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Understanding Dopaminergic Cell Death Pathways in Parkinson Disease

Patrick P. Michel,^{1,2,3,4,5} Etienne C. Hirsch,^{1,2,3,4,5,*} and Stéphane Hunot^{1,2,3,4,5}

¹Inserm, U 1127, F-75013, Paris, France

²CNRS, UMR 7225, F-75013, Paris, France

³Sorbonne Universités, UPMC Univ Paris 06, UMR S 1127, F-75013, Paris, France

⁴Institut du Cerveau et de la Moelle Epinière, ICM, F-75013, Paris, France

⁵Equal contributions

Running Title: Cell Death in Parkinson Disease

*Correspondence: etienne.hirsch@upmc.fr

INSERM UMR 1127 - CNRS UMR 7225 - Université Pierre et Marie Curie

Institut du cerveau et de la moelle épinière - ICM

Thérapeutique Expérimentale de la Maladie de Parkinson

Hôpital de la Salpêtrière

47 boulevard de l'Hôpital

75651 Paris CEDEX 13

France

Phone +33 (0) 1 57 27 45 57

SUMMARY

Parkinson's disease (PD) is a multifactorial neurodegenerative disorder, the etiology of which remains largely unknown. Progressive impairment of voluntary motor control, which represents the primary clinical feature of the disease, is caused by a loss of midbrain substantia nigra dopamine (DA) neurons. We present, here, a synthetic overview of cell autonomous mechanisms that are likely to participate in DA cell death in both sporadic and inherited forms of the disease. In particular, we describe how damage to vulnerable DA neurons may arise from cellular disturbances produced by protein misfolding and aggregation, disruption of autophagic catabolism, endoplasmic reticulum (ER) stress, mitochondrial dysfunction or loss of calcium homeostasis. Where pertinent, we show how these mechanisms may mutually cooperate to promote neuronal death.

INTRODUCTION

Parkinson disease (PD) is the second most frequent neurodegenerative disorder of aging. It is mostly sporadic, less than 10% of PD cases being inherited. The disease is diagnosed clinically based on the presence of typical motor symptoms that include bradykinesia, rigidity, abnormal posture, and resting tremor. Although pathological stigmata are detectable in several areas of the brain, motor symptoms result primarily from the death of substantia nigra (SN) dopamine (DA) neurons (Damier et al., 1999). This paper is a comprehensive review of how cell-autonomous mechanisms may participate in DA cell death in both sporadic and hereditary forms of the disease. Readers are referred to specific reviews for the description of non-cell-autonomous mechanisms involving activated glial cells and peripheral immune cells in PD progression (Hirsch and Hunot, 2009; Perry, 2012).

More specifically, we present evidence showing that the progressive deterioration of vulnerable SN DA neurons may arise from cellular disturbances produced by misfolding and aggregation of the synaptic protein alpha synuclein (α -syn), disruption of the autophagy-lysosome system, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, or dysregulation of calcium homeostasis. Where relevant, we will indicate how some of these mechanisms may act in concert to promote the degeneration of DA neurons.

Synucleinopathy and dysproteostasis.

Pathogenic role of α -syn aggregation. The discovery in 1997 that point mutation in the α -syn gene was responsible for the inherited form of PD, together with the finding that Lewy bodies (LBs), a key neuropathological feature of sporadic PD, are primarily composed of misfolded/aggregated α -syn, has dramatically changed our view of PD pathogenesis, which has become in many respects “synuclein-centric” (Polymeropoulos et al., 1997; Spillantini et al., 1997). Genetic evidence for a role of α -syn in sporadic PD has emerged from genome-wide

association studies (GWAS) that reported an association between the *SNCA* locus and the risk of developing PD (Simon-Sanchez et al., 2009). These studies failed to identify protein-coding variants at this locus, suggesting that the risk alleles are likely to affect expression levels of α -syn rather than its function. In line with this, the identification of inherited forms of PD with duplication or triplication of the *SNCA* gene suggests that an increased expression level of the normal protein is sufficient to trigger the pathogenic mechanism (Chartier-Harlin et al., 2004; Singleton et al., 2003).

Alpha-syn is a small 140aa protein normally enriched in the presynaptic compartment (Iwai et al., 1995), where it is thought to promote the formation of the SNARE complex, thereby regulating vesicle dynamics and trafficking and, hence, neurotransmitter release (Burré et al., 2010). Whether this physiological function is lost and plays any role in PD pathogenesis is still uncertain. While α -syn-deficient mice display impaired evoked DA release in the striatum, these alterations are not associated with obvious features of neuronal distress and degeneration, suggesting that the pathogenic role of α -syn in sporadic PD is unlikely to be linked to a loss of function but rather to a gain of pathological function (Abeliovich et al., 2000). In fact, accumulating evidence shows that aggregation of α -syn is necessary for its pathogenicity. In model systems with α -syn overexpression, aggregation and deposition of α -syn precede neuronal cell death and strategies to reduce the aggregative process were shown to reduce neurodegeneration and improve motor deficits in many species, including invertebrates (nematodes and flies), rodents and non-human primates (Lashuel et al., 2013). It is also worth noting that familial PD-associated *SNCA* mutations share a common property of accelerated aggregation of α -syn leading to early-onset and rapidly progressive forms of PD (Kim and Lee, 2008). Finally, aggregate forms of α -syn generated from recombinant protein were shown to be pathogenic when administrated directly into the brain of mice, further supporting the toxic gain-of-function hypothesis (Luk et al., 2012).

There is much debate about the toxic aggregate species of α -syn involved in neurodegeneration. While it is now well accepted that under physiological conditions α -syn may exist in various conformational and oligomeric states, the full spectrum of toxic α -syn species and their precise role in the mechanism of neuronal cell death has not been completely identified. Nonetheless, soluble oligomers and protofibrils, which are formed relatively early during the fibrillation process of α -syn, seem to be particularly toxic compared to the insoluble amyloid-like fibrils accumulating into LBs (Danzer et al., 2007; Winner et al., 2011) (Figure 1).

Interconnection between synucleinopathy and dysfunction of the autophagy lysosome pathway. What causes accumulation and aggregation of misfolded α -syn are important questions that will lead to major breakthroughs in our understanding of disease development if solved. Similarly, why some neurons show α -syn accumulation earlier than others during the course of the disease remains to be understood. Among mechanisms possibly responsible for α -syn misfolding and aggregation, dysfunction of degradation pathways, notably the autophagy-lysosome system, has attracted much attention recently (Figure 1).

Macroautophagy (referred hereafter as autophagy), is a self-destructive process whereby double-membrane-bound structures called autophagosomes engulf cytosolic cargo (damaged organelles, aggregates, lipid droplets, ...) before delivery to the hydrolytic milieu of lysosomes for degradation (Figure 2). The resulting metabolites are transported into the cytoplasm and used either for the synthesis of new macromolecules or as a source of energy (Boya et al, 2013). In SN DA neurons from PD patients, autophagosomes and lysosomal marker proteins are increased and decreased, respectively, suggesting that the autophagic flux is profoundly disrupted in these patients (Anglade et al., 1997; Chu et al., 2009). Interestingly, several PD-related genes have been linked to dysfunctional autophagy. Indeed, the loss of DJ-1 that triggers mitochondrial dysfunction related to oxidative stress (Kim et al, 2005; Guzman et al., 2010),

leads to the accumulation of microtubule-associated protein 1 light chain 3 (LC3), a reliable marker for autophagosomes (Thomas et al., 2011) suggesting that oxidative stress represents a potential trigger for autophagy (Filomeni et al., 2015) (Figure 2). Furthermore, overexpression of the PD-related protein LRRK2, another PD-related protein shown to exacerbate α -syn pathology (Lin et al., 2009,) increases autophagosome numbers and lysosomal pH thereby reducing lysosomal hydrolytic enzyme activities (Gómez-Suaga et al, 2012).

Involvement of dysfunctional autophagy in α -syn accumulation is supported by *in vivo* gene-targeting experiments in mouse showing that whole-brain specific loss of Atg7 (an enzyme required for autophagosome formation) leads to presynaptic neuronal accumulation of α -syn (Friedman et al., 2012). Note that the presence of aggregates containing K48-linked polyubiquitin and the ubiquitin binding protein p62 were detected after conditional *Atg7* deletion in DA neurons (Ahmed et al., 2012), suggesting that the autophagy-lysosome pathway and the proteasomal pathway cooperate to reduce misfolded protein burden. These observations also indicate that selective impairment of one pathway can lead to a compensatory upregulation of the other as already noted by Ebrahimi-Fakhari and colleagues (2011). Interestingly, mouse brains deficient in Atg7 also showed increased levels of LRRK2 (Friedman et al., 2012). Also coherent with these observations, conditional deletion of *Atg7* in mouse midbrain DA neurons resulted in early dendritic and axonal dystrophy associated with delayed neurodegeneration and late-onset locomotor impairments (Friedman et al., 2012).

Besides macroautophagy, chaperone-mediated autophagy (CMA) has also been implicated in PD (Cuervo et al., 2014) (Figure 1). CMA is a selective type of autophagy responsible for lysosomal degradation of a highly specific subset of soluble cytosolic proteins containing a KFERQ-like motif recognized by the cytosolic chaperone heat shock cognate protein 70 (HSC70) (Figure 2). Of interest, wild-type α -syn is a substrate for CMA (Cuervo et al., 2004) suggesting a possible link between impaired CMA activity and PD. Furthermore, pathogenic

mutant forms of PD genes *SNCA* and *LRRK2* (Martinez-Vicente et al., 2008; Orenstein et al., 2013) or post-transcriptionally modified α -syn (Martinez-Vicente et al., 2008) exert inhibitory effects on CMA (Figure 2). Despite being recognized by cytosolic HSC70 and normally delivered to the lysosomal membrane, mutant forms of α -syn and LKCR2 proteins are unable to reach the lysosomal lumen for CMA-mediated degradation (Orenstein et al., 2013). This is due to aberrant interactions of these proteins with the CMA receptor LAMP-2A. Providing additional evidence for decreased CMA activity in PD, both LAMP2A and HSC70 levels are reduced in PD patients (Alvarez-Erviti et al, 2010). Unexpectedly, however, LAMP-2A is augmented in patients carrying pathogenic mutations in *LRRK2*, which is indicative of a possible compensatory response to CMA dysfunction in these patients (Orenstein et al, 2013).

The implication of the autophagy-lysosome pathway in PD is also supported by the discovery that mutations in genes encoding proteins of the endosomal/lysosomal system, vacuolar protein sorting-35 (VPS35), type 5 P-type ATPase and glucocerebrosidase (GBA), result in parkinsonian syndromes (Figure 2).

VPS35 is part of the retromer, a protein complex that associates with the cytosolic face of early endosomes to regulate retrograde transport of cargo proteins (plasma membrane receptors, transporters) to the trans-Golgi network, to rescue them from lysosome-mediated turnover (Tsika et al, 2014). Mutations in VPS35 have been identified in patients with autosomal dominant PD (Vilariño-Güell et al. 2011; Zimprich et al. 2011). Confirming that VPS35 dysfunction has a profound impact on the autophagic process, VPS35 deficient DA neurons in culture contained enlarged late endosomes/lysosomes and exhibited impaired endosome-to-Golgi retrieval of the CMA receptor LAMP-2A (Tang et al, 2015). Aged VPS35^{+/-} mice developed PD-related deficits including loss of DA and accumulation of α -syn in SN DA neurons (Tang et al, 2015). Besides, AAV mediated gene transfer of the VPS35 D620N mutation in the SN of adult rats resulted in marked degeneration of DA neurons (Tsika et al,

2014). Of note, another PD gene *eukaryotic translation initiation factor 4 gamma, 1 (eIF4G)* involved in autosomal-dominant forms of parkinsonism was reported to genetically and functionally interact with *VPS35* (Dhungel et al, 2015).

ATP13A2, which is localized in acidic membrane compartments of lysosomes is a P-type ATPase that is expected to function as a cation metal transporter (Gitler et al., 2009) (Figure 2). *ATP13A2* mutations cause familial Kufor-Rakeb syndrome, characterized by early onset parkinsonism associated with pyramidal degeneration and dementia (Ramirez et al., 2006). Experiments carried out in cultured fibroblasts from patients with Kufor-Rakeb syndrome not only confirmed that ATP13A2 loss of function impairs lysosomal catabolism but also revealed that it favors the accumulation of α -syn (Usenovic and Krainc, 2012). Conversely, the loss of DA neurons induced by α -syn overexpression in animal and cellular models of PD is rescued by coexpression of ATP13A2 (Gitler et al., 2009). The presence of ATP13A2 is also dramatically reduced within the bulk cytosol of DA SN neurons from patients with sporadic PD (Dehay et al., 2012), and partially redistributed towards LBs (Dehay et al., 2012; Murphy et al., 2013), confirming indirectly that the function of the autophagy-lysosome system is impaired in sporadic forms of PD. Note that cells expressing mutated ATP13A2 were also reported to accumulate the mutant protein in the ER, making them predisposed to ER-stress induced degeneration (Ugolino et al, 2011).

The lysosomal enzyme GBA cleaves glucocerebroside (also called glucosylceramide, GC) into a sugar, glucose and a simpler fat molecule, ceramide. Pathogenetic mutations in both alleles of GBA result in Gaucher disease, a disorder in which a severe loss of GBA activity leads to unprocessed GC that accumulates in the bone marrow, lungs, spleen, liver and sometimes the brain (Platt et al., 2014). Some patients developing a non-neuronopathic form of Gaucher disease present parkinsonian symptoms (Neudorfer et al., 1996) but, quite unexpectedly, individuals carrying a single mutant GBA allele who do not develop Gaucher

disease, have a 5-fold increase in PD risk (Sidransky et al., 2009), pointing to a possible link between GBA dysfunction and sporadic PD. Consistent with this view, the analysis of cerebrospinal fluid and brain tissue samples from PD patients revealed that GBA activity is reduced regardless of whether or not these patients harbor mutations in GBA (Balducci et al., 2007; Parnetti et al., 2014). Of note, GBA protein levels and activity are specifically decreased in brain areas where α -syn levels are elevated in early stage PD, suggesting that GBA dysfunction may be a consequence of the accumulation and aggregation of α -syn (Murphy et al, 2014). In keeping with this idea, Mazzulli and colleagues demonstrated that α -syn oligomeric species interrupt ER-Golgi trafficking of GBA, resulting ultimately in defective storage of the enzyme within lysosomes (Mazzulli et al, 2011). In addition, α -syn can operate as a direct inhibitor of GBA, at the internal membrane surface of lysosomal vesicles (Yap et al, 2013). Inversely, experimental evidence suggests that a reduction in GBA lysosomal activity may promote α -syn misprocessing as AAV-mediated expression of GBA decreases α -syn aggregation in a presymptomatic mouse model of Gaucher-related synucleinopathy (Sardi et al., 2013). Overall, these observations suggest the existence of a positive feedback loop in which reduced GBA lysosomal activity leads to accumulation of α -syn and vice versa, leading ultimately to neurodegeneration (Mazzulli et al, 2011) (Figure 2). The stabilization of α -syn soluble oligomers by unprocessed GC may be a possible reason why GBA dysfunction facilitates α -syn-mediated aggregation (Mazzulli et al, 2011). Overall, a valuable therapeutic strategy to halt PD progression may be to augment brain GBA activity as recently evidenced in PD fibroblasts from a GBA carrier using Ambroxol, a secretolytic agent that works as a GBA activity enhancer (McNeill et al., 2014).

As developed above, if accumulation and subsequent aggregation of α -syn can originate from dysfunctional cellular degradation pathways, α -syn aggregates can also profoundly affect on their own the ubiquitin–proteasome system (Lindersson et al., 2004) and the autophagy-

lysosomal pathway (Winslow et al., 2010). This pathological feedback loop may therefore reinforce the synucleinopathic mechanisms and their consequences (Figure 1). Yet, the pathogenic mechanisms associated with α -syn aggregates are likely to be even more diverse since aggregate species were reported to cause membrane permeabilization (van Rooijen et al., 2010), golgi fragmentation (Gosavi et al., 2002), mitochondrial dysfunction (Parihar et al., 2008) and activation of the unfolded protein response associated with endoplasmic reticulum stress (Colla et al., 2012a) (Figure 1).

Synucleinopathy-associated endoplasmic reticulum stress response. The endoplasmic reticulum (ER) is central to protein folding in eukaryote cells and any perturbations altering ER homeostasis can result in the disruption of the folding process and accumulation of misfolded or unfolded proteins. This cellular condition, also called ER stress, usually induces a physiological protective response termed unfolded protein response (UPR) which is aimed at restoring ER homeostasis and normal cell function if it is induced transiently (for review, see Hetz, 2012) (Figure 3). Yet, under chronic ER stress, the accumulation of unfolded proteins and sustained UPR activity triggers activation of pro-apoptotic pathways and cell death, thereby eliminating damaged cells. Among the factors possibly involved in triggering the deleterious chronic ER stress response in PD, accumulation and aggregation of misfolded α -syn is an obvious candidate. In support of this view, markers of UPR activation have been consistently observed in mammalian and invertebrate models of synucleinopathy (for review, see Matus et al., 2011). Some of these findings revealed that α -syn conformers, and in particular toxic oligomers, accumulate at the ER early during the disease process concomitantly with upregulation of ER chaperones, including Bip and PDIp (Colla et al., 2012b) (Figure 3). Interestingly, a similar ER accumulation of α -syn has also been observed in PD brain tissue (Colla et al., 2012b). In line with this, evidence from postmortem studies suggests that both ER

stress and engagement of the UPR machinery occur in nigral dopaminergic neurons from patients with PD (Hoozemans et al., 2007; Selvaraj et al., 2012; Conn et al., 2004). Apart from synucleinopathy-associated chronic ER stress response, a number of other reports have also suggested a link between PD-related genes and ER function/alteration. For instance, dopaminergic neuronal cell death induced by human α -syn in the nematode *C. elegans* was shown to be attenuated by LRRK2 through a mechanism involving upregulated expression of the ER chaperone Bip (Yuan et al., 2011), suggesting that deficiency in LRRK2 may sensitize neurons to ER stress-induced neurodegeneration associated with α -syn misfolding and accumulation (Figure 3).

As suggested by the above data, if chronic ER stress plays a central role in neuronal cell death in PD, targeting key elements of the UPR pathway would likely be neuroprotective. Surprisingly, however, increasing rather than suppressing UPR components was shown to ameliorate disease phenotypes in both toxic- and genetic-based models of PD (Sado et al., 2009; Colla et al., 2012a; Gorbatyuk et al., 2012). This apparently counterintuitive result has led to the concept that a mild ER stress response causing a sustained, yet moderate UPR, may improve the resistance of dopaminergic neurons during PD progression. This type of response called ER hormesis was observed with the ER stress inducer, tunicamycin which conferred neuroprotection in mouse and *Drosophila* PD models (Fouillet et al, 2012). Thus, fine-tuning of the UPR, may be a possible therapeutic strategy for PD.

A Disturbance in Mitochondrial Function

Mitochondria, once simply seen as the ‘powerhouses’ of the cell, are now considered to work as initiators and transducers of signaling pathways associated with a number of cell functions, including apoptosis (Tait and Green, 2013). The crucial implication of mitochondria in PD progression is therefore not surprising.

The most direct evidence for the implication of mitochondria in PD relies on seminal observations made in autopsy brain tissue and other tissue samples from PD patients, as well as on experiments carried out in non-human primates and rodents. In particular, a deficit in mitochondrial complex I activity is found in autopsy brains from PD patients (Schapira et al., 1990) and in cytoplasmic hybrid (cybrid) cell lines that contain mitochondrial DNA (mtDNA) from patients' platelets (Swerdlow et al., 1996). Besides, the mitochondrial complex I inhibitor, 1-methyl-4-phenylpyridinium (MPP⁺) induces a selective destruction of SN DA neurons in mice (reviewed by Przedborski et al., 2004) and non-human primates (Langston et al., 1984) receiving systemic injections of its prodrug MPTP. This explains why accidental intravenous self-administration of MPTP in humans led to an irreversible neurodegenerative condition that was clinically and histologically very similar to sporadic PD (Langston et al., 1984). In line with these findings, other complex I inhibitors, with a chemical structure that is, however, unrelated to MPP⁺, such as the alkaloid rotenone (Sherer et al., 2003) and the acetogenin annonacin (Champy et al., 2004) can also induce irreversible lesions of the dopaminergic nigro-striatal pathway when systemically administered to rodents. Remarkably, annonacin is present in soursop, a tropical plant whose consumption is linked to an atypical form of parkinsonism in the Caribbean island of Guadeloupe (Lannuzel et al., 2008).

Apart from postmortem evidence for complex I deficiency in PD and the parallel drawn with parkinsonism-induced mitochondrial poisons targeting the electron transport chain (ETC), major breakthroughs in the field of mitochondrial dysfunction in PD have been made thanks to the discovery that some of the PD-related genes are involved in key biological processes that govern mitochondrial homeostasis and stress-response. In particular, it is now well established that the recessive PD-linked genes *PINK1* and *Parkin* likely play a central role in mitochondrial quality control and dynamics (Narendra et al., 2012) (Figure 4). Mitochondrial quality control refers to cellular mechanisms aimed at ensuring proper functioning of the mitochondrial

network, which is critical for cell physiology and survival. It involves several functional aspects including damage prevention and repair mechanisms, autophagic elimination of dysfunctional/damaged organelles (known as mitophagy) and neosynthesis of mtDNA- and nuclear DNA-encoded mitochondrial proteins for mitochondria biogenesis. To properly perform these tasks, the mitochondrial network evolves dynamically from elongated to fragmented states (and vice versa) thanks to a highly specialized molecular machinery that tightly controls fusion and fission events, which in turn facilitate repair and autophagic degradation of defective organelles, respectively (Ono et al., 2001). Finally, mitochondrial dynamics and transport are also essential for suitable distribution of organelles, especially at neuronal terminal ends, where high energy demand is required to sustain synaptic activity (Verstreken et al., 2005) (Figure 4).

PINK1 and parkin were shown not only to participate in the functional and morphological maintenance of the mitochondrial network (Palacino et al., 2004; Gautier et al., 2008) but also, to critically regulate the removal of dysfunctional mitochondria through mitophagy (Deas et al., 2011). Remarkably, parkin and PINK1 fulfill these functions collectively, with parkin being recruited to damaged (i.e., depolarized) mitochondria by PINK1 and acting downstream to promote their degradation by autophagy (Clark et al., 2006; Park et al., 2006; Narendra et al., 2010; Vives-Bauza et al., 2010; Bertolin et al., 2013). Although the functional interplay of parkin and PINK1 on altered mitochondria is still not fully elucidated, it has been shown that PINK1 accumulation at the outer mitochondrial membrane (OMM) of dysfunctional mitochondria is required and sufficient to recruit and activate parkin. Parkin in turn drives the ubiquitination and degradation of mitochondrial substrates, including, mitofusin (Mfn) 1 and 2, which regulate OMM fusion, and the OMM-associated Miro protein, a Ca^{2+} -binding GTPase that controls the anterograde transport of mitochondria along the axons (Narendra et al., 2010; Tanaka et al., 2010; Matsuda et al., 2010; Gegg et al., 2010; Wang et al., 2011) (Figure 4).

Overall, the parkin/PINK1 pathway-mediated degradation of Mfn1/2 and Miro is believed to facilitate the elimination of bioenergetically compromised mitochondria by promoting both their isolation from the filamentous and healthy mitochondrial network and their immobilization at the somatic compartment away from high-energy-consuming end terminals. Ubiquitinated OMM proteins then bind to the ubiquitin- and LC3-binding adaptor protein p62 for engulfment by autophagosomes (Geisler et al., 2010; Rakovic et al., 2013). On the basis of these data, it has been proposed that impairment of mitophagy by mutations of PINK1 or Parkin may become toxic by accumulation of dysfunctional mitochondria (Narendra et al., 2010; Vives-Bauza et al., 2010) (Figure 2 and 4). However, chemical uncouplers that are used at relatively high concentrations to activate mitophagy through mitochondrial depolarization in immortalized cell lines failed to activate this process in rat primary neuronal cell cultures (Van Laar et al., 2011) or in human induced pluripotent stem cell-derived neurons (Rakovic et al., 2013). This has led to the speculation that mitophagy may be controlled by distinct mechanisms in neuronal cells, possibly because of the specific bioenergetic requirements of these cells. Yet, a more recent study suggests that, unlike chemical uncouplers, the short mitochondrial isoform of ARF (smARF), previously identified as an alternate translation product of the tumor suppressor p19ARF, has the capacity to depolarize mitochondria and to promote mitophagy in neuronal cells through the canonical Parkin/PINK1-dependent pathway (Grenier et al., 2014). It still remains intriguing that, in contrast to the mechanism in invertebrates such as flies, the control of mitochondrial motility and fusion machinery by the parkin/PINK1 pathway in mammalian cells appears to be minimal under normal physiological conditions and only unmasked under stress conditions (Wang et al., 2011; Tanaka et al., 2010). This difference may underscore evolutionary changes in mitochondrial quality control mechanisms which may be more tightly regulated in higher eukaryotes that live longer and in which mitochondrial ageing is a heavy burden for cell survival. In line with this, redundant mechanisms may be important

and there is evidence to suggest that mitophagy is far from being the only process involved in mitochondrial quality control. Other lines of defense such as activation of mitochondrial matrix proteases –that degrade unfolded and oxidized soluble proteins, as well as removal of mitochondrial bits containing damaged cargos through the generation of mitochondrial-derived vesicles (MDVs) transiting to lysosomes, are rapidly induced mechanisms that could prevent irreversible mitochondrial damage and elimination by mitophagy (for review see Sugiura et al., 2014). Interestingly, PINK1, Parkin and Vps35 have been involved in the biogenesis of MDVs but the functional contribution of MDVs in PD pathophysiology has not been demonstrated so far.

Enlarged mitochondria with disrupted cristae have been observed in nigral neurons from PD patients, which confirm the view that mitochondrial quality control is defective in sporadic PD (Anglade et al., 1997). Similar changes in mitochondrial morphology can be recapitulated in experimental setups using the mitochondrial complex I inhibitors MPTP/MPP⁺ and rotenone, implying that ETC dysfunction and as a corollary to that, oxidative stress and bioenergetic deficits might primarily impair mitochondrial dynamics in PD (for review, see van Laar and Berman, 2013) (Figure 4). Yet, other candidates such as α -syn accumulation and aggregation might be involved as well. In support of this statement, many reports have shown that α -syn can not only interact with and accumulate in mitochondria but also trigger morphological alterations, including swelling and cristae disruption (Martin et al., 2006). More recently, Kamp and colleagues showed that α -syn overexpression in cultured cells and in the nematode *C. elegans* results in mitochondrial fusion inhibition and fragmentation, a phenotype that is rescued by parkin and PINK1 (Kamp et al., 2010). Interestingly, this effect may not rely on inhibition of the fusion/fission machinery but rather could involve the direct interaction of α -syn oligomers with mitochondrial membranes resulting in their fragmentation (Nakamura et al., 2011). Completing these observations and further reinforcing the link between α -syn pathology

and defective mitochondrial quality control, is the finding that neuronal expression of the A53T synuclein mutant is associated with increased mitophagy, mitochondrial loss and cell death. Intriguingly, stimulating mitochondrial fusion (i.e., inhibiting Drp1 activity or overexpressing Mfn2) or silencing autophagy-related genes could reverse the mitochondrial loss and protect neurons against A53T α -syn-induced cell death (Choubey et al., 2011). This result contrasts therefore with other reports showing that stimulation instead of inhibition of macroautophagy and of the PINK/Parkin-associated mitophagy pathway is actually neuroprotective in different synucleinopathy models including mice overexpressing wild-type α -Syn (Lo Bianco et al., 2004; Spencer et al., 2009; Winslow et al., 2010). The discrepancy may be explained by the fact that, unlike wild-type α -syn, mutated A53T α -syn inhibits the CMA pathway resulting in compensatory over activation of macroautophagy which in turn would lead to excessive elimination of mitochondria including those that are not depolarized (Xilouri et al., 2009). These data underline that excessive macroautophagy activity may have negative consequences and that - fine tuning of mitophagy is required for proper neuronal function and for therapeutic purposes. These studies underscore also the potential of strategies aimed at stimulating mitochondrial elongation in distressed neurons. Indeed, filamentous mitochondrial networks ensure a better response to oxidative stress and other insults through dilution of stress molecules across the network and induction of compensatory mechanisms (Youle and van der Bliek, 2012). Such an approach has recently been illustrated in toxic-based models of PD where neuroprotection afforded by the X protein, a virus-derived protein with mitochondrial targeting and anti-apoptotic properties, was associated with mitochondrial filamentation, i.e., mitochondria elongation forming a reticular network (Szelechowski et al., 2014).

Dysregulated Calcium Homeostasis

Dysregulation of calcium homeostasis may also intervene crucially in the preferential loss of DA neurons in PD. Defects in calcium-handling may be due principally to changes in the discharge activity of DA neurons but may also occur as indirect consequence of multiple PD-related events such as α -syn aggregation, mitochondrial deficits or ER dysfunction. DA neuron survival may be compromised in situations where levels of calcium exceed the upper (Blandini et al., 2004; Chan et al., 2007) or fall below the lower limit (Salthun-Lassalle et al., 2004) of the physiological range in the cytosol and subcellular organelles.

Cytosolic calcium ($\text{Ca}^{2+}_{\text{cyt}}$) overload in DA neurons may result from sustained engagement of N-methyl-D-aspartate (NMDA) glutamate receptors as a consequence of the overactivity of subthalamic nucleus glutamatergic inputs (Blandini, et al., 2004). This hypothesis which presupposes that glutamate is increased extracellularly in the vicinity of SN DA neurons (Assous et al., 2014) is challenged, however, by the concept of a slowly occurring excitotoxic process, in which calcium overload and ensuing neurodegenerative events including nitrosative and oxidative stress (Blandini et al., 2004), may occur in spite of normal extracellular concentrations of glutamate. Indeed, mitochondrial bioenergetic deficits occurring in PD may reduce the Mg^{2+} block of the NMDA channel pore and as a consequence may increase the sensitivity of DA neurons to glutamate-mediated excitotoxic stress (Schapira and Gegg, 2011).

SN DA neurons are also characterized by Ca^{2+} -dependent pacemaking, an autonomous mode of discharge that elevates $\text{Ca}^{2+}_{\text{cyt}}$ through L-type voltage-dependent calcium channels, most likely a subset of them having a Cav1.3 pore (Chan et al., 2007). This type of activity which serves to maintain a basal DA tone in the striatum may confer a specific vulnerability to SN DA neurons, which have low intrinsic calcium buffering capacity (Hirsch et al., 1992). Indeed, calcium influx through autonomous pacemaking results in elevation of basal mitochondrial oxidative stress in SN DA neurons, presumably as a direct consequence of an increased mitochondrial Ca^{2+} load (Guzman et al., 2010). One of the consequences of mitochondrial

oxidative stress is to reduce the bioenergetic reserve capacity of DA neurons which in turn makes them particularly vulnerable in conditions of increased metabolic demand (Rivero-Rios et al., 2014). This probably explains why Isradipine an L-type Cav channel blocker with some specificity for Cav1.3 over Cav1.2 and Cav1.1 channels not only reduced basal mitochondrial oxidant stress in SN DA neurons (Guzman et al., 2010) but also protected these neurons from mitochondrial toxins such as MPP⁺ and rotenone that impair mitochondrial respiration and energy production (Chan et al., 2007). Isradipine did not stop pacemaking, however, most probably because calcium supports, but is not necessary to preserve this mode of activity in DA neurons.

Mitochondrial oxidative stress generated by calcium currents during pacemaking was reported to be enhanced in SN DA neurons that lack the PD gene *DJ-1* (Guzman et al., 2010) in agreement with the putative role of this gene in regulating oxidant defenses. This type of stress was also augmented in perinuclear and dendritic compartments of DA neurons exhibiting intracellular α -syn LB-like inclusions formed *in vitro* by recruitment of preformed fibrils of α -syn (Dryanovski et al., 2013), providing a new link between dysregulated proteostasis and mitochondrial dysfunction in PD pathogenesis. In line with this last observation, Subramaniam and colleagues (2014) also reported a progressive and sustained increase in spike rate in mouse nigral DA neurons overexpressing A53T mutant α -syn. Overall, these findings suggest that L-type calcium channel blockers may be helpful in PD. Epidemiological studies of dihydropyridine class L-type calcium channel blockers for association with PD have so far yielded conflicting results, however (Rees et al., 2011).

There are alternative scenarios by which high Ca²⁺ levels may become deleterious for SN DA neurons. The calcium elevation may increase DA synthesis through the activation of tyrosine hydroxylase (TH) (Rittenhouse and Zigmond, 1999), thus causing intracellular damage via autooxidation of the neurotransmitter (Michel and Hefti, 1990) or post-translational

modifications of the PD gene α -syn (Martinez-Vicente et al., 2008). Calcium overload may also exert adverse effects for DA neurons through activation of calpains, a family of calcium-dependent cysteine proteases. Dufty and colleagues (2007) showed the presence of C-terminally calpain-cleaved α -syn in LB in brains of PD patients. Most interestingly, overexpression of the endogenous and specific inhibitor of calpain calpastatin in human A30P α -syn transgenic mice caused a reduction of α -syn-positive aggregates (Diepenbroek et al, 2014). In keeping with these findings, calpains are known to induce cleavage of cdk5, a protein that has a facilitating role in DA cell death (Smith et al, 2003). Thus, calpains and possibly cdk5 inhibitors may be attractive candidate targets for therapeutic intervention in PD.

Regardless of the nature of the mechanism actually causing $\text{Ca}^{2+}_{\text{cyt}}$ overload, the capacity of mitochondria to handle calcium through uptake or efflux mechanisms remains probably crucial to prevent PD-related neurodegenerative changes (Celardo, 2014). Illustrating this point is the demonstration that deficiency in the PD protein PINK1 leads to mitochondrial calcium ($\text{Ca}^{2+}_{\text{mit}}$) overload due to a reduction of calcium efflux via the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Gandhi et al., 2009). Mitochondrial Ca^{2+} overload and ensuing PD-neurodegenerative events may result from direct accumulation of Ca^{2+} from the cytosol to mitochondria through the mitochondrial Ca^{2+} uniporter (MCU) (Guzman et al, 2010; Qiu et al, 2014). Mitochondria, often in close apposition to the ER, can also accumulate Ca^{2+} into the matrix through the coordinated activation of ER inositol-1,4,5-triphosphate (IP3) receptors and MCU (Celardo et al, 2014). Therefore, $\text{Ca}^{2+}_{\text{mit}}$ overload and mitochondrial oxidative stress in DA neurons may also be due to the fact that too much Ca^{2+} is pumped out of the cytosol into the ER through the high affinity sarco-endoplasmic reticulum Ca^{2+} -ATPase (Guzman et al., 2010; Rivero Rios et al., 2014), a process energetically costly which may as such further amplify mitochondrial dysfunction in a vicious cycle.

Despite large evidence implicating stressful pacemaking and calcium overload in PD progression, it appears that the survival of these neurons may be similarly compromised when pacemaking is reduced and intracellular calcium drops below threshold levels (Michel et al., 2013). This notion is actually supported by the following observations; (i) The dopaminergic toxins MPP⁺ and rotenone cause an early reduction of DA neuron firing in rodent midbrain slices (Liss et al, 2005; Yee et al., 2014) and preserving this activity by genetic inactivation of Kir6.2, the pore forming subunit of K_{ATP} channels favors the survival of these neurons (Liss et al, 2005); (ii) Toxic effects of MPP⁺'s prodrug MPTP are reduced *in vivo* by the brain-penetrant blocker of small conductance calcium-activated potassium channels apamin (Alvarez-Fischer et al, 2013) and by the alkaloid nicotine (NIC) (for review see Quik et al, 2012), two compounds that raise Ca²⁺_{cyt} levels (Toulorge et al, 2011) in DA neurons and stimulate them electrically (Wolfart et al, 2001; Teo et al, 2004); (iii) Tobacco smoking reduces PD risk, presumably through an effect of its major component NIC (Quik et al, 2012). Still in line with this hypothesis, Hirsch and colleagues (1988) found that the rate-limiting enzyme in DA synthesis, TH whose expression is activity- and calcium-dependent (Brosenitsch and Katz, 2001), disappears entirely from a large population of neuromelanized SN DA neurons in PD. Interestingly, SN DA neurons demonstrated decreased discharge frequencies in aged mice that express disease relevant levels of wild-type α -syn from the complete human *SNCA* locus (Janezic et al., 2013). Because DA neurons in these mice die in the absence of overt aggregation pathology, one may assume that pre-aggregates of α -syn were the cause of reduced spiking and that this deficit may represent an early biomarker of neurodegeneration. One way to reconcile this set of results with data implicating activity-dependent calcium entry in DA cell death would be to assume that DA neurons go successively during degeneration through hypo- and hyperactive phases during which they endure calcium deficiency and calcium overload, respectively. Such interpretation would be coherent with data reported in the MitoPark mouse,

a genetic mitochondrial model of PD where silent DA neurons appear physiologically less compromised than hyperactive ones (Good et al, 2011).

The possible contribution of activity and calcium deficits to the death of DA neurons may imply that these neurons have a fundamental need for proteins regulated by calcium. PI3K may represent one of the cytosolic proteins required for survival (Ries et al., 2009; Toulorge et al., 2011). The concept that calcium needs to remain above a threshold level to enable survival of DA neurons is perhaps best illustrated by the mechanism whereby the depolarizing alkaloid NIC provides protection to spontaneously dying DA neurons in midbrain cultures. Indeed, NIC was protective only if $\text{Ca}^{2+}_{\text{cyt}}$ was raised above threshold levels by concurrent depolarizing stimuli, presumably to keep $\alpha 7\text{nAChRs}$, which intervene in the protective action of NIC both *in vitro* (Toulorge et al., 2011) and *in vivo* (Bordia et al., 2014), in an active conformational state (Changeux, and Edelstein, 1998). Moreover, under adequate depolarizing conditions NIC-mediated neuroprotection in itself required calcium influx.

Calcium stored in the ER may also enable DA neurons to regulate their own survival. In particular, elevating basal $\text{Ca}^{2+}_{\text{cyt}}$ through ER ryanodine receptor channel (RyR) activation provided protection to midbrain DA neurons in several culture paradigms where neurodegeneration is either spontaneous or induced by trophic support deprivation or MPP^{+} intoxication (Guerreiro et al., 2008). The beneficial action of ER calcium mobilization was attributed to the action of $\text{Ca}^{2+}_{\text{cyt}}$ on a putative protein target required for DA cell survival but it was also proposed that the resultant decrease in ER Ca^{2+} load may be beneficial by limiting mitochondrial Ca^{2+} accumulation occurring through ER calcium mobilization (Rivero-Rios et al., 2014). Reducing calcium shuttling from ER to mitochondria through blockade of ER IP3R or inhibition of the mitochondrial calcium uniporter (MCU) was, however, detrimental for DA neurons (Rousseau et al., 2013), indicating that calcium transfer between these two compartments has to remain at a physiological level, presumably to maintain the mitochondrial

bioenergetic machinery functional in these neurons and ultimately preserve their survival (Cali et al., 2013). Consistent with this interpretation, an early and concomitant depletion in $\text{Ca}^{2+}_{\text{mit}}$ (Rousseau et al., 2013) and ATP (Höglinger et al., 2003) preceded DA neuronal loss in midbrain cultures exposed to the mitochondrial toxin MPP^+ . These findings also need to be placed in perspective with data showing that the PD protein α -syn facilitates $\text{Ca}^{2+}_{\text{mit}}$ transients elicited by IP3R activation whereas α -syn loss of function in addition to reducing Ca^{2+} fluxes into mitochondria also results in increased autophagy (Cali et al., 2012). Coherent with these observations, α -syn was reported to associate with mitochondria-associated ER membranes (Guardia-Laguarta et al., 2014), a structurally and functionally distinct subdomain of the ER. Two other PD-associated proteins, DJ-1 (Ottolini et al., 2013) and parkin, were also reported to facilitate ER-mitochondria tethering upon stimulation of IP3Rs (Cali et al., 2013), whereas mutated forms of DJ-1 and parkin siRNA impaired this process. Figure 5 summarizes how calcium-related events could participate in DA cell loss in PD.

Concluding Remarks

Within a period of just 20 years, we have witnessed tremendous progress in our understanding of PD pathogenesis. Before the synuclein era, research efforts on the pathomechanisms of PD were mostly focused on mitochondrial bioenergetic deficits, oxidative stress and death pathways involved in apoptosis (Jenner and Olanow, 1996; Mizuno et al., 1995). They have now expanded to embrace a myriad of molecular and biochemical defects affecting key cellular functions such as autophagic pathways, mitochondrial quality control and dynamics and the unfolded protein response. The identification of genes linked to inherited forms of PD has unquestionably contributed massively to this explosion of new knowledge (Table 1), which in turn should foster the discovery of more efficient therapeutic tools. Yet, the other side of the coin of this success is the increasing complexity of the molecular pathways

involved in neurodegeneration which are interconnected and influence each other in a still poorly defined dynamic cascade. Adding to this complexity is the fact that non-cell autonomous mechanisms are likely to be important in participating and/or modulating disease mechanisms as well. For instance, neuroinflammatory processes orchestrated by both innate and adaptive immune cells have emerged as important contributors of pathogenesis in PD (Hirsch and Hunot, 2009). Non-cell autonomous mechanisms are likely also involved in the spreading of α -syn pathology. Indeed, mounting evidence suggests that α -syn can undergo a toxic templated conformational change and spread from cell to cell to seed and initiate the formation of aggregates in the newly “contaminated” neurons (for review see Tyson et al., 2015). According to such a scenario, the seeding-prone exogenous α -syn aggregates can be viewed as non-cell autonomous culprits that could drive disease progression. Yet, despite strong experimental evidence for α -syn spreading, the possibility that such spreading actually happens in a pathophysiological context and at disease relevant level of α -syn is still lacking. Such knowledge would however have far reaching therapeutic consequences since trapping and subsequent degradation of exogenous neuron-released aggregates by antibodies through active or passive immunization has emerged as a powerful neuroprotective approach in preclinical settings (for review see Dehay et al., 2015).

To better capture disease complexity, systems biology, which relies on the computational and mathematical modeling of complex biological systems, has emerged as a powerful approach to understand in a comprehensive way the intricate interactions between the dysregulated pathways involved in disease mechanisms (Funke et al., 2013; Lausted et al., 2014; Fujita et al., 2014). Such approaches have the potential not only to improve our understanding of disease initiation and progression but also to help the discovery and implementation of more effective therapeutic strategies. While well advanced in the cancer research field (Du and Elemento, 2014), systems biology in PD is still in its infancy, with very

few studies that have started to develop and use bioinformatic tools for therapeutic target and biomarker identification (Büchel et al., 2013; Chandrasekaran and Bonchev, 2013; Ouzounoglou et al., 2014; Dusancho et al., 2014). However, with the rapid growth of PD-related knowledge and technological advances enabling massive databases to be generated from high-throughput multi-omics profiling, it is predicted that such global and integrated approaches will gradually be needed and will prove useful in identifying new therapeutic targets, predicting disease outcome of treatments, and guiding treatment strategies. In line with the latter point, it is increasingly recognized that there is not a single PD but several PD forms caused by different factors, all leading to a common pathological denominator that is the massive and preferential loss of nigral dopaminergic neurons and, to a lesser extent, synucleinopathy. Genetic and environmental factors are the two major classes of etiological culprits involved in PD, with pure genetic or environmental forms representing ~10% of all cases (Lesage and Brice, 2012). For most PD forms, however, it is generally accepted that disease results from a complex combination of genetic and environmental factors. Yet, their respective influence on disease initiation could likely vary from one individual to another. For instance, occupational exposure to pesticides may outweigh genetic burden in PD development in farmers (Elbaz et al., 2009). On the other hand, carriers of loss-of-function mutations in glucocerebrosidase (GBA) that have been reliably identified as having a significant risk factor for sporadic PD (Lesage and Brice, 2012), may develop the disease mostly because of this genetic defect affecting key cellular functions in neuronal homeostasis. Thus, notwithstanding the convergence mode of death mechanisms leading to nigral dopaminergic degeneration in PD, it is conceivable that the number and respective importance of the death pathways described here may vary from one patient to another. Consequently, therapeutic strategies for preventing or slowing disease progression might be more efficient if personalized to each PD case. Such individualized medicine will only be possible if one can accurately identify and integrate factors

involved in the development of the disease in a given subpopulation of patients and will greatly benefit from the characterization and validation of biomarkers than can be used to establish a molecular diagnosis (Gotovac et al., 2014). Associated with a state-of-the-art clinical evaluation personalized molecular diagnostic tests have the potential to identify PD patients more likely to respond to a given treatment.

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Table 1. Cell Autonomous Mechanisms By Which PD-Related Proteins Cause Degeneration of DA Neurons

Gene	Protein normal activity	Cellular localization	Inherited pathology	Pathomechanisms linked to PD protein dysfunction	References ^d
<i>α-synuclein/SNCA</i>	Vesicle dynamics and trafficking	Synaptic vesicles, Mitochondria-associated ER membranes	AD PD	↑LB formation ↑Mitochondria dysfunction ↓UPS activity ↓macroautophagy ↓CMA ↑Golgi fragmentation ↑ER stress and UPR ↓ER-mitochondria Ca ²⁺ transfer ↓Pacemaking ^b ; ↑Pacemaking ^c	Gosavi et al., 2002; Lindersson et al., 2004; Martin et al., 2006; Parihar et al., 2008; Devi et al., 2008; Martinez-Vicente et al., 2008; Kamp et al., 2010; Winslow et al., 2010; Nakamura et al., 2011; Zhu et al., 2011; Colla et al., 2012a; Cali et al., 2012; Janezic et al., 2013; Dryanovski et al., 2013 ; Guardia-Laguarta et al., 2014 ; Subramaniam et al., 2014
<i>Parkin/PARK2</i>	E3 ubiquitin protein ligase	Cytosol	AR PD	↓mitophagy ↓proteasomal activity ↓ER-mitochondria Ca ²⁺ transfer	Kitao et al., 2007; Geisler et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010 ; Boumann et al., 2011; Imai et al., 2011; Cali et al., 2013.
<i>PINK1</i>	Serine/threonine protein kinase	Mitochondria outer membrane	AR PD	↓mitophagy ↑Ca ²⁺ _{mit} (possibly by reduced extrusion)	Gandhi et al., 2009; Vives-Bauza et al., 2010;
<i>DJ-1/PARK7</i>	Oncogene	Mitochondria	AR PD	↑mitochondrial oxidant stress ↓autophagy ↓ER-mitochondria tethering ↓Ca ²⁺ uptake by mitochondria	Aron et al., 2010 ; Guzman et al., 2010; Thomas et al., 2011; Kim et al., 2005; Ottolini et al., 2013.
<i>LRRK2</i>	GTPase/kinase	Cytosol, intracellular membranes and organelles	AD PD	↓CMA ↑α-syn pathology ↑ER stress (?) ↑lysosomal pH	Lin et al., 2009 ; Tong et al., 2009; Yuan et al., 2011; Gómez-Suaga et al., 2012 ; Orenstein et al., 2013.
<i>ATP13A2</i>	5 P-type ATPase cation metal transporter	Lysosomes	AR PD-related disorder with dementia	↑α-syn pathology ↓lysosomal function	Gitler et al., 2009; Usenovic and Krainc, 2012; Dehay et al., 2012; Murphy et al., 2013.

<i>GBA</i>	Glucocerebrosidase	Lysosomes	Gaucher disease + Parkinsonism ^a (AR)	↑ α -syn pathology ↓lysosomal function ↑PD risk in single mutant <i>GBA</i> allele carriers	Balducci et al., 2007; Sidransky et al., 2009 ; Sardi et al., 2013; Murphy et al., 2014 ; Parnetti et al., 2014.
<i>VPS35</i>	Part of the retromer complex	Endosomes	AD PD	↑ α -syn ↑size of endosomes/lysosomes ↓ retromer function ↓ LAMP-2A retrieval by trans-Golgi	Vilariño-Güell et al. 2011; Zimprich et al. 2011; Tsika et al., 2014; Tang et al., 2015.
<i>eIF4G</i>	Translation initiation factor	Cytosol	AD PD	Genetically and functionally interacts with VPS35	Dhungel et al, 2015

^a In non-neuronopathic forms of Gaucher disease; ^b In DA neurons expressing the human protein under the control of its native promoter and regulatory elements; ^c In DA neurons overexpressing A53T mutant α -syn under the control of an heterologous promoter; ^dReferences related to pathomechanisms.

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Figure Legends

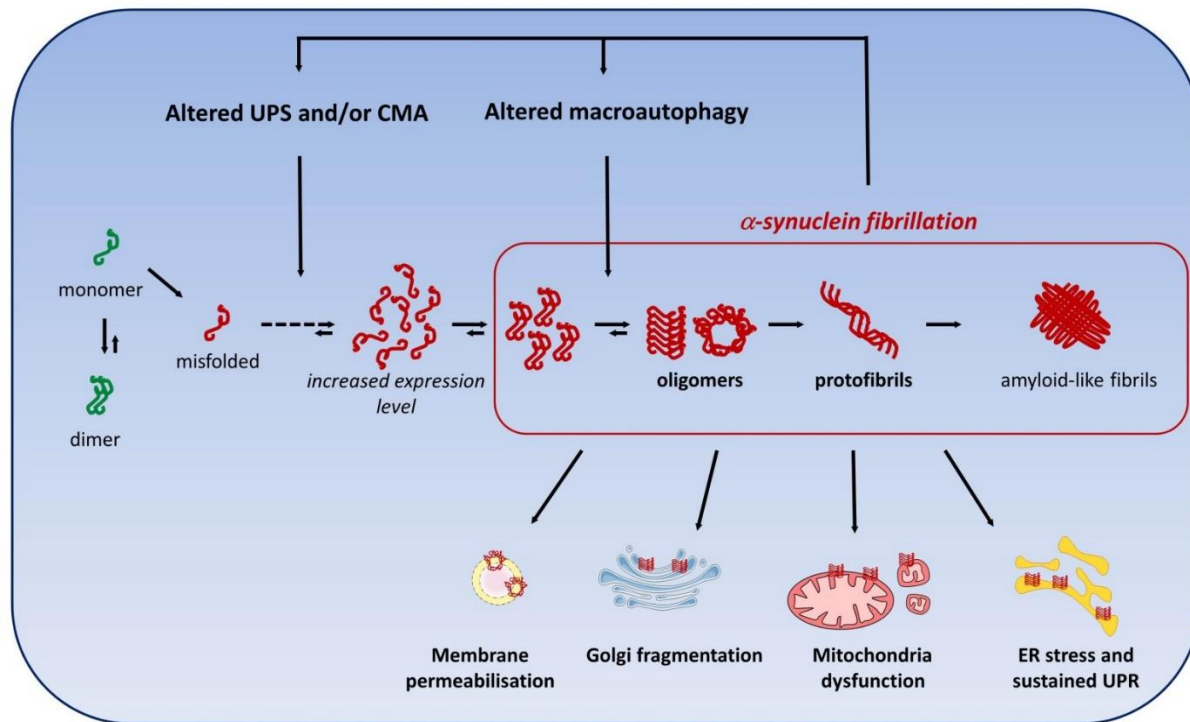


Figure 1. Possible causes and consequences of synuclein aggregation in PD

Compelling and still growing evidence have pointed out the central role of α -synuclein misfolding and aggregation in neuronal cell death in PD. The fibrillation process of α -synuclein involves the aggregation of unfolded or misfolded monomers into dimers then small oligomeric species that can be stabilized by β -sheet-like interactions. At this stage, synuclein aggregation evolves to higher molecular weight insoluble protofibrils and culminates in the formation of amyloid-like fibrils building-up into pathological structures such as Lewy bodies. Among the factors initiating and/or favoring α -synuclein fibrillation, imbalance between expression and degradation can cause progressive accumulation of full-length wild-type α -synuclein leading to oligomer formation. In particular, dysfunction of the ubiquitin proteasome system (UPS) and chaperone-mediated autophagy (CMA) degradation pathways may be involved in accumulation of misfolded and/or unfolded α -synuclein favoring subsequent fibrillation. Similarly, impairment of the autophagy-lysosome pathway may be involved in inefficient elimination of higher molecular weight of α -synuclein (such as oligomers and fibrils)

through macroautophagy. Importantly, evidence suggests that α -synuclein assemblies, in particular, can directly impair these degradation pathways thus amplifying the fibrillation process. In turn, oligomers and protofibrils of α -synuclein are believed to be particularly toxic for neurons. They can alter key cellular functions including mitochondrial and ER homeostasis as well as promote fragmentation of the Golgi apparatus and the permeabilization of membranes.

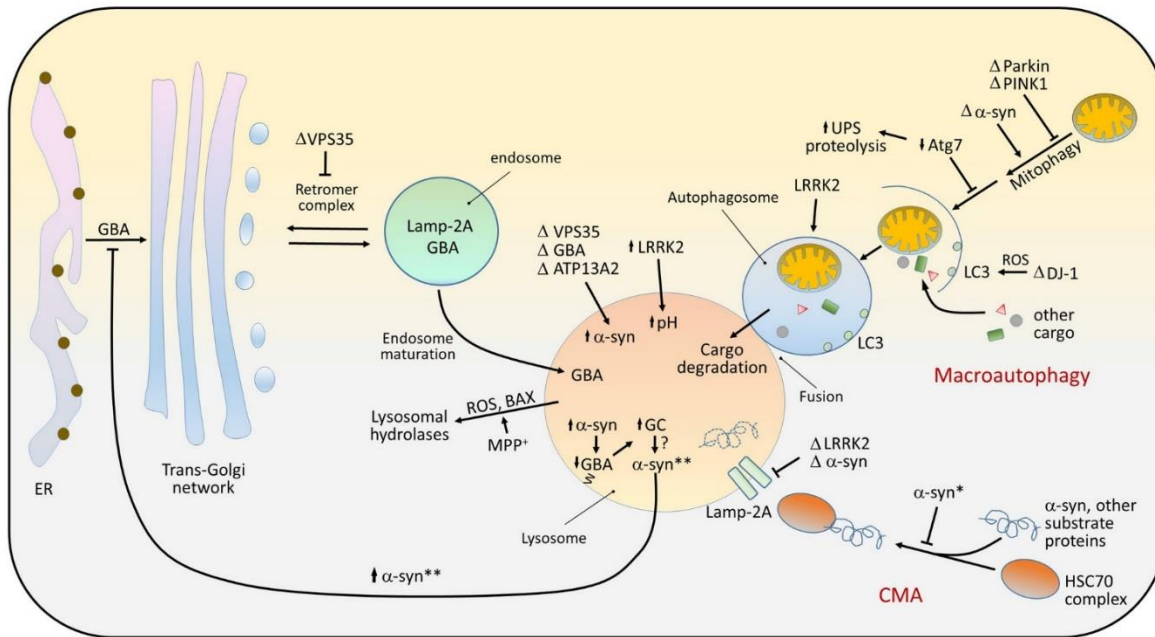


Figure 2. Dysfunction of the autophagy-lysosome system in PD.

Both chaperone-mediated autophagy (CMA) and macroautophagy (referred as autophagy) have a pathogenic role in PD. CMA is a selective type of autophagy responsible for lysosomal degradation of a highly specific subset of soluble cytosolic proteins containing a motif recognized by the cytosolic chaperone heat shock cognate protein 70 (HSC70). Wild-type α -syn is a substrate for CMA and post-transcriptionally modified α -syn (*) exert inhibitory effects on this process. The function of the CMA receptor LAMP-2A is compromised by LRRK2 and α -syn mutations. Bulk degradation of unwanted cellular components such as misfolded proteins or damaged organelles including mitochondria occurs, however, by macroautophagy, a self-destructive process whereby double-membrane-bound structures called autophagosomes engulf cytosolic materials before delivery to the hydrolytic milieu of lysosomes for degradation. Autophagosomes are increased in vulnerable DA neurons from PD patients. Conditional deletion of the autophagy gene *Atg7* (the protein Atg7 being an enzyme required for autophagosome formation) in DA neurons results in delayed neurodegeneration of these neurons. Ubiquitin Proteasome System (UPS) proteolysis is induced to compensate autophagy deficits induced by Atg7 dysfunction. Loss of the recessive PD-related protein DJ-1 that triggers mitochondrial dysfunction related to oxidative stress results in the accumulation of LC3, an inner component of autophagosomal membranes. Mutant A53T α -syn stimulates mitophagy and cell death. PINK1

and Parkin operate cooperatively to eliminate bioenergetically compromised mitochondria whereas mutated forms of these proteins impair this process. The PD-related protein LRRK2 modulates autophagosome numbers and regulates the acidic environment in lysosomes. Ectopic release of lysosomal proteases is observed after treatment with the dopaminergic toxin MPP⁺. ROS and the pro-apoptotic protein BAX may intervene as mediators of this process. Mutations in genes encoding proteins of the endosomal/lysosomal system, vacuolar protein sorting-35 (VPS35), type 5 P-type ATPase ATP13A2 and glucocerebrosidase (GBA), result in PD syndromes. VPS35 is part of the retromer, a protein complex that associates with the cytosolic face of early endosomes to regulate retrograde transport of cargo proteins (membrane receptors, transporters) to the trans-Golgi network, to rescue them from lysosome-mediated turnover. VPS35 deficient DA neurons accumulate α -syn, contain enlarged late endosomes/lysosomes and exhibit impaired endosome-to-Golgi retrieval of the CMA receptor LAMP-2A. ATP13A2, is localized in acidic membrane compartments of lysosomes. ATP13A2 loss of function impairs lysosomal catabolism and promotes accumulation of α -syn. GBA is involved in the lysosomal degradation of glucosylceramide (GC). Individuals carrying a single mutant *GBA* allele have an increased PD risk. GBA activity is reduced in PD patients regardless of whether or not these patients harbor mutations in the enzyme. Two mechanisms may account for the reduced activity of GBA in sporadic PD; (i) oligomeric species of α -syn (***) impair ER-Golgi trafficking of GBA and ultimately its storage within lysosomes; (ii) α -syn operates as a direct inhibitor of GBA bound at the internal membrane surface of lysosomal vesicles. Unprocessed GC that accumulates in lysosomes when GBA activity is reduced, may also promote the stabilization of α -syn soluble oligomers.

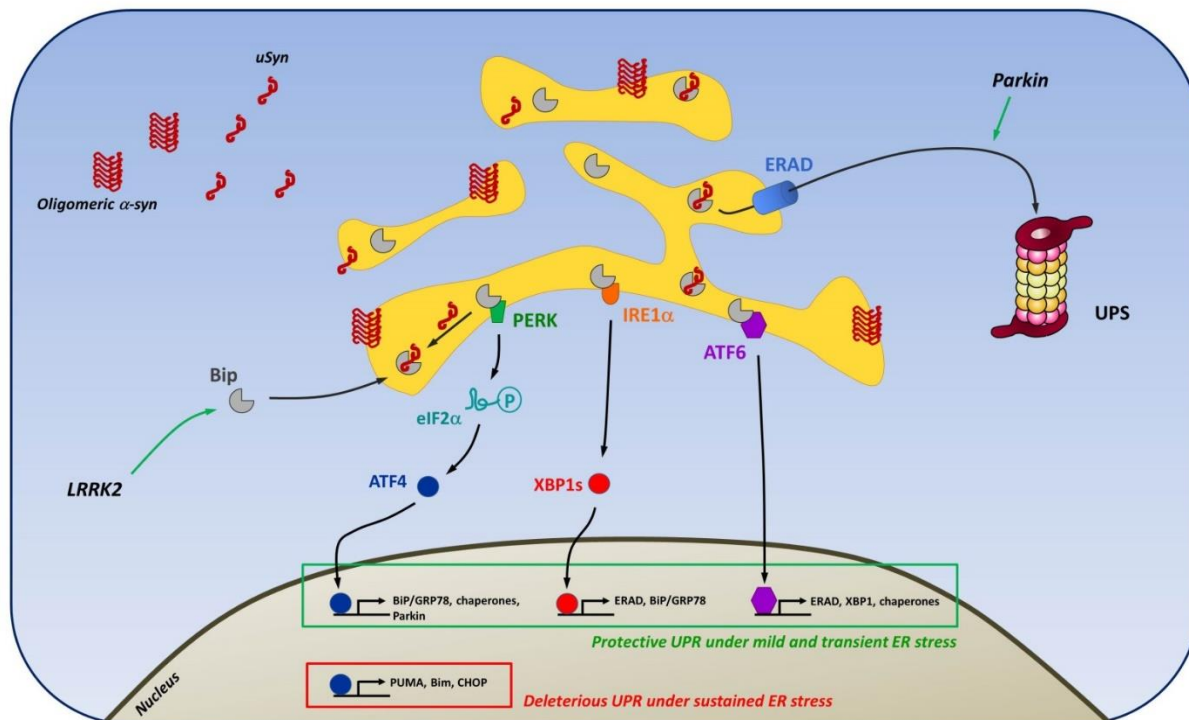


Figure 3. Endoplasmic reticulum stress and unfolded protein response in PD.

In PD, accumulation and aggregation of misfolded/unfolded α -syn may promote sustained endoplasmic reticulum (ER) stress resulting in the activation of death mechanisms. ER stress usually induces a protective response termed unfolded protein response (UPR) that involves a complex molecular network in which stress sensors lying at the ER membrane (PERK, IRE1 α , ATF6) transduce an unfolded protein signal to the nucleus through different mechanisms and intermediate factors. Notably, IRE1 α promotes X-box binding protein 1 (XBP1) splicing (XBP1s) while PERK activation results in eIF2 α phosphorylation that in turn induces Activating transcription factor 4 (ATF4). XBP1s, ATF4 and ATF6 translocate into the nucleus where upon binding to specific DNA responsive elements they regulate gene transcription. The ER chaperone Bip, a heat shock protein 70 kDa family member, maintains the stress sensors in an inactivated state upon binding to their luminal domains but dissociates from and activates them upon binding to unfolded α -syn (uSyn). In turn, Bip-uSyn complexes are targeted for degradation by the ER-associated degradation (ERAD) and ubiquitin proteasome (UPS) systems. Of note, Parkin, an E3 ubiquitin ligase and UPS component, is up-regulated upon

ER stress presumably to participate in the degradation of unfolded proteins through the ERAD pathway. Moreover, LRRK2 could also be involved in the protective UPR by stimulating the expression of Bip. At the nucleus, gene regulation by UPR results in suppression of protein synthesis, degradation of unfolded proteins by the ERAD system and induction of ER chaperones. Yet, under chronic ER stress, the accumulation of unfolded proteins and sustained UPR activity triggers activation of pro-apoptotic pathways and cell death, thereby eliminating damaged cells. In particular, the transcription factor ATF4, a downstream effector of the PERK/eIF2a pathway, regulates the expression of pro-apoptotic factors PUMA and BIM as well as other transcription factors involved in the induction of apoptosis, including C/EBP homologous protein (CHOP).

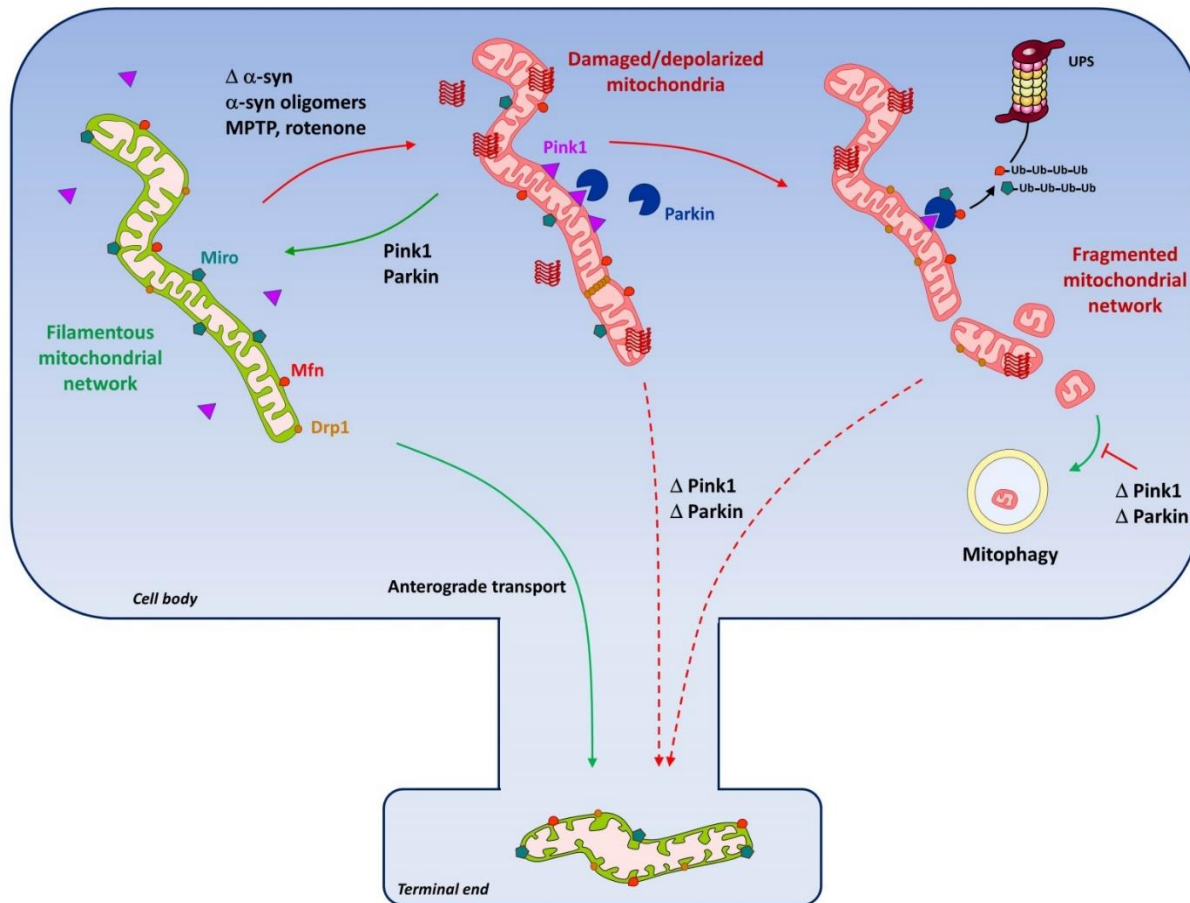


Figure 4. Mitochondrial dysfunction in PD.

At the mitochondrial level, α -syn oligomer accumulation induces mitochondrial fragmentation. Fragmented mitochondrial network is a hallmark of dysfunctional mitochondria and can also be induced by the PD-related mitochondrial poisons MPTP and rotenone and more generally by oxidative stress. Compelling evidence suggest that Pink1 and Parkin play a critical role in conducting the fragmentation and elimination of compromised mitochondria through mitophagy. Damaged/depolarized mitochondria induces the recruitment and accumulation of PINK1 at the outer mitochondrial membrane (OMM) which in turn recruit and activate Parkin, a E3 ubiquitin ligase involved in the ubiquitin proteasome system (UPS)-dependent degradation pathway. Parkin-dependent ubiquitination of the OMM fusion-prone protein Mitofusin (Mfn) results in its degradation by the UPS and favors the fragmentation of the damaged mitochondrial network together with Dynamin related protein 1 (Drp1) that controls the last step of mitochondrial fission. Fragmented mitochondria are then eliminated through their isolation into autophagosomes and subsequent delivery to lysosomes for degradation (mitophagy). Besides Mfn, another important OMM protein involved in the anterograde transport of mitochondria along the axon, Miro, is also degraded through Parkin-and UPS-

dependent mechanisms. Miro degradation abates damaged mitochondria transport to terminal ends and concurs to the elimination of fragmented organelles by mitophagy. Mutations in Parkin and PINK1 may compromise mitochondria quality control pathway and mitochondria dynamics leading to reduced mitophagy and the accumulation of dysfunctional mitochondria.

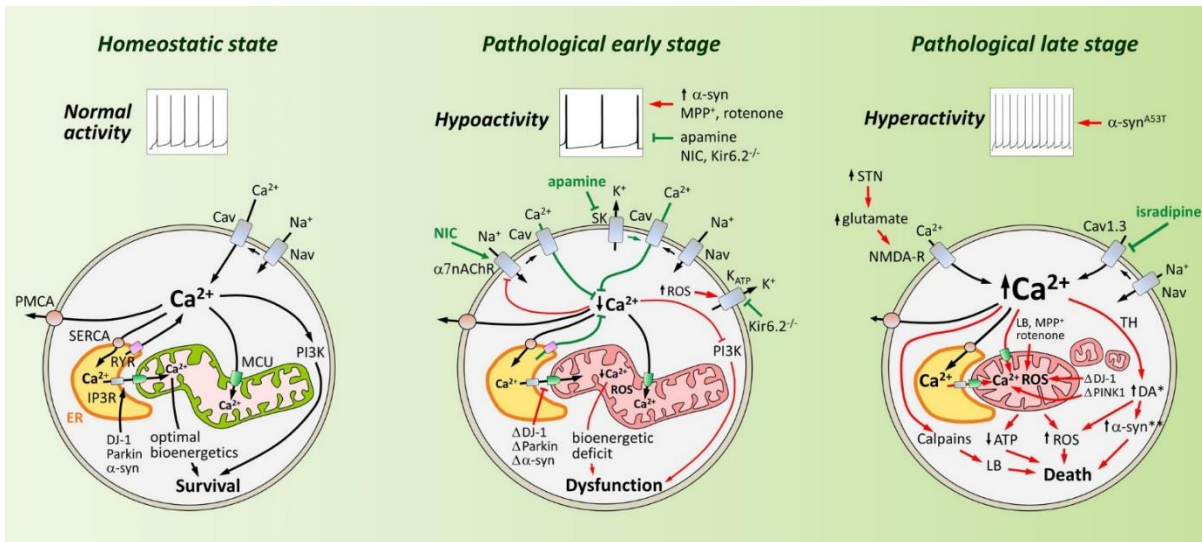


Figure 5. Role of activity- and calcium-dependent mechanisms in DA cell loss in PD: possible link with the fibrillation process of α -synuclein.

DA neurons are particularly vulnerable to alterations that affect their mode of discharge and their capacity to handle calcium. Therefore, during degeneration they undergo hypo- and hyperactive states during which they endure calcium deficiency and calcium overload, respectively. *Homeostatic state*: DA neurons do not rely only on Na⁺ channels (Nav) to drive autonomous pacemaking but also engage L-type voltage-dependent calcium channels some of them having a Cav1.3 pore. Pacemaking causes an increase in Ca²⁺_{cyt} and secondarily in Ca²⁺_{mit}. Transfer of calcium between the cytosol and mitochondria occurs through the mitochondrial uniporter (MCU), either directly or indirectly. In the latter case, Ca²⁺ is first pumped out of the cytosol into the ER through the high affinity sarco-endoplasmic reticulum Ca²⁺-ATPase (SERCA) before being transferred to mitochondria by sequential activation of ER IP3R and MCU. The increase in mitochondrial calcium stimulates basal mitochondrial oxidant stress in SN DA neurons and places them under an increased demand for ATP to maintain proper Ca²⁺_{cyt} concentrations via the SERCA and the plasma membrane Ca²⁺ ATPase (PMCA). *Hypoactive state*: DA neurons are particularly vulnerable when they become less active and basal Ca²⁺_{cyt} drops below threshold levels. These changes may occur early during degeneration and may precede a phase of hyperactivity. A reduction in the activity of DA neurons can be experimentally produced by a treatment with mitochondrial toxins such as MPP⁺ and rotenone or by expression of α -syn, at disease-relevant levels. Rescue against PD toxins can be achieved by preserving the

activity of DA neurons through genetic inactivation of K_{ATP} channels, e.g., channels that become activated in response to oxidative stress or by stimulation of DA neurons by the alkaloid nicotine (NIC) or the bee venom component apamine. Protection by apamine and NIC occurs by blockade SK channels and by activation of $\alpha 7$ nAChRs, respectively. The maintenance of Ca^{2+}_{cyt} above threshold levels (and below toxic levels) reveals the protection by NIC and also permits the activation of protein targets such as PI3K required for survival. Preserving calcium shuttling from ER to mitochondria through sequential activation of IP3R and MCU appears also critical for DA neuron survival because this transfer is required for optimal mitochondrial bioenergetics. ER mitochondria calcium shuttling is favored by wild-type PD proteins Parkin, α -syn and DJ-1 whereas mutated forms of DJ-1 and α -syn or parkin siRNA silencing impaired this process. Reduced Ca^{2+}_{mit} levels below threshold levels may be a trigger for DA cell death whereas treatments that restore mitochondrial calcium towards optimal levels provide neuroprotection. *Hyperactive state*: Mitochondrial oxidant stress evoked by pacemaking is enhanced by lack of the PD protein DJ-1 and accumulation of intracellular α -syn LB-like inclusions. Overexpression of A53T mutant α -syn also increases the spike rate in nigral DA neurons. Deficiency in the PD protein PINK1 leads to Ca^{2+}_{mit} overload by reduction of mitochondrial calcium efflux. The rise in basal mitochondrial oxidant stress also reduces the bioenergetic reserve capacity of DA neurons and makes them particularly vulnerable to mitochondrial toxins (MPP^{+} , rotenone). Isradipine, an L-type Ca^{2+} channel blocker with some specificity for Cav1.3 channels reduces basal mitochondrial oxidant stress in SN DA neurons and as a consequence lowers their sensitivity to mitochondrial toxins. Alternatively, the elevation in Ca^{2+}_{cyt} may become deleterious for SN DA neurons by stimulating DA synthesis through the activation of TH, thus causing intracellular damage via autoxidation of the neurotransmitter (*) and post-translational modifications of α -syn (**). Calcium overload may also exert adverse effects through activation of calpains, (i.e., calcium-activated proteases). Indeed, calpain-cleaved α -syn fragments were identified within LBs. Note that abnormal calcium influx may be aggravated by subthalamic nucleus (STN)-mediated excitotoxicity through a mechanism involving NMDA glutamate receptors.