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# Alpha-Synuclein, Oxidative Stress and Autophagy Failure: Dangerous Liaisons in Dopaminergic Neurodegeneration

Giovanni Stefanoni<sup>1,2</sup>, Gessica Sala<sup>1</sup>, Lucio Tremolizzo<sup>1,2</sup>,  
Laura Brighina<sup>1,2</sup> and Carlo Ferrarese<sup>1,2</sup>

<sup>1</sup>*Department of Neuroscience and Biomedical Technologies, University of Milano-Bicocca*

<sup>2</sup>*Department of Neurology, San Gerardo Hospital, Monza.*

*Italy*

## 1. Introduction

The molecular mechanisms of neurodegeneration in Parkinson's disease and the cause of the selective dopaminergic neuronal loss are mostly unknown. Many pathogenetic factors have been found to play a role but the relationships among these factors, together with the reasons of the high vulnerability of dopaminergic neurons to them, have not been completely defined. Only a small fraction of Parkinson's disease cases have a defined etiology: this fraction include the monogenic hereditary variants of the disease and the sporadic cases determined by prolonged exposition to toxic agents inhibiting mitochondrial complex I, such as 1,1'-dimethyl-4,4'-5 bipyridinium (paraquat), rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Parkinson's disease-related toxins and pathogenetic mutations have been indispensable to create cell and animal models with the aim to clarify the molecular physiopathology of the disease. Little is known about the primitive causes of idiopathic Parkinson's disease, that probably represents a multi-factorial disease influenced by various genetic and environmental factors, all characterized by high incidence in general population. The different risk factors together would contribute to initiate the complex pathogenetic sequence of events leading to the death of dopaminergic neurons.

Recently, Parkinson's disease has been placed in the large category of neurodegenerative diseases caused by protein misfolding. In particular, alpha-synuclein has been proposed as the central and most specific factor implied in the pathogenesis of this syndrome, which, as a consequence, has been classified among synucleinopathies, together with dementia with Lewy bodies and multiple system atrophy, other neurodegenerative diseases having alpha-synuclein pathology as a major feature.

Aim of this chapter is to provide an organic revision of evidences for the involvement of alpha-synuclein in the pathogenesis of Parkinson's disease. We will define the mechanisms responsible for the toxic gain of function of  $\alpha$ -synuclein and the processes triggered by aberrant alpha-synuclein and mediating its neurotoxic effect. Particular attention will be paid to establish the links that correlate the deleterious action of alpha-synuclein with oxidative stress and with the efficiency of the processes involved in the clearance of aberrant

proteins; these last include the ubiquitin-proteasome system and the autophagic-lysosomal pathways. Both oxidative stress and impairment of protein degradation machinery exert a neurotoxic effect contributing to the pathogenesis of Parkinson's disease and partially mediated, in dopaminergic neurons, by qualitative and quantitative alterations in alpha-synuclein. This protein in turn is able to boost the processes responsible for its toxic gain of function. Therefore, a physiopathologic circuit emerges constituted by a complex interaction among different pathogenetic mechanisms, everyone able to support the others; the death of dopaminergic neurons represents the ultimate and irreversible outcome of all these events. This chapter aims at clarifying this complex dynamics responsible for the neurodegenerative process, with particular regard to mechanisms both determining and mediating the deleterious effect of alpha-synuclein. Furthermore, we will report data on the development of biochemical tests helpful for diagnosis and prognosis and useful as objective criteria to determine the neuroprotective effectiveness of drugs in clinical trials: in this regard, levels and modifications of  $\alpha$ -synuclein, as well as parameters of oxidative stress and autophagy, could represent suitable peripheral biomarkers of disease risk and progression. Finally, we will discuss about the opportunity of interfering with all the reported pathogenetic mechanisms as putative neuroprotective pharmacologic strategy.

## 2. Alpha-synuclein toxicity

Alpha-synuclein is a 144aa protein encoded by the gene SNCA, localized at 4q chromosome. Three different SNCA transcripts can be detected in neurons. The physiological functions of alpha-synuclein is still not fully understood. It is abundantly expressed in nervous tissues and it localizes in the cytoplasm or associated with lipid membranes. In particular, this protein is mainly localized in the pre-synaptic compartment, where it seems to have a role in regulating neurotransmitter release, vesicle turnover, membrane stability and neuronal plasticity.

The hypothesis of an active involvement of alpha-synuclein in the pathogenesis of Parkinson's disease was proposed when this protein was identified as the main component of Lewy bodies, intraneuronal aggregates constituting a constant report in the neuropathology of Parkinson's disease (Spillantini et al 1997). Beside alpha-synuclein, Lewy bodies also contain components of proteasome machinery, chaperone proteins, alpha-tubulin, synphilin-1, glyceraldehyde 3-phosphate dehydrogenase and several other proteins known to be involved in nigral neurodegeneration. Residues 71 to 82 of alpha-synuclein are essential for the assembly of Lewy bodies. The exact role of these inclusions in the disease physiopathology was unknown for several years.

The key function of alpha-synuclein protein in the pathogenesis of Parkinson's disease was first elucidated by genetic studies: the finding of familial cases of Parkinson's disease genetically linked to missense mutations in the SNCA gene and to genomic triplication of the wild type gene gave evidence of the potential neurotoxic effect of this protein. The first causal mutation of hereditary Parkinson's disease was identified in a large Italian family ("Contursi kindred", from the name of the town): it was the missense mutation Ala53Thr in the SNCA gene (Polymeropoulos et al 1997). Only two other pathogenetic missense mutations have been identified in SNCA: Ala30Pro and Glu46Lys. All these variants are located in the N-terminus of the protein and are linked to autosomal dominant forms of Parkinson's disease. Anyway, the missense mutation frequency in SNCA in different populations is very low, whereas SNCA multiplications are a bit more frequent. The

Ala53Thr alpha-synuclein mutant is responsible for the greater *in vivo* neurotoxicity in transgenic animal models.

The deleterious effects of these genetic alterations on dopaminergic neurons have been demonstrated in cellular and animal models, confirming the hypothesis that both qualitative and quantitative alterations of alpha-synuclein are able to trigger its toxic effect: mutant alpha-synuclein protein exerts a selective toxicity in dopaminergic neurons when expressed in rats; at the same time, the mutant protein causes aggregation and formation of Lewy bodies in dopaminergic neurons of mouse models.

The toxicity of both mutant and wild type alpha-synuclein seems to require the acquisition of a misfolded conformation which prevents alpha-synuclein degradation and favors its fibrillization, firstly into protofibrillar oligomeric species and then to fibrillar aggregates. Recently, it has become clear that insoluble aggregates probably do not have an intrinsic toxic function, as suggested by the finding that Lewy bodies-positive dopaminergic neurons are less vulnerable to degeneration. Furthermore, the increased size of Lewy bodies and the decreased levels of soluble alpha-synuclein correlate with a higher resistance to cytotoxic agents. Therefore, formation of Lewy bodies could represent a protective phenomenon favoring the removal of soluble oligomers. Different mechanisms can explain the negative effects exerted by alpha-synuclein oligomers, which seem to be directly responsible for the neurotoxic effect of the protein (Vekrellis et al 2004):

- Binding to microtubules and dysregulation of cytoskeleton functions.
- Damaging of mitochondrial and other cellular membranes.
- Interaction with other proteins and modulation of their activity.

Alpha-synuclein oligomers directly interfere with proteasome subunits. Furthermore, alpha-synuclein impairs microtubule stability through binding to alpha-tubulin and to the microtubule associated protein TAU. Alpha synuclein induces the aggregation of TAU, which in turn favors alpha-synuclein fibrillization; interestingly, polymorphisms in the TAU gene have proved to modify the risk of developing Parkinson's disease.

In monogenic forms of Parkinson's disease the direct neurotoxic effect of mutant alpha-synuclein may depend on specific conformational modifications, modulating the propensity of the protein to aggregate, to interact with other proteins and to be processed by clearance mechanisms. Pathogenetic mutations of alpha-synuclein protein are known to confer a tendency to acquire abnormal conformation substantially easier than the wild type protein, which accounts for the development of Parkinson's disease in patients having these mutations.

The fact that over-expressed wild type alpha-synuclein is responsible for neurotoxicity suggests that even simple protein accumulation is able to trigger the misfolded conformation and to promote protein aggregation.

As well as in familial cases, even in sporadic Parkinson's disease the toxic gain of function of alpha-synuclein could derive from the intraneuronal accumulation of the protein or from biochemical modifications enhancing the propensity of the protein to aggregate. Anyway, the ubiquitous expression and the high levels of alpha-synuclein in the brain suggests that its simple presence in dopaminergic neurons is not sufficient to explain the selective degeneration of these cells, which must have specific characteristics able to induce or to accelerate alpha-synuclein misfolding, oligomerization and aggregation. Oxidative stress and production of highly reactive aldehydes, both depending on high levels of dopamine, can modify alpha-synuclein, partially explaining the high tendency of this protein to acquire

a misfolded conformation in dopaminergic neurons. Indeed, dopamine promotes alpha-synuclein protofibrils formation. Oxidative stress, which is known to be one of the main pathogenetic factors leading to the death of dopaminergic neurons in Parkinson's disease, has been identified as a major responsible for alpha-synuclein post-translational modifications, consisting in oxidation and nitration of specific aminoacid residues; these alterations in alpha-synuclein have been detected in nigral neurons of brain samples from patients with Parkinson's disease (Giasson et al 2000) and from animal models of disease (Gao et al 2008).

An increase in alpha-synuclein levels, correlated with the degree of nigrostriatal dopamine depletion, has been demonstrated in the substantia nigra of patients with sporadic Parkinson's disease (Chu et al 2007). It is conceivable to suppose that this accumulation of the toxic protein might derive from an increase in transcription and translation of the SNCA gene or from a decrease in the protein degradation.

The variability in codifying and non-codifying SNCA gene sequences has demonstrated to modify the risk of developing sporadic Parkinson's disease, probably through a mild modulatory effect on transcription activity and, as a consequence, on intraneuronal levels of alpha-synuclein. SNCA duplication was found in sporadic cases of Parkinson's disease. Increasing length in the dinucleotide repeat sequence (REP1) of the SNCA gene promoter has been demonstrated to be correlated with an increased risk of developing sporadic Parkinson's disease (Maraganore et al 2006; Brighina et al 2008). Furthermore, single nucleotide polymorphisms at the promoter region and at the 3' end of SNCA gene have been found to modify the risk of disease.

Anyway, aging remains the most evident risk factor for sporadic Parkinson's disease. An interesting hypothesis suggests that senescence of dopaminergic neurons could promote the accumulation of misfolded alpha-synuclein through two main mechanisms: slowing alpha-synuclein turnover through the impairment of clearance machinery; impairing the function of mitochondria and antioxidant systems, with consequent increase in oxidative stress.

Great efforts have been done in order to identify genetic and environmental factors able to modulate the aging process. Caloric restriction represents the only environmental condition which has demonstrated to delay aging in a wide range of organisms. This evidence suggests that caloric restriction might guarantee protection from developing Parkinson's disease and could delay the disease progression. As it was supposed, caloric restriction has shown a protective effect in MPTP-induced animal models of Parkinson's disease, even if an epidemiologic correlation between diet and incidence of Parkinson's disease has not been demonstrated. The molecular basis of the protective role of caloric restriction in animal models has been elucidated in yeast, where a family of NAD<sup>+</sup>-dependent protein deacetylases, called sirtuins (SIRTs), have been identified as mediators of the changes induced by starvation in cells; many sirtuins analogs have been discovered in mammals. These proteins regulate cell homeostasis partially through an epigenetic mechanism: they modulate histones acetylation and chromatin condensation. SIRT1 is a major responsible for protective cell modifications during starvation, whereas other SIRTs seem to exert a specific deleterious effect on dopaminergic neurons. In particular, SIRT2, an alpha-tubulin deacetylase, promotes formation of alpha-tubulin oligomers, which destabilize microtubules and form toxic complexes with alpha-synuclein oligomers. The interaction of alpha-synuclein oligomers with alpha-tubulin plays an important role in mediating alpha-

synuclein toxicity. The NAD<sup>+</sup> concentration-dependency of SIRT2 activity suggests a possible modulation of alpha-synuclein misfolding and aggregation by energy metabolism.

### **3. Oxidative stress, mitochondrial dysfunction and excitotoxicity**

An impairment in multiple steps of the mitochondrial respiratory chain has been demonstrated in Parkinson's disease (Schapira et al 1998). The importance of this pathogenetic mechanism mainly depends on its role of main source of reactive oxygen species. At the same time, the loss of mitochondrial function and the consequent energetic deficit interfere with glutamate uptake and increase neuronal vulnerability to glutamate excitotoxicity.

#### **3.1 Mitochondrial dysfunction**

Several epidemiologic and experimental data confirm the importance of mitochondrial derangement in the pathogenesis of Parkinson's disease. First of all, electron transport complex I represents the main molecular target of paraquat, rotenone and MPTP, the exogenous substances known to determine degeneration of human dopaminergic neurons. The selective toxicity of MPTP on dopaminergic neurons derives from its transformation to the metabolite 1-methyl-4-phenyl-pyridium (MPP<sup>+</sup>), which is concentrated within dopaminergic neurons by dopamine transporters. Even paraquat, which is structurally similar to MPP<sup>+</sup>, is carried into dopaminergic cells by the same transporter. In contrast to MPTP and paraquat, rotenone is not concentrated in nigral neurons, nevertheless it produces selective death of these cells. The development of parkinsonism after exposure to rotenone represents a further proof of the high susceptibility of dopaminergic neurons to mitochondrial impairment. Toxic derangement in complex I induces alpha-synuclein aggregation and formation of Lewy body-like inclusions (Betarbet et al 2000).

A reduced activity of the mitochondrial complexes I and IV has been observed in the substantia nigra of patients with Parkinson's disease and in animal models of disease (Palacino et al 2004). Decreased levels of coenzyme Q10 together with a higher ratio of oxidized vs. reduced Coenzyme Q10 have been observed in patients. An increase of cerebral lactate levels, suggesting an alteration in aerobic metabolism as a consequence of derangement of mitochondrial respiratory chain, has been detected by brain magnetic resonance spectroscopy in patients with Parkinson's disease (Henchcliffe et al 2008). Experiments performed on cytoplasmic hybrid cell lines from patients with Parkinson's disease suggest that the complex I deficit associated to the disease might be genetically determined by defects in mitochondrial DNA. A single nucleotide polymorphism within the gene encoding NADH dehydrogenase 3 of complex I has been associated to a decrease in the risk of sporadic Parkinson's disease, providing genetic evidence of the pathogenetic relevance of complex I activity (van der Walt et al 2003).

An endogenous substance able to impair mitochondrial function is salsolinol, which is synthesized from dopamine and acetaldehyde by salsolinol synthase. This molecule has been found in various regions of the brain, including striatum and substantia nigra. Salsolinol inhibits mitochondrial complex II and this effect may explain the fact that high levels of this molecule are able to induce degeneration of dopaminergic neurons (Storch et al 2000).

Alpha-synuclein contains a mitochondrial targeting sequence. Over-expression of this protein favors its translocation to the mitochondria. The damage of these organelles seems to be one of the mechanisms mediating the toxic effects of alpha-synuclein oligomers.

An additional confirmation of the importance of mitochondrial function for the homeostasis of dopaminergic neurons is the finding that some proteins linked to hereditary forms of Parkinson's disease exert their function within mitochondria; furthermore, pathogenetic mutations of the genes encoding for some of these proteins are associated with the impairment of mitochondrial function, which might be the main neurotoxic action exerted by these mutations:

- PTEN induced putative kinase 1 (PINK1/PARK6) is a kinase that localizes in the mitochondrial membrane. Its loss of function is associated with a decrease in complex I activity determining an increase in oxidative stress. Mutations of PINK1 gene are responsible for recessive forms of Parkinson's disease.
- DJ-1 (PARK7) is a protein found to be mutated in recessive forms of Parkinson's disease. It plays a role in cell protection during oxidative stress, which induces the translocation of the protein to the mitochondrial matrix and the intermembrane space. The loss of DJ1 has been associated to depolarization and fragmentation of mitochondria.
- Leucine-rich repeat kinase 2 (LRRK2/PARK8) is a kinase linked to dominant forms of Parkinson's disease. It is mainly localized in the cytosol but a fraction can be found in the outer mitochondrial membrane. Pathogenetic mutations determine an increase in the kinase activity of the protein.
- High temperature requirement proteinA2 (HTRA2/Omi/PARK13) is a mitochondrial protease which has been associated with recessive forms of Parkinson's disease. Its loss of function has revealed to produce mitochondrial damage.

### 3.2 Oxidative stress

Free radicals include reactive oxygen species and reactive nitrogen species. During cell metabolism some of these substances are normally produced: superoxide anion, hydrogen peroxide, nitric oxide, peroxynitrite, nitroxyl and hydroxy radical. These products can be responsible for the damage of protein, DNA and lipid.

The brain contains a high amount of substances, such as phospholipids and free fatty acids, which are vulnerable to oxidative modifications and, as a consequence, are responsible for the high vulnerability of the central nervous system to the deleterious action of free radicals. Lipid peroxidation represents a key mechanism mediating the toxicity of free radicals on a wide range of cell organelles and functions. Free radicals initiate peroxidation of the membrane lipids making them lose a hydrogen atom from a methylene group, with formation of a diene; this product mediates the formation of a peroxy radical through reaction with oxygen; the peroxy radical abstracts a hydrogen atom from another lipid to form hydroperoxides, which mediates the propagation of lipid peroxidation.

In physiologic conditions free radicals are rapidly converted into non toxic molecules by antioxidants, which prevent oxidation of other molecules, thus protecting cell from oxidative stress. The cell antioxidant system include four main molecules:

- Catalase is a heme-protein localized in peroxisomes, which catalyzes the decomposition of hydrogen peroxide to water and oxygen.

- Superoxide dismutase is a cytosolic, mitochondrial and extracellular metallo-protein which catalyzes the transformation of superoxide into  $O_2$  and  $H_2O_2$ .
- Reduced glutathione is a tripeptide which exerts an important reducing activity of reactive oxygen species through its conversion to glutathione disulfide.
- Glutathione peroxidase is an enzyme which catalyzes the reduction of  $H_2O_2$  to water and  $O_2$  by reduced glutathione.

A large amount of studies have demonstrated that oxidative damage plays a major pathogenetic role in Parkinson's disease and represents a key contributor to the loss of dopaminergic neurons (Jenner & Olanow 1998). The high concentration of dopamine is presumed to be essential to determine the high vulnerability of dopaminergic cells to oxidative stress. Dopamine itself does not seem to exert direct toxic effects at physiologic concentrations, but toxic intermediates derive from its catabolism:

- Auto-oxidation of dopamine leads to the production of semiquinones, which have an intrinsic toxic effect and generate reactive oxygen species.
- The enzymatic metabolism of dopamine, mediated by monoamine oxidase B, leads to the generation of  $H_2O_2$ ; therefore, the inhibition of monoamine oxidase B by selective drugs may protect against production of some toxins and free radicals deriving from dopamine oxidation.

The experimental stimulation of dopamine catabolism by monoamine oxidase B leads to an increase in oxidative stress levels, thus confirming the deleterious role of dopamine derivatives on neuronal oxidoreductive equilibrium.

Extensive studies performed on postmortem brain samples have provided evidence supporting the involvement of oxidative stress in the pathogenesis of Parkinson's disease. Elevated levels of reactive oxygen species have been detected by assessment of lipid peroxidation, protein oxidation and DNA damage in nigrostriatal regions of patients with Parkinson's disease and in animal models:

- Malondialdehyde, 4-hydroxy-2,3-nonenal and thiobarbituric acid reactive substances (TBARS) are markers of lipid peroxidation which are elevated in the substantia nigra and striatum of diseased patients (Yoritaka et al 1996).
- Markers of oxidative damage to proteins, such as carbonyl modifications of aminoacid residues, are increased in substantia nigra of patients with Parkinson's disease (Alam et al 1997).
- An increase in oxidative alterations of nucleic acids was detected in midbrain of diseased patients (Zhang et al 1999).

On the other hand, a dysregulation of multiple antioxidant systems has been demonstrated in Parkinson's disease:

- An increase in superoxide dismutase and catalase levels has been detected in striatum and midbrain of animal models (Keeney et al 2006). This feature probably represents a compensatory reaction of cell control systems, aimed at neutralizing the increasing levels of reactive oxygen species.
- A decrease of reduced glutathione levels was detected in the substantia nigra and corpus striatum of patients with Parkinson's disease and in animal models (Pearce et al 1997). Glutathione deficiency could be a consequence of the high levels of free radicals, which rapidly oxidize glutathione to glutathione disulfide, or, alternatively, it might represent an early pathogenetic feature which contributes to the high vulnerability of dopaminergic neurons to oxidative stress.

The iron-catalyzed conversion of  $H_2O_2$  into hydroxyl radicals is supposed to contribute to oxidative stress and neurodegeneration in Parkinson's disease; this remark is based on the finding of increased iron content in postmortem brain samples from diseased patients.

Various proteins linked to recessive hereditary forms of Parkinson's disease play an important role in cell response to oxidative stress. In particular, an increase in protein and lipid peroxidation has been demonstrated in parkin defective animal models, suggesting that a defect in the ubiquitin-proteasome pathway or in formation of Lewy bodies can favor the generation of free radicals.

The mechanisms mediating the neurotoxic effects of oxidative species in dopaminergic neurons are complex and only partially understood, but a central role seems to be played by the alterations that free radicals produce on alpha-synuclein conformational status and degradation. Indeed, it has been demonstrated that oxidative stress induces intraneuronal accumulation and aggregation of alpha-synuclein and formation of Lewy bodies-like inclusions. This effect might be produced through a double mechanism:

- Reactive oxygen species directly generate post-translational modifications in alpha-synuclein residues, enhancing the tendency of the protein to aggregate.
- High levels of free radicals determine a down-regulation of both the ubiquitin-proteasome system and the autophagy-lysosomal pathway, thus impairing the entire degradative machinery of alpha-synuclein.

The deleterious effect of oxidative stress on the function of clearance systems might partially depend on direct alteration of proteins regulating and mediating these pathways; at the same time, oxidative stress produces ATP depletion through damage of mitochondrial membranes, thus interfering with all energy-dependent processes, such as the clearance of proteins and organelles.

### 3.3 Excitotoxicity

Several lines of experimental evidence indicate that a toxic effect exerted by high levels of glutamate in the synaptic cleft contributes to the neurodegeneration of dopaminergic neurons in Parkinson's disease. Excitotoxicity is a pathogenetic mechanism that has demonstrated a role in the neuronal death in different neurologic diseases, both acute and chronic (Ferrarese & Beal 2004).

The molecular processes that mediate glutamate effects on neuronal survival consist on a complex cascade of events that ultimately results in neuronal death. Overactivation of the N-methyl-D-aspartate (NMDA) receptors seem to mediate a great part of this neurotoxic effect through generation of a calcium overload. Indeed, NMDA receptors are permeable to calcium ions, so that their activation results in an increase in the cytoplasmatic concentration of this element; high intracellular levels of calcium are responsible for the activation of several pro-apoptotic pathways and for induction of oxidative stress through activation of oxygenases and perturbation of mitochondrial homeostasis.

Oxidative and nitrosative stress represent important processes both favoring and mediating excitotoxic damage. An increase in the production of free radicals has been demonstrated in neurons exposed to excitotoxic insults. Nitric oxide is produced in response to NMDA receptors activation thanks to the interaction of nitric oxide synthase with these receptors; nitric oxide contributes to the generation of free radicals and is involved in the activation of the pro-apoptotic cascade. These data suggest that oxidative stress gives an important contribution to the excitotoxic process downstream of glutamate receptor activation.

Furthermore, reactive oxygen species have been identified as agents able to induce the increased release of glutamate during pathologic conditions. In fact, lipid peroxidation of presynaptic membrane seems to impair the function of transporters involved in the maintenance of calcium homeostasis, resulting in sustained elevation of this ion in the presynaptic terminal, which is depolarized and so releases glutamate into the synaptic cleft; moreover, oxidative stress directly impairs glutamate transporter function in astrocytes and neurons, leading to an increase in concentrations of extracellular glutamate, which can bind postsynaptic receptors and mediate excitotoxicity.

Glutamate transport is an energy-dependent process, which explains, together with oxidative stress, the fact that mitochondrial dysfunction determine a decrease in the reuptake of glutamate and, as a consequence, an increase in neuron vulnerability to excitotoxicity.

Based on this strong relationship among oxidative stress, mitochondrial dysfunction and excitotoxicity, it is easy to imagine an involvement of glutamate toxicity in the pathogenesis of Parkinson's disease, where free radicals and mitochondrial impairment are known to play a major role. In animal models of disease modifications in the abundance and phosphorylation status of the different NMDA receptor subunits have been detected (Dunah et al 2000). Anyway, the importance of excitotoxicity in the degeneration of nigral dopaminergic neurons might be increased by the complex set of changes that nigrostriatal dopamine depletion triggers in functional anatomy of basal ganglia circuitry. In particular, the reduction of the nigrostriatal dopaminergic transmission produces a strong increase in the glutamatergic activity of the subthalamic projection, normally inhibited by nigral neurons. This hyperactivity critically contributes to the onset of parkinsonian motor symptoms, as confirmed by the evidence that electric suppression of subthalamic nucleus through deep brain stimulation improves motor functions. Furthermore, these functional alterations in basal ganglia circuitry might have pathogenetic implications: overactivation of subthalamic glutamatergic transmission to substantia nigra could promote the excitotoxic damage of dopaminergic neurons, favoring neuronal death and accelerating disease progression; this concept has been confirmed by the beneficial effects obtained by blockade of subthalamic activity in animal models of Parkinson's disease (Blandini et al 2001).

Therefore, excitotoxicity appears to be an important contributor to neuronal death in the substantia nigra of patients with Parkinson's disease; this mechanism correlates with oxidative stress and is amplified by subthalamic overactivation.

#### **4. Role of clearance mechanisms**

Emerging data support the view that dysregulation of alpha-synuclein clearance machinery might represent the key feature leading to the intraneuronal accumulation of this protein. These mechanisms include the ubiquitin-proteasome system and the autophagic-lysosomal pathways; the autophagic pathways involved in the degradation of alpha-synuclein are chaperone-mediated autophagy and macroautophagy (Webb et al 2000). Anyway, the accumulation of alpha-synuclein does not represent the only negative consequence of clearance systems failure.

##### **4.1 Impairment of the ubiquitin-proteasomal system**

Increasing evidences indicate that the ubiquitin-proteasome pathway is defective in Parkinson's disease. The finding that Lewy bodies are ubiquitin-positive aggregates has

suggested that a dysfunction in proteasome might contribute to the accumulation and aggregation of alpha-synuclein and other neurotoxic proteins. The first confirmation of this hypothesis has derived from the identification of hereditary forms of Parkinson's disease linked to two genes within the ubiquitin-proteasome system: parkin and UCHL1.

Parkin (PARK2) is an ubiquitin E3 ligase, an enzyme that catalyzes the addition of ubiquitin chains to substrate proteins which must be degraded by the proteasome. Mutations in parkin gene are responsible for almost half of autosomal recessive cases of hereditary Parkinson's disease (Kitada et al 1998); most of the pathogenetic mutations are associated with a defect in the E3 ubiquitin ligase activity of the protein. Furthermore, parkin is responsible for K63 ubiquitination of alpha-synuclein; this function is not involved in the degradation of substrate proteins through proteasome but favors protein aggregation and generation of insoluble inclusions. Mutations in the parkin gene prevent the formation of Lewy bodies in the substantia nigra, thus suggesting that the K63 ubiquitin ligase function of parkin is essential for the fibrillization of alpha-synuclein and formation of insoluble inclusions; the degeneration of dopaminergic neurons deriving from parkin loss of function supports the hypothesis that Lewy bodies might contrast the toxic effects of the protofibrillar protein.

Ubiquitin C-terminal hydrolase L1 (UCHL1/PARK5) is an enzyme that cleaves peptide-ubiquitin bonds and recycles ubiquitin monomers. Variants of UCHL1 gene have been associated to familial forms of Parkinson's disease. The I93M mutation was identified in a family with probable autosomal dominant Parkinson's disease; *in vitro* studies have demonstrated that this mutation results in partial loss of UCHL1 hydrolytic activity. In addition to the hydrolase activity, UCHL1 exerts a dimerization-dependent ubiquitin ligase activity that promotes alpha-synuclein aggregation. The single nucleotide polymorphism S18Y, which has been found to decrease the susceptibility to sporadic disease, seems to be associated with reduced ligase activity.

A direct proof of proteasome dysfunction in Parkinson's disease has been the finding of structural and functional alterations in the 20S proteasome subunit in the substantia nigra of patients with sporadic disease. The pathogenetic relevance of proteasome impairment has been reinforced by the observation that administration of proteasomal inhibitors to animals can produce the neuropathological and motor manifestations of Parkinson's disease, including selective nigral cell loss, Lewy-bodies-like inclusions and typical clinical signs.

Two mechanisms are known to be responsible for a dysfunction in the ubiquitin-proteasome system: aging is associated with a physiologic decrease of proteasome efficiency and ubiquitination activity; oxidative species and alpha-synuclein protofibrils exert a deleterious effects on proteasome subunits.

Alpha-synuclein oligomers have demonstrated to inhibit proteasome function through direct interaction with 20S subunit; this effect might favor further accumulation of alpha-synuclein, which in turn may worsen proteasome impairment.

The mechanisms behind proteasome inhibition by oligomeric alpha-synuclein require further investigation in order to be clarified. Synphilin-1 has been associated to the pathogenesis of Parkinson's disease since its identification as an alpha-synuclein-interacting protein, a component of Lewy bodies and a substrate of the E3 ligase parkin. This protein has been found to interact with the protein S6 ATPase, which exerts a regulatory function on proteasome. Furthermore, recent studies indicate that synphilin-1 exerts an inhibitory effect on proteasome activity partially due to the interaction with alpha-synuclein (Alvarez-Castelao et al 2010); mutations in synphilin-1 gene have been detected in patients with sporadic Parkinson's disease.

Chronic oxidative stress alters the subunits of proteasome, so that even reactive oxygen species might be responsible for proteasome impairment in Parkinson's disease.

## 4.2 Autophagy dysfunction

Autophagy is a finely regulated intracellular process that mediates lysosomal degradation of proteins and organelles. Its function allows the clearance of substrates characterized by alterations limiting their physiologic function or responsible for a cytotoxic effect. This degradative process exerts a cytoprotective role that is probably dependent on the clearance of toxic intracellular structures and the catabolism of substrates in order to obtain energy during starvation. Anyway, in particular situations autophagy seems to mediate a specific pathway of programmed cell death; this function requires a strong activation of autophagy and until now, *in vivo*, has been identified only during involutinal physiologic processes in embryonic tissues (Nixon et al 2006).

In mammalian cells autophagy encompasses three main processes: microautophagy, macroautophagy and chaperone-mediated autophagy. Microautophagy is a constitutive, non selective process consisting on endocytosis of small amounts of cytoplasm into lysosomes through invagination of lysosomal membrane. Macroautophagy and chaperone-mediated autophagy are inducible processes: the first one allows lysosomal degradation of organelles and proteins after their sequestration within a double-membrane-limited vacuole called autophagosome; the second one is a selective device for degradation of aberrant proteins, which are directly transported into the lysosomal lumen by a translocation system constituted by specific carrier proteins.

Recent research has revealed the existence of a dysfunction in autophagy pathways in the cerebral regions involved in neurodegenerative processes. A decrease in the activity of both autophagic-lysosomal pathway and the ubiquitin-proteasome system has been reported during aging in every tissue, included neurons (Martinez-Vicente et al 2005). This deficit might be a major responsible for the intracellular accumulation of misfolding proteins and aberrant mitochondria, representing factors leading to oxidative stress and neuronal damage. Therefore, autophagy impairment might be a *primum movens* of the neurodegenerative processes and, at the same time, a limiting factor that is necessary for preventing early onset of massive neuronal loss.

In the last years the pathogenetic role of altered clearance machinery has become a central subject of research in Parkinson's disease: indeed, the impairment of chaperone-mediated autophagy and macroautophagy, together with the dysfunction of ubiquitin-proteasome system, seems to give a major contribute to the development and progression of nigral degeneration. The loss of function of these degradative pathways might produce a deleterious effect through induction of the pathogenetic processes more directly responsible for neuronal death, such as the accumulation and oligomerization of alpha-synuclein, the persistence of damaged mitochondria and the consequent production of reactive oxygen species. In turn, these key mediators of dopaminergic neurodegeneration can further worsen the impairment of clearance machinery and, in particular, of autophagic pathways (Cuervo et al 2010).

### 4.2.1 Impairment of chaperone-mediated autophagy

Chaperone mediated autophagy mediates the translocation of proteins containing a Lys-Phe-Glu-Arg-Gln (KFERQ) motif into lysosomes. This process requires the presence of three

main proteins: cytosolic heat shock protein 70 (hsc70), lysosomal hsc70, lysosomal-associated membrane protein 2A (lamp2A). Cytosolic hsc70 binds the KFERQ sequence of substrate proteins and carries them to the lysosomal membrane, where lamp2A, after interaction with cytosolic hsc70, multimerizes and forms a translocation complex with lysosomal hsc70, thus mediating the transport of the substrate protein into the lysosomal lumen. Several proteins with neuropathologic relevance, such as alpha-synuclein, amyloid precursor protein  $\beta$  and huntingtin, contain a KFERQ motif.

The binding of the substrate protein to lamp2A represents the limiting step of chaperone mediated autophagy. Oxidative stress, accumulation of substrates and the lack of nutrients and growth factors are all conditions determining a compensatory and cytoprotective activation of chaperone mediated autophagy through an increase of lamp2A levels on lysosomal membrane.

The hypothesis that a dysfunction of chaperone mediated autophagy might be involved in the pathogenesis of Parkinson's disease was proposed when this process was demonstrated to be the main degradative pathway for alpha-synuclein; indeed, this protein accumulates when chaperone-mediated autophagy is suppressed by down-regulation of lamp2A expression. Therefore, the efficiency of this clearance pathway appears to be crucial in regulating the intraneuronal levels of alpha-synuclein. The evidence of a strong correlation between the functional state of chaperone mediated autophagy and the deleterious action of alpha-synuclein has been reinforced by demonstration that both pathogenetic mutations and overexpression of  $\alpha$ -synuclein inhibit this process (Cuervo et al 2004, Xilouri et al 2009). Experiments performed on animal models over-expressing alpha-synuclein have revealed that down-regulation of chaperone mediated autophagy is responsible for part of the alpha-synuclein toxicity in dopaminergic neurons. A reduced turnover of proteins directly involved in the neuronal survival and in the apoptotic machinery may mediate the deleterious effect of alpha-synuclein-mediated inhibition of this clearance pathway.

Therefore, it is conceivable to assume that other substrates of chaperone mediated autophagy contribute to neuronal death through their accumulation. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tatton 2000) and myocyte enhancer factor 2D (MEF2D) (Yang et al 2009) are substrates of this degradative pathway and, at the same time, are specifically involved in the molecular physiopathology of Parkinson's disease; these factors could represent key mediators of the neuronal death when chaperone mediated autophagy is impaired.

Besides mutations and multiplications of alpha-synuclein gene, other mutations linked to hereditary Parkinson's disease are responsible for the impairment of chaperone mediated autophagy. Pathogenetic mutation I93M of PARK5 gene has been demonstrated to determine the inhibition of chaperone mediated autophagy and the accumulation of alpha-synuclein (Kabuta et al 2008).

The finding of low levels of lamp2A and total hsc70 in post-mortem substantia nigra of patients with sporadic disease indicates that a reduced activity of chaperone mediated autophagy is likely to be a pathogenetic mechanism even in idiopathic Parkinson's disease (Alvarez-Erviti 2010).

In conclusion, the failure of chaperone mediated autophagy seems to be a pathogenetic mechanism favoring the death of dopaminergic neurons and, as a consequence, contributing to the development and progression of Parkinson's disease.

#### 4.2.2 Macroautophagy failure

Macroautophagy starts with sequestration of a region of cytoplasm containing proteins and organelles designed for degradation within a double-membrane vacuole called autophagosome. Once formed, the autophagic vacuole undergoes a process of maturation, which is essential for the subsequent fusion with lysosome and the degradation of substrates. The proper function of macroautophagy allows the removal of misfolded proteins and aberrant organelles, which are unsuitable for degradation through other pathways.

Macroautophagy is regulated by the mammalian target of rapamycin (mTOR), a serine/threonine kinase on which two main signal transduction pathways converge:

- The AKT pathway, modulated by neurotrophic growth factors.
- The AMPK pathway, modulated by intracellular levels of aminoacids and ATP.

Macroautophagy is suppressed by mTOR in presence of high levels of nutrients or when growth factors stimulate the cell.

Several exogenous molecules are known to activate macroautophagy independently of mTOR activity. These include drugs actually used in the treatment of neurologic and non neurologic diseases, such as calcium antagonists, lithium and valproate.

Macroautophagy proceeds through various steps, each requiring the presence of specific autophagy related genes (Atg). Four main phases can be distinguished:

1. Nucleation of the autophagosome.
2. Sequestration of a region of cytoplasm containing substrates to be degraded.
3. Maturation of the autophagosome.
4. Fusion with lysosome and degradation of substrates.

Therefore, regulation and biological function of macroautophagy appear to be characterized by a remarkable complexity. Since different variants of this process exist, each one characterized by specific activators, regulatory pathways and biological effects.

Although macroautophagy, chaperone mediated autophagy and ubiquitin-proteasome system are responsible for the degradation of different preferential substrates, a functional correlation among them have been demonstrated. In particular, macroautophagy is able to modulate its own activity depending on the efficiency of the other two pathways. Both proteasome and chaperone mediated autophagy inhibition determine a cytoprotective activation of macroautophagy.

Macroautophagy has been implied in the pathogenesis of several neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, Huntington's disease and frontotemporal dementia, as well as acute injuries. A role of macroautophagy in the development of these pathologies was proposed when intraneuronal accumulation of autophagic vacuoles was detected in postmortem brains of diseased patients; increasing experimental data indicate that this feature is likely to mirror a dysfunction in maturation and lysosomal clearance of autophagosomes.

Macroautophagy seems to play a relevant role in the clearance of alpha-synuclein. Both pathogenetic mutations and over-expression of alpha-synuclein determine an induction of macroautophagy, which is dependent on the inhibition of chaperone mediated autophagy (Xilouri et al 2009).

Anyway, the impairment of alpha synuclein degradation seems not to have a major role in mediating the deleterious effect of macroautophagy impairment: the accumulation of other substrates might be more decisive. In this regard, it is important to remind that

macroautophagy represents the only mechanism able to mediate the clearance of damaged mitochondria through a process named mitophagy. The intraneuronal accumulation of aberrant mitochondria determines neurotoxic effects linked to the generation of reactive oxygen species and the release of pro-apoptotic mediators. Increasing evidence from transgenic models of disease suggests that a defect in the mitophagy pathway might exert an key pathogenetic role in Parkinson's disease. In fact, genes responsible for hereditary disease are essential components of mitophagy machinery: PINK1 and parkin, two genes linked to recessive forms of Parkinson's disease, encode proteins that work synergistically to ensure the sequestration of aberrant mitochondria within the autophagic vacuole (Narendra et al 2010).

Even DJ1, a gene linked to autosomal recessive Parkinson's disease, encodes for a protein that activates macroautophagy and favors mitochondrial turnover (Krebiel et al 2010).

Loss of function of PINK1, parkin or DJ1 causes hereditary Parkinson's disease and the death of dopaminergic neurons in cell and animal models: the decreased efficiency of macroautophagy and mitophagy might be responsible for part of this neurotoxic effect.

Based on these assumptions, the efficacy of mitochondria turnover should be evaluated as a putative defective step linked to macroautophagy dysfunction even in idiopathic Parkinson's disease.

#### **4.2.3 Loss of lysosomal function**

The hypothesis that macroautophagy might be impaired in Parkinson's disease only apparently contrasts with the accumulation of autophagosomes and the increase of LC3II levels that have been demonstrated in postmortem substantia nigra of diseased patients. As previously reported, recent data suggest that a defect in maturation of autophagosome and in its fusion with lysosome could be the major responsible for the accumulation of autophagic vacuoles. Indeed, a depletion of the intraneuronal lysosomal pool and decreased levels of lysosome associated proteins, such as LAMP1, cathepsin D and heat shock protein 73, have been detected in nigral neurons of patients with sporadic Parkinson's disease. Furthermore, lysosomal depletion has been found in dopaminergic neurons of a MPTP mouse model (Dehay et al 2010); in this study the loss of lysosomes has been identified as an early alteration preceding the accumulation of autophagosomes and the neuronal degeneration. Oxidative injury has been found to favor lipid peroxidation of lysosomal membrane and, at last, to reduce the pool of lysosomes. Therefore, a strong and synergistic correlation seems to exist between oxidative stress and the dysfunction of autophagic-lysosomal pathway.

An additional proof of the protective role of lysosomal function in dopaminergic neurons has derived from the finding that mutations of genes encoding lysosomal proteins can modify the risk of developing Parkinson's disease. ATP13A2 (PARK9) is a lysosomal transmembrane cation transporting ATPase that is necessary to maintain acidity in the lysosomal lumen; low pH is indispensable for the function of lysosomal hydrolases and, as a consequence, for degradation of autophagy substrates. Homozygous mutations of ATP13A2 gene causes Kufor-Rakeb syndrome, characterized by early-onset parkinsonism with pyramidal degeneration and dementia. In vitro experiments have demonstrated that loss of ATP13A2 impairs lysosomal degradation of alpha-synuclein and cause intraneuronal accumulation of this protein (Gitler et al 2009).

Glucocerebrosidase (GBA) is a lysosomal enzyme that catalyzes the hydrolysis of the lipid glucosylceramide into glucose and ceramide. Homozygous mutations of GBA gene are responsible for Gaucher syndrome, a multisystem lysosomal storage disease characterized by neurodegenerative manifestations. Recently, carriers of GBA pathogenetic mutations have been identified at higher risk of sporadic Parkinson's disease (Sidransky et al 2009). This confirms the great importance of lysosomal function for the survival of dopaminergic neurons.

## **5. Neuropathologic outcome of molecular physiopathology: More than nigral degeneration**

In the previous paragraphs we have reported evidences regarding the occurrence of specific deleterious processes in degeneration of dopaminergic neurons; furthermore, we have highlighted the functional correlations that have been demonstrated among these processes. A wide body of data confirms that each of these mechanisms can be responsible for degeneration of dopaminergic neurons and involucional modifications of the substantia nigra. These modifications are responsible for the motor symptoms that represent the major criteria for diagnosis of Parkinson's disease. The reasons of the selective damage of nigral neurons is not currently known; particular biological conditions might favor the onset of pathogenetic mechanisms in these neurons or could be responsible for an extreme vulnerability to systemic biochemical alterations.

However, the selectiveness of nigral involvement in Parkinson's disease is not absolute. In fact, the cardinal symptoms of Parkinson's disease are often preceded by a series of early manifestations, such as depression, sleep disturbances, eye movement disorders, hyposmia, constipation and loss of cardiac and vasomotor reflexes, which can start several years before the motor disturbances. The neuropathologic substrate of these aspecific manifestations does not seem to be ascribable to the degeneration of substantia nigra, rather they would derive from the involvement of other monoaminergic neurons in central and peripheral nervous system. Postmortem studies in patients with Parkinson's disease have provided evidence of a typical temporal pattern of pathology progression from peripheral neurons, spinal cord and brainstem to basal ganglia and cortical regions. These studies have revealed the precocious degeneration of autonomic ganglia of mesenteric plexus, dorsal motor nucleus of vagus, rafe nuclei, locus ceruleus and ventral tegmental area; the involvement of these centers is consistent with the clinical symptoms preceding parkinsonian signs. Based on these premises, the degeneration of substantia nigra appears as a late manifestation of the neuropathologic process; moreover, the onset of the characteristic motor manifestations is usually insidious and can be appreciated only when about 50% to 60% of dopaminergic neurons have been lost. These remarks indicate that the diagnosis of Parkinson's disease is always made when the underlying neuropathologic process has already reached an advanced phase; this limits the benefits that can be expected from putative neuroprotective therapies. Hence, it would be desirable to be endowed of diagnostic tools useful to make diagnosis of Parkinson's disease during early or even pre-clinical stages. This assumption has promoted the pre-clinical and clinical research aimed at identifying biomarkers of disease.

### **5.1 Peripheral biomarkers**

Different rationales justify the use of biomarkers for Parkinson's disease:

- To study disease mechanisms.
- To identify individuals with an increased risk of disease.
- To diagnose patients before or at the threshold of motor symptoms.
- To monitor disease progression throughout its course.
- To establish drug mechanisms, assess drug dosing and secondary outcomes in clinical studies.

Neuroimaging biomarkers include detection of dopamine transporter (DAT) levels in basal ganglia with radiolabeled tracers; <sup>123</sup>I-FP-CIT SPECT, commonly known as DATscan, is actually used in clinical practice to assist in the differential diagnosis of movement disorders. Anyway, DAT depletion follows the death of nigral neurons; furthermore, the exposure to radiations and the high costs limit the use of this test in screening and follow up.

Peripheral biochemical markers would offer the advantage of being easily accessible for repeated measurements, feature that makes them particularly useful not only for diagnosis but even for monitoring the course of the disease. Various peripheral cell types share with neurons the molecular machinery for several biochemical processes; this provides the chance of testing peripheral ex-vivo models to seek for disease-specific biochemical alterations. The ideal biomarker should reflect a pathogenetic mechanism operative at central level (core biomarker) and playing a major role in the pathophysiology of Parkinson's disease, such as alpha-synuclein aggregation, mitochondrial dysfunction, oxidative stress and impairment of the clearance machinery. Therefore, it is essential to identify peripheral models reproducing the molecular alterations detected in the affected neurons. Up to now, a wide range of putative peripheral models of disease have been proposed: platelets, peripheral blood mononuclear cells and fibroblasts represent ex vivo tissues which are actually under investigation with the aim of disclosing differences in biochemical parameters between patients with Parkinson's disease and controls; even plasma is currently studied with the same purpose. The accessibility of peripheral tissues represent the clearest advantage of their use, significantly compensating the fact that they are not the main target of the disease.

Cerebrospinal fluid (CSF) cannot be considered a peripheral sample, because it is not easily accessible; anyway, CSF represents a more direct window on central nervous system than blood, so that it can be analysed in pilot studies to determine biochemical alterations which could be more extensively assessed in peripheral models.

The development of therapies demonstrating a pre-clinical neuroprotective effect has raised the need of peripheral biomarkers more and more pressing. In fact, new drugs tested to delay the progression of Parkinson's disease are targeted to specific biochemical alterations occurring in the central nervous system; in this context, a great help would derive from the availability of reliable biochemical parameters linked to the targeted pathway, useful to monitor therapies in clinical studies. Biomarkers expected to predict the effect of a therapeutic intervention and assessed as substitutes for a clinically relevant endpoint are named surrogate markers; their use would allow to overcome some difficulties that often occur in clinical trials of neuroprotective therapies: a biomarker could give short-term information on drug biochemical efficacy, whereas clinical outcome can be evaluated only after years of treatment; a biomarker would not be influenced by a symptomatic effect that could complicate the demonstration of the neuroprotective effect of the drug.

Actually, among the putative peripheral biochemical markers which have been investigated, no one has a reliability which could justify its use in clinical practice.

Alpha-synuclein levels in peripheral blood cells from patients with Parkinson's disease do not seem to differ from controls (Brighina et al 2010), even if an increased expression of the alpha-synuclein gene has been detected in fibroblasts from patients (Hoepken et al 2008). Data on total plasma level of the protein in patients compared to controls are contrasting but new alpha-synuclein assays able to detect oligomeric protein have demonstrated changes in plasma and cerebrospinal fluid from diseased patients. Recently, the concentration of alpha-synuclein oligomers and the ratio of oligomers on total alpha-synuclein have been found significantly increased in cerebrospinal fluid from diseased patients compared to controls (Tokuda et al 2010). The oligomeric soluble fraction of alpha-synuclein has been reported to be increased in plasma samples obtained from patients.

A reduction of glutamate uptake has been observed in platelets from patients and the decrease has showed a positive correlation with clinical severity of the disease (Ferrarese et al. 1999, 2001). This finding indicates that a systemic defect in glutamate transport occurs in patients with Parkinson's disease.

Some studies have reported a decrease in proteasome function in lymphocytes from diseased patients (Blandini et al 2006), but this finding has not been confirmed by subsequent experiments (Brighina et al 2010).

Oxidative stress is induced in lymphocytes from patients with Parkinson's disease and inversely correlates with L-dopa daily doses (Prigione et al 2006, 2009). Increased nitrotyrosine modifications of alpha-synuclein have been found in peripheral blood mononuclear cells obtained from individuals with idiopathic Parkinson's disease compared to controls; moreover, the amount of nitrotyrosine-modified alpha-synuclein has been demonstrated to positively correlate with intracellular levels of reactive oxygen species; in the same *ex vivo* model a significant increase in LC3II level have been demonstrated in diseased patients (Prigione et al 2010). Therefore, in patients with sporadic Parkinson's disease, a systemic dysregulation of autophagy and oxidative stress seem to exist and correlate with alpha-synuclein post-translational modifications. This confirms the chance of identifying peripheral biomarkers able to mirror the activation of pathogenetic processes in the central nervous system: biochemical parameters of autophagy and oxidative stress, together with qualitative and quantitative alterations of alpha-synuclein, could be useful for diagnostic or prognostic purposes and to monitor the biochemical effects of putative neuroprotective therapies. Moreover, the physiopathologic correlation among these mechanisms could offer the possibility of a combined use of these parameters to increase the accuracy of single biomarkers. In particular, the specificity of autophagy dysregulation in peripheral models, not confirmed in other neurodegenerative diseases, qualifies autophagy machinery as a new and promising target of research in the field of peripheral biochemical markers of disease.

It is conceivable that neuroprotective drugs would have more chance of success if they are administered during early stages of disease, when the molecular pathogenetic processes occurring in dopaminergic neurons could be more easily blocked; therefore, the availability of tools useful to make early diagnosis would increase the potential neuroprotective effects of old and new putative therapies. Therefore, development of disease biomarkers is strongly related to the identification of drugs able to delay the progression of Parkinson's disease or even to prevent its onset if diagnosis is made in preclinical stages.

## 5.2 Therapeutic perspectives

Several pharmacological approaches have been tested in order to prevent dopaminergic neurodegeneration. It is obvious that the most promising targets of neuroprotective strategy for Parkinson's disease are represented by the molecular pathogenetic mechanisms known to contribute to nigral degeneration.

An ideal therapeutic approach would consist in preventing alpha-synuclein misfolding, accumulation and aggregation in dopaminergic neurons. Means to achieve this aim include the suppression of alpha-synuclein expression through siRNA technology, the development of agents able to rescue the formation of toxic oligomeric intermediates, the induction of the neuronal pathways involved in alpha-synuclein degradation. The first two strategies will be tested in the near future, whereas the stimulation of clearance pathways is actually subjected to intense research.

Different drugs have been successfully used to induce macroautophagy in cell and animal models of disease. Anyway, the use of these interventions to prevent neurodegeneration is limited by the lack of specificity; indeed, most of the tested drugs affect major intracellular pathways, thus explaining a series of undesired side effects. Molecules that activate the transcriptional factor EB, responsible for the induction of lysosomal biogenesis, have proved a neuroprotective effect in cell and animal models of disease (Dehay et al 2010). In particular, rapamycin and trehalose, two largely used macroautophagy inducers, have demonstrated to rescue neurons from the toxicity of various stimuli related to Parkinson's disease. The effectiveness of these two molecules seems to derive from their property to modulate the two most critical steps of macroautophagy: they induce the formation of autophagosomes through an mTOR-dependent (rapamycin) or mTOR-independent (trehalose) mechanism; furthermore, they activate the transcription factor EB, determining an increase of lysosomal biogenesis, so that the increased sequestration of cargoes within the autophagosomes is balanced by an increase of lysosomal function, thus preventing the intracellular accumulation of autophagic vacuoles. This might represent a reasonable model to develop therapeutic tools aimed at activating macroautophagy. Ongoing screenings to identify novel specific macroautophagy enhancers should provide, in coming years, novel agents useful to modulate macroautophagy for therapeutic purposes.

No effective strategies are available to activate the ubiquitin-proteasome system; pharmacologic research has focused on increasing the proteolytic activity of proteasome subunits, but ubiquitin targeting and substrate deubiquitization are all possible target of future therapies which could be tested.

The importance of chaperone mediated autophagy in the degradation of alpha-synuclein makes it a priority to identify compounds able to stimulate this pathway. In this regard, the pharmacologic increase of lamp2a levels on lysosomal membrane appears as the most reasonable approach. Genetic overexpression of lamp2A has been demonstrated to upregulate chaperone mediated autophagy in animal models but pharmacologic tools able to obtain the same effect are currently not available; cholesterol-depleting agents has shown to enhance the activity of chaperone mediated autophagy through disruption of lysosome membrane lipid microdomains, where inactive lamp2A is stored.

As previously reported, sirtuins have been identified as mediators of modifications induced by starvation in dopaminergic neurons of animals; in particular, SIRT2 seems to enhance the toxic effect of alpha-synuclein, whereas SIRT1 has demonstrated to exert a protective role in dopaminergic neurons. These discoveries have led to the search for potential therapeutic

targets among SIRT1s. Promising pharmacologic approaches include stimulation of SIRT1, which imitates the protective effects produced by caloric restriction in animals, and inhibition of SIRT2.

Stimulation of mitochondrial function might represent another target of putative neuroprotective drugs for Parkinson's disease. Resveratrol is a molecule that has been demonstrated to induce genes involved in mitochondrial biogenesis and aerobic metabolism; this drug rescues dopaminergic neurons in MPTP mouse models.

Several studies have evaluated the putative neuroprotective role of treatments aimed at reducing oxidative stress. An evidence of the potential benefit of antioxidant treatment in Parkinson's disease has been the finding that induction of glutathione peroxidase expression determines a protective effect against 6-hydroxydopa toxicity in dopaminergic neurons of mice. Similarly, overexpression of glutathione peroxidase, as well as Cu, Zn-superoxide dismutase, protects murine brain against paraquat-induced neurodegeneration.

Several foods and natural molecules are supposed to exert a neuroprotective effect thanks to their antioxidant properties. Soy component genistein increases cellular reduced glutathione. Green tea contains polyphenols and flavonoids that prevent lipid peroxidation; another substance contained in green tea, epigallocatechin 3-gallate, has been demonstrated to stimulate nigrostriatal transmission and prevent dopaminergic neuronal loss through suppression of nitric oxide levels in a MPTP mouse model; this finding has qualified nitrosative stress as a putative target of neuroprotective therapies: 7-nitroindazole, a synthetic inhibitor of nitric oxide synthase, has been shown to prevent neurodegeneration in MPTP animal models. Administration of supplements of coenzyme Q10, an endogenous antioxidant that acts as an electron transporter for mitochondrial complexes I and II, has been found to increase the activity of mitochondrial complex I in patients with Parkinson's disease and to prevent dopaminergic neuronal degeneration in a mouse model of disease.

Different plant extracts have antioxidant properties and produce positive effects on experimental models of Parkinson's disease. *Withania somnifera* belongs to the solanaceae family, extensively used in Indian Ayurvedic medicine. This plant contains various alkaloids which might mediate its putative therapeutic effects. Recent studies have shown that administration of extract from *w. somnifera* increases the levels of antioxidant molecules in the midbrain and corpus striatum of mouse models of Parkinson's disease; furthermore, this treatment has produced an improvement in dopaminergic transmission and motor function in the same models (Rajasankar et al 2009).

Use of non-steroidal anti-inflammatory medications seems to reduce the risk of Parkinson's disease, probably as a consequence of a decreased generation of free radicals and nitric oxide in dopaminergic neurons.

Antioxidant therapies have demonstrated to be effective at preventing alpha-synuclein aggregation and neuronal death in many cellular and animal models of PD but clinical studies have failed to demonstrate any significant effect on disease progression in patients. Propargylamines (selegiline and rasagiline) are drugs actually used in clinical practice for the treatment of Parkinson's disease. These molecules seem to positively modify the natural history of the disease thanks to their antioxidant effect, partially related to the inhibition of MAO-B-mediated dopamine catabolism (Magyar et al 2010). The neuroprotective effect of propargylamines has been demonstrated in various models of disease but it has not been definitely proved in patients. In fact, MAO-B inhibition is also responsible for an

improvement in motor symptoms, based on the increase of dopaminergic nigrostriatal transmission, so that it is difficult to distinguish symptomatic from neuroprotective effect. This confirms the need of a peripheral, pathology-related biomarker, useful as a surrogate endpoint in clinical trials of neuroprotective drugs. Anyway, molecular mechanisms different from MAO-B inhibition are supposed to contribute to the putative neuroprotective effect of selegiline and rasagiline: propargylamines have demonstrated to prevent mitochondrial permeabilization, cytochrome c release, caspase activation and nuclear translocation of glyceraldehyde 3-phosphate dehydrogenase; moreover, rasagiline induces the expression of anti-apoptotic proteins, such as Bcl-2 and glial cell-line derived neurotrophic factor (GDNF).

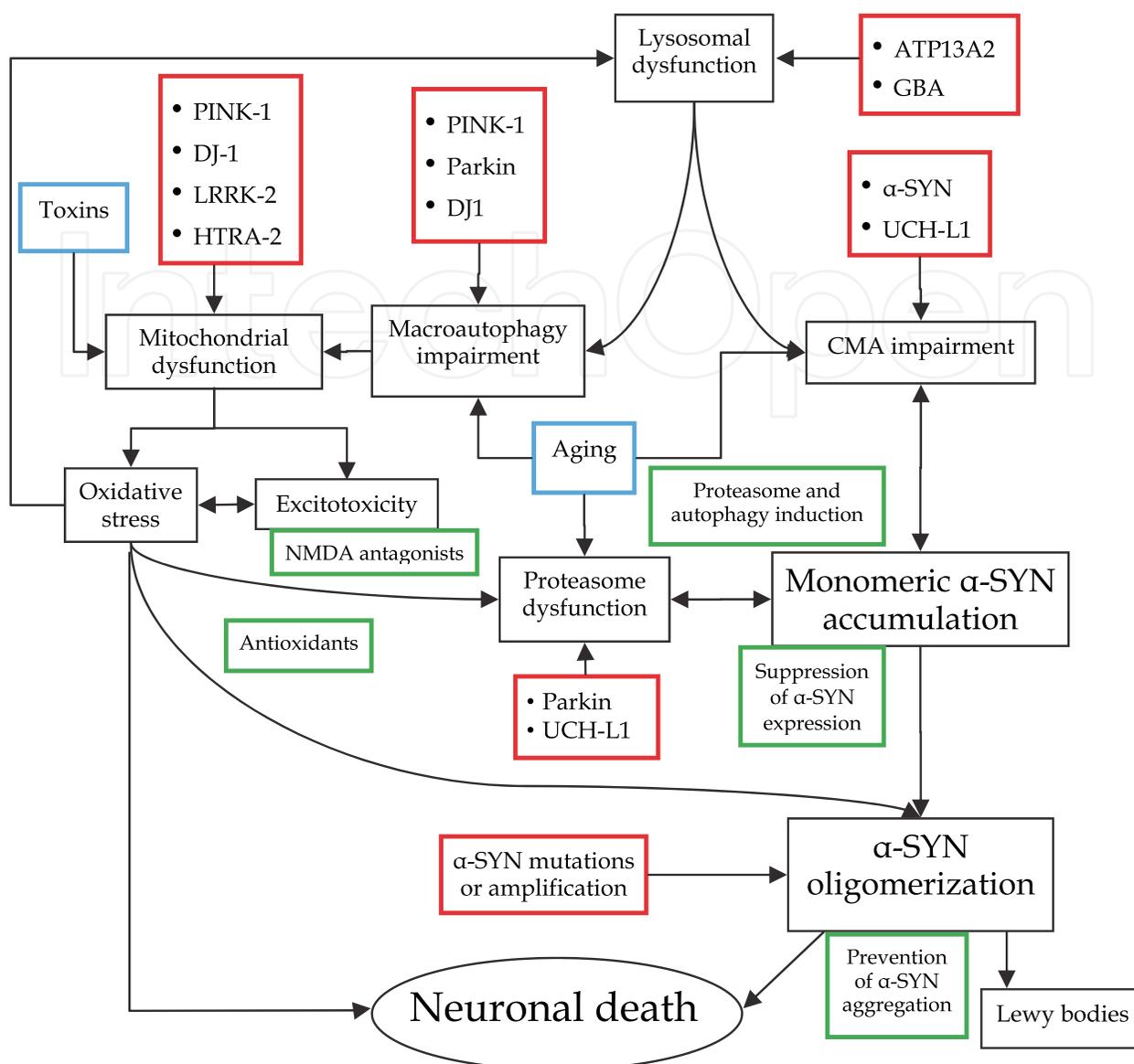
Drugs interfering with excitotoxic insult, such as the NMDA antagonist amantadine, produce little improvement in motor parkinsonian symptoms and significantly reduce motor side effects of L-dopa treatment. Although NMDA antagonists have been found to prevent nigral degeneration in animal models of Parkinson's disease (Blandini et al 2001), no neuroprotective effect has been demonstrated in patients.

Dopaminergic drugs, such as L-dopa and dopamine agonists, as well as deep brain stimulation, might determine neuroprotective effects based on the hypothesis that an increase of dopaminergic transmission should inhibit the glutamatergic projection from subthalamus to the substantia nigra.

## 6. Conclusions

Molecular physiopathology of Parkinson's disease represents an intricate cascade of events that researchers have only begun to clarify (Fig. 1). The association that has been established between nigral degeneration and alpha-synuclein modifications has found its confirmation in a growing body of experimental evidences. Anyway, the primitive conditions that are responsible for the toxic gain of function of this protein, as well as the molecular properties of aberrant alpha-synuclein that are responsible for its deleterious effect on neuronal homeostasis, are mostly unknown and must be further explored. The precise assessment of the sequence of events leading to alpha-synuclein oligomerization and to neuronal death is undoubtedly an essential step in the research of neuroprotective tools; an ideal goal would consist in the identification of critical nodes in the molecular physiopathology of Parkinson's disease, which may provide novel promising targets of therapies aimed at blocking the degenerative process.

Mitochondrial impairment and oxidative stress seem to be early features responsible for alpha-synuclein modifications favoring protein misfolding and aggregation, but the same mechanisms can be induced by the same alpha-synuclein oligomers that they contribute to produce, so that a linear sequence of events cannot be established. Glutamatergic overstimulation of nigral neurons seems to exert a major role in the genesis of motor symptoms, whereas excitotoxicity probably plays a secondary role that only accelerates the death of neurons when these are exposed to energetic defects, oxidative stress and other toxic features. Therefore, it is possible to argue that a significant neuroprotective effect is unlikely to be obtained through pharmacologic interference with only one of these mechanisms, as it is confirmed by the failure of multiple selective strategies aimed at stimulating mitochondrial function or at contrasting oxidative stress or excitotoxicity.



□ : genetic factors; □ : environmental factors; □ : therapeutic strategies.

Fig. 1. Scheme illustrating genetic and environmental factors involved in alpha-synuclein (α-SYN) toxicity and possible therapeutic targets. CMA: chaperone mediated autophagy

Therapeutic tools able to remove alpha-synuclein oligomers or to favor the protein refolding are not currently available. Anyway, several data support a critical role for the efficiency of the intracellular clearance machinery in the maintenance of neuronal integrity; in fact, strong links have been established between abnormal functioning of surveillance pathways and the pathogenesis of Parkinson's disease. Ubiquitin-proteasome system represents the first degradative pathway which has been investigated, however its importance has been downsized by the finding of its secondary role in the degradation of alpha-synuclein, which remains the most disease-specific pathogenetic factor. In this context, the scientific attention has switched to autophagy. Alpha-synuclein, UCH-L1, ATP13A2, DJ1 and PINK1 have all been demonstrated to regulate the clearance of proteins and mitochondria through autophagic-lysosomal pathway. Furthermore, the loss of a single allele of the glucocerebrosidase gene seems to be sufficient to increase the risk of developing the disease.

Pathogenetic mutations of genes responsible for recessive forms of Parkinson's disease, such as DJ1, PINK1 and ATP13A2, inhibit autophagy or lysosomal function through a loss of function of the encoded protein, whereas mutations of genes linked to dominant forms of the disease, such as alpha-synuclein and UCH-L1, impair autophagy through a gain of an aberrant function. Therefore, a correspondence exists between the type of transmission of the hereditary disease and the mechanism through which mutated proteins impair autophagy. Furthermore, several studies performed on postmortem brains and on cell and animal models of disease have confirmed that a failure of the autophagic-lysosomal pathway is likely to be a key feature of the disease physiopathology. This defect can trigger all the well-known pathogenetic mechanisms involved in Parkinson's disease, which in turn can worsen the efficiency of autophagy. Together, these evidences sustain the hypothesis of a central involvement of autophagic and lysosomal dysfunction in the pathogenesis of Parkinson's disease.

The fact that the intraneuronal surveillance machinery has been demonstrated to contrast the onset of most pathogenetic mechanisms of Parkinson's disease makes the recovery and the improvement of this cell quality-control a priority in the development of new therapeutic strategies. Pharmacologic approaches aimed at promoting the removal of aberrant proteins and organelles could reveal as the most rationale and global solution applicable to delay the progression of Parkinson's disease and other neurodegenerative disease related to protein misfolding.

In conclusion, restoring the normal function of autophagic-lysosomal pathway, together with reducing oxidative stress and favouring alpha-synuclein refolding, represent promising strategies of neuroprotective therapies. Anyway, all these approaches still face some limitations that need to be overcome; the identification of early diagnostic biomarkers remains an absolute priority that could allow to overcome part of these limitations.

An accurate knowledge of the wide range of mechanisms involved in the physiopathology of Parkinson's disease and, firstly, the definition of the complex interactions connecting these mechanisms represent basic requirements to develop diagnostic instruments and therapeutic tools able to modify the natural course of this disease.

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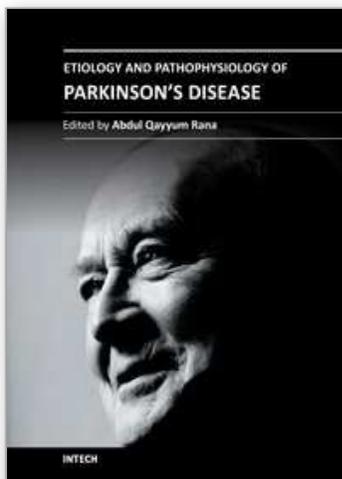
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## **Etiology and Pathophysiology of Parkinson's Disease**

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This book about Parkinson's disease provides a detailed account of etiology and pathophysiology of Parkinson's disease, a complicated neurological condition. Environmental and genetic factors involved in the causation of Parkinson's disease have been discussed in detail. This book can be used by basic scientists as well as researchers. Neuroscience fellows and life science readers can also obtain sufficient information. Beside genetic factors, other pathophysiological aspects of Parkinson's disease have been discussed in detail. Up to date information about the changes in various neurotransmitters, inflammatory responses, oxidative pathways and biomarkers has been described at length. Each section has been written by one or more faculty members of well known academic institutions. Thus, this book brings forth both clinical and basic science aspects of Parkinson's disease.

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51000 Rijeka, Croatia  
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Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

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