowersky O., Klein C., Golbe L.I., Mark M.H., Growdon J.H., Wooten G.F., Watts R.L., et al. (2008). The Gly2019Ser mutation in LRRK2 is not fully penetrant in familial Parkinson's disease: the GenePD study. BMC Medicine 6: 32. doi: 10.1186/1741-7015-6-32.

LaVoie MJ, Hastings TG. (1999). Peroxynitrite- and nitrite-induced oxidation of dopamine: implications for nitric oxide in dopaminergic cell loss. Journal of Neurochemistry 73: 2546-2554.

LaVoie MJ, Ostaszewski BL, Weihofen A, Schlossmacher MG, Selkoe DJ. (2005). Dopamine covalently modifies and functionally inactivates parkin. Nature Medicine 11: 1214-1221.

Lees A.J. (2009). The Parkinson chimera. Neurology 72: S2-11.

Leroy E., Boyer R., Auburger G., Leube B., Ulm G., Mezey E., Harta G., Brownstein M.J., Jonnalagada S., Chernova T., et al. (1998). The ubiquitin pathway in Parkinson's disease. Nature 395: 451-452.

Li Y., Huang T.T., Carlson E.J., Melov S., Ursell P.C., et al. (1995). Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. Nature Genetics 11: 376–381.

Lin, M.T. and Beal, M.F. (2006) Mitochondrial dysfunction and
oxidative stress in neurodegenerative diseases. Nature, 443, 787–795.

Lincoln, S., Vaughan, J., Wood, N., Baker, M., Adamson, J., Gwinn-Hardy, K., Lynch, T., Hardy, J., and Farrer, M. (1999). Low frequency of pathogenic mutations in the ubiquitin carboxy-terminal hydrolase gene in familial Parkinson's disease. Neuroreport 10: 427-429.

Lotharius J., and Brundin P. (2002). Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. Nature Reviews Neuroscience 3: 932-942.

Maguire JJ, Kagan VE, Packer L. Electron transport between cytochrome c and alpha tocopherol. Biochem Biophys Res Commun. 1992;188:190–197.

Manáková S., Puttonen K.A., Raasmaja A., and Männistö P.T. (2004). The roles of dopamine transporter and Bcl-2 protein in the protection of CV1-P cells from 6-OHDA-induced toxicity. Toxicology Letters 154: 117–123.

Mann VM, Cooper JM, Daniel SE, Srai K, Jenner P, Marsden CD, Schapira AH. (1994). Complex I, iron, and ferritin in Parkinson's disease substantia nigra. Annals of Neurology 36: 876-881.

Margis R., Dunand C., Teixeira F.K., and Margis-Pinheiro M. (2008). Glutathione peroxidase family—an evolutionary overview. FEBS Journal 275: 3959–3970.

McGeer PL, Itagaki S, Boyes BE, McGeer EG. (1998). Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology 38: 1285-91.

McNaught K.S., Olanow C.W., Halliwell B., Isacson O, and Jenner P. (2001). Failure of the ubiquitin–proteasome system in Parkinson's disease. Nature Reviews Neuroscience 2: 589-594.
Miao L., and Clair D.K.S. (2009). Regulation of superoxide dismutase genes: implications in disease. Free Radical Biology and Medicine 47: 344–356.

Miranda M, Botti D. (1983). Harding-passey mouse-melanoma tyrosinase inactivation by reaction products and activation by L-epinephrine. General Pharmacology 14: 231-237.

Mitsumoto A., and Nakagawa Y. (2001). DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radical Research 35: 885-93.

Mitsumoto A., Nakagawa Y., Takeuchi A., Okawa K., Iwamatsu A., Takanezawa Y. (2001). Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radical Research 35: 301-310.

Mochizuki H., Goto K., Mori H., Mizuno Y., (1996). Histochemical detection of apoptosis in parkinson's disease. J. Neurol sci 137: 120-123.

Mollereau C., Zajac J.M., and Roumy M. (2007). Staurosporine differentiation of NPFF2 receptor-transfected SH-SY5Y neuroblastoma cells induces selectivity of NPFF activity towards opioid receptors. Peptides 28: 1125-1128.

Moore D. J., West A. B. Dawson V. L. and Dawson T. M. (2005). Molecular pathophysiology of Parkinson’s disease. Annual review of neuroscience 28: 57-87.

Mythri RB, Venkateshappa C, Harish G, Mahadevan A, Muthane UB, Yasha TC, Srinivas Bharath MM, Shankar SK. (2011). Evaluation of markers of oxidative stress, antioxidant function and astrocytic proliferation in the striatum and frontal cortex of Parkinson's disease brains. Neurochemical research 36: 1452-63.

Nagakubo D., Taira T., Kitaura H., Ikeda M., Tamai K., Iguchi-Ariga S.M, and Ariga H. (1997). DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. Biochemical and Biophysical Research Communications 231: 509-13.

Nakayama K, Okamoto F, Harada Y. Antimycin A: isolation from a new Streptomyces and activity against rice plant blast fungi. J Antibiot (Tokyo) 1956;9:63–66.

neurons in aging and Parkinson disease. Nat. Genet., 38, 515–517.

Nicklas W.J., Vyas I., and Heikkila R.E. (1985). Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life sciences 36: 2503-2508.

Pahlman S., Hoehner J.C., Nanberg E., Hedborg F., Fagerstrom S., Gestblom C., Johansson I., Larsson U., Lavenius E., Ortoft E., et al. (1995). Differentiation and survival influences of growth factors in human neuroblastoma, European Journal of Cancer 31A: 453–458.

Pahlman S., Mamaeva S., Meyerson G., Mattsson M.E., Bjelfman C., Ortoft E., Hammerling U. (1990). Human neuroblastoma cells in culture: a model for neuronal cell differentiation and function. Acta physiologica Scandinavica. Supplementum 592: 25–37.

Påhlman S., Odelstad L., Larsson E., Grotte G., Nilsson K. (1981). Phenotypic changes of human neuroblastoma cells in culture induced by 12-O-tetradecanoyl-phorbol-13-acetate. International Journal of Cancer 28: 583-589.

Panchision D. M., Chen H., Pistollato F., Papini D., Ni H., Hawley T. S. (2007). Optimized Flow Cytometric Analysis of Central Nervous System Tissue Reveals Novel Functional Relationships Among Cells Expressing CD133, CD15, and CD24. Sem cell 25: 1560–1570.

Park C.B., and Larsson N.G. (2011). Mitochondrial DNA mutations in disease and aging. The Journal of Cell Biology 193: 809–818.

Parkinson J. (1817). An essay on the shaking palsy, vol London: Neely & Jones (Sherwood).

Perry, R.H., Jaros, E., Hersheson, J.S., Betts, J., Klopstock, T. et al.
Pollanen M.S., Dickson D.W., Bergeron C. (1993). Pathology and biology of the Lewy body. Journal of Neuropathology & Experimental Neurology 52: 183-191.

Polymeropoulos M.H., Lavedan C., Leroy E., Ide S.E., Dehejia A., Dutra A., Pike B., Root H., Rubenstein J., Boyer R., et al. (1997). Mutation in the alpha synuclein gene identified in families with Parkinson's disease. Science 276: 2045- 2047.

Poon H.F., Calabrese V., Scapagnini G., and Butterfield D.A. (2004). Free radicals: key to brain aging and heme oxygenase as a cellular response to oxidative stress. The journals of gerontology. Series A. Biological sciences and medical sciences. 59: 478–493.

Poorkaj P. (2004). Parkin Mutation Analysis in Clinic Patients With Early-Onset Parkinson’s Disease. American Journal of Medical Genetics 129A: 44–50.

Ragland M., Hutter C., Zabetian C., and Edwards K. (2009). Association between the ubiquitin carboxyl-terminal esterase L1 gene (UCHL1) S18Y variant and Parkinson's Disease: a HuGE review and meta-analysis.American Journal of Epidemiology 170: 1344-1357.

Robinson, P.A. (2008). Protein stability and aggregation in Parkinson's disease. Biochem J 413, 1-13.

Ross O.A., Soto A.I., Vilarino-Guell C., Heckman M.G., Diehl N.N., Hulihan M.M., Aasly J.O., Sando S., Gibson J.M., Lynch T., et al. (2008). Genetic variation of Omi/HtrA2 and Parkinson's disease. Parkinsonism & Related Disorders 14: 539-543.

Rothman RB, Mele A, Reid AA, Akunne HC, Greig N, Thurkauf A, de Costa BR, Rice KC, Pert A. (1991). GBR12909 antagonizes the ability of cocaine to elevate extracellular levels of dopamine. Pharmacology Biochemistry and Behavior 40: 387-397.

Santiago M., Machado A., Cano J. (1996). Nigral and striatal comparative study of the neurotoxic action of 1-methyl-4-phenylpyridinium ion: involvement of dopamine uptake system, Journal of Neurochemistry 66: 1182–1190.

Santoro L., Breedveld G.J., Manganelli F., Iodice R., Pisciotta C., Nolano M., Punzo F., Quarantelli M., Pappatà S., Di Fonzo A., Oostra B.A., and Bonifati V. (2011). Novel ATP13A2 (PARK9) homozygous mutation in a family with marked phenotype variabilyty. Neurogenetics 12: 33–39.

Schapira A.H. (2008). Mitochondria in the aetiology and pathogenesis of Parkinson's disease. The Lancet Neurology 7: 97-109.

Schapira A.H. (2009). Neurobiology and treatment of Parkinson's disease. Trends in Pharmacological Sciences 30: 41-47.

Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. (1990). Mitochondrial complex I deficiency in Parkinson's disease. Journal of Neurochemistry 54: 823-827.

Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD. (1989). Mitochondrial complex I deficiency in Parkinson's disease. The Lancet 1: 1269.

Schober A. (2004). Classical toxin-induced animal models of Parkinson’s disease: 6-OHDA and MPTP. Cell tissue research 318: 215-224.

Schultheis P.J, Hagen T.T, O'Toole K.K., Tachibana A., Burke C.R., McGill D.L., Okunade G.W., Shull G.E. (2004). Characterization of the P5 subfamily of P-type transport ATPases in mice. Biochemical and Biophysical Research Communications 323: 731–738.

Schultheis P.J., Hagen T.T., O'Toole K.K., Tachibana A., Burke C.R., McGill D.L., Okunade G.W.,

and Shull GE. (2004). Characterization of the P5 subfamily of P-type transport ATPases in mice. Biochemical and Biophysical Research Communications 323: 731-8.

Seibler P, Graziotto J, Jeong H, Simunovic F, Klein C, Krainc D. (2011). Mitochondrial Parkin recruitment is impaired in neurons derived from mutant PINK1 induced pluripotent stem cells. The journal of neuroscience. 31: 5970-5976.

Shen J., and Cookson M.R. (2004). Mitochondria and dopamine: new insights into recessive parkinsonism. Neuron 43: 301-304.

Sherman M.Y., and Goldberg A.L. (2001). Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron 29:15-32.

Shimura H., Hattori N., Kubo S., Mizuno Y., Asakawa S., Minoshima S., Shimizu N., Iwai K., Chiba T., Tanaka K., et al. (2000). Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nature genetics 25: 302-305.

Shimura H., Schlossmacher M.G., Hattori N., Frosch M.P., Trockenbacher A., Schneider R., Mizuno Y., Kosik K.S., and Selkoe D.J. (2001). Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science 293: 263-269.

Shin, J.H., Dawson, V.L., and Dawson, T.M. (2009). SnapShot: pathogenesis of Parkinson's disease. Cell 139: 440 e441-442.

Shults C.W. (2006). Lewy bodies. Proceedings of the National Academy of Sciences USA 103: 1661-1668.

Singh J., and Kaur G. (2007). Transcriptional regulation of polysialylated neural cell adhesion molecule expression by NMDA receptor activation in retinoic acid-differentiated SH-SY5Y neuroblastoma cultures. Brain Research 1154: 8-21.

Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K. (2003). alpha-Synuclein

locus triplication causes Parkinson's disease. Science 302: 841

Smeyne R.J., and Jackson-Lewis V. (2005). The MPTP model of Parkinson's disease. Brain Research. Molecular Brain Research 134: 57-66.

Spillantini M.G., Schmidt M.L., Lee V.M., Trojanowski J.Q., Jakes R., and Goedert M. (1997). Alpha-synuclein in Lewy bodies. Nature 388: 839-840.

Stocco A., Lebiere C., and AndersonJ.R. (2010). "Conditional Routing of Information to the Cortex: A Model of the Basal Ganglia's Role in Cognitive Coordination". Psychological Review 117: 541–74.

Stokes A. H., Brown B. G., Lee C. K., Doolittle D. J. and Vrana K. E. (1996) Tyrosinase enhances the covalent modification of DNA by dopamine. Brain Research Molecular Brain Research 42: 167-170.

Strauss K.M., Martins L.M., Plun-Favreau H., Marx F.P., Kautzmann S., Berg D., Gasser T., Wszolek Z., Muller T., Bornemann A., et al. (2005). Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. Human Molecular Genetics 14: 2099-2111.

Sulzer D, Zecca L. (2000). Intraneuronal dopamine-quinone synthesis: a review. Neurotoxicity research 1: 181-195.

Swerdlow RH, Parks JK, Miller SW, Tuttle JB, Trimmer PA, Sheehan JP, Bennett JP Jr, Davis RE, Parker WD Jr. (1996). Origin and functional consequences of the complex I defect in Parkinson’s disease. Annals of Neurology 40: 663–671.

Tanner C. M., Ottman R., Goldman S. M., Ellenberg J., Chan P., Mayeux R., and Langston J.W. (1999). Parkinson disease in twins: An etiologic study . Journal of the American Medical Association 281: 341–346.

Tatton NA. (2000). Increased caspase 3 and Bax immunoreactivity accompany nuclear GAPDH

translocation and neuronal apoptosis in Parkinson's disease. Experimental Neurology 166: 29-43.

Tatton W. G., Chalmers-Redman R., Brown D., and Tatton N. (2003). Apoptosis in Parkinson’s disease: signal for neuronal degradation. Annals of Neurology 53: S61-S70 and S70-S72.

Thomas K. J. and Cookson M. R. (2009). The role of PTEN-induced kinase 1 in mitochondrial dysfunction and dynamics. International journal of biochemistry and cell biology 41: 2025-2035.

Tief K., Schmidt A. and Beermann F. (1998) New evidence for presence of tyrosinase in substantia nigra, forebrain and midbrain. Brain Research Molecular Brain Research 53: 307-310.

Tompkins MM, Basgall EJ, Zamrini E, Hill WD. (1997). Apoptotic-like changes in Lewy-body-associated disorders and normal aging in substantia nigral neurons. American Journal of Pathology 150: 119-131.

Tse DC, McCreery RL, Adams RN. (1976). Potential oxidative pathways of brain catecholamines. Journal of medicinal chemistry 19: 37-40.

Vallone D., Picetti R., and Borrelli E. (2000). Structure and function of dopamine receptors. Neuroscience & Biobehavioral Reviews 24: 125-132.

Van Laar V.S., Dukes A.A., Cascio M., and Hastings T.G. (2008). Proteomic analysis of rat brain mitochondria following exposure to dopamine quinone: implications for Parkinson disease. Neurobiology of Disease 29: 477–489.

Van Laar V.S., Mishizen A.J., Cascio M., and Hastings T.G. (2009). Proteomic identification of dopamine-conjugated proteins from isolated rat brain mitochondria and SH-SY5Y cells. Neurobiology of Disease 34: 487–500.

Vermesa I., Haanena C., and Reutelingspergerb C. (2000). Flow cytometry of apoptotic cell death. Journal of Immunological Methods 243: 167–190.

West A.R. , Floresco S.B. , Charara A., Rosenkranz J.A. (2003). Electrophysiological interaction between striatal glutamatergic and dopaminergic systems. Annals of the New York Academy of

Sciences 1003: 53–74.

Wüllner U, Kornhuber J, Weller M, Schulz JB, Löschmann PA, Riederer P, Klockgether T. (1999). Cell death and apoptosis regulating proteins in Parkinson's disease--a cautionary note. Acta Neuropathologica 97: 408-412.

Xie W., Li X., Li C., Zhu W., Jankovic J., and Le W. (2010). Proteasome inhibition modeling nigral neuron degeneration in Parkinson's disease. Journal of Neurochemistry 115: 188-99.

Xu Y., Stokes A. H., Freeman W. M., Kumer S. C., Vogt B. A. and Vrana K. E. (1997). Tyrosinase mRNA is expressed in human substantia nigra. Brain Research Molecular Brain Research 45: 159-162.

Xu Y., Stokes A. H., Roskoski R., Jr. and Vrana K. E. (1998) Dopamine, in the presence of tyrosinase, covalently modifies and inactivates tyrosine hydroxylase. Journal of Neuroscience Research 54: 691-697.

Zarranz J.J., Alegre J., Gomez-Esteban J.C., Lezcano E., Ros R., Ampuero I., Vidal L., Hoenicka J., Rodriguez O., Atares B., et al. (2004). The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Annals of Neurology 55: 164-173.

Zimprich A., Biskup S., Leitner P., Lichtner P., Farrer M., Lincoln S., Kachergus J., Hulihan M., Uitti R.J., Calne D.B., et al. (2004). Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron 44: 601-607.

Ziv I, Barzilai A, Offen D., Stein R., Achiron A., Melamed E. (1996). Dopamine-induced, genotoxic activation of programmed cell death: a role in nigrostriatal neuronal degeneration in Parkinson's dissease? Adv Neurol 69: 229-233