

## Mitochondrial Ca<sup>2+</sup> and neurodegeneration

Tito Cali<sup>a</sup>, Denis Ottolini<sup>b</sup>, Marisa Brini<sup>a,\*</sup>

<sup>a</sup> Department of Comparative Biomedicine and Food Science, University of Padova, Padova, Italy

<sup>b</sup> Department of Biomedical Sciences, University of Padova, Padova, Italy

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### ABSTRACT

Mitochondria are essential for ensuring numerous fundamental physiological processes such as cellular energy, redox balance, modulation of Ca<sup>2+</sup> signaling and important biosynthetic pathways. They also govern the cell fate by participating in the apoptosis pathway.

The mitochondrial shape, volume, number and distribution within the cells are strictly controlled. The regulation of these parameters has an impact on mitochondrial function, especially in the central nervous system, where trafficking of mitochondria is critical to their strategic intracellular distribution, presumably according to local energy demands. Thus, the maintenance of a healthy mitochondrial population is essential to avoid the impairment of the processes they regulate: for this purpose, cells have developed mechanisms involving a complex system of quality control to remove damaged mitochondria, or to renew them. Defects of these processes impair mitochondrial function and lead to disordered cell function, i.e., to a disease condition. Given the standard role of mitochondria in all cells, it might be expected that their dysfunction would give rise to similar defects in all tissues. However, damaged mitochondrial function has pleiotropic effects in multicellular organisms, resulting in diverse pathological conditions, ranging from cardiac and brain ischemia, to skeletal muscle myopathies to neurodegenerative diseases. In this review, we will focus on the relationship between mitochondrial (and cellular) derangements and Ca<sup>2+</sup> dysregulation in neurodegenerative diseases, emphasizing the evidence obtained in genetic models. Common patterns, that recognize the derangement of Ca<sup>2+</sup> and energy control as a causative factor, have been identified: advances in the understanding of the molecular regulation of Ca<sup>2+</sup> homeostasis, and on the ways in which it could become perturbed in neurological disorders, may lead to the development of therapeutic strategies that modulate neuronal Ca<sup>2+</sup> signaling.

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### 1. Mitochondria in neuronal Ca<sup>2+</sup> homeostasis

Ca<sup>2+</sup> is the main second messenger that helps the biochemical machinery of neurons to connect their depolarization status with synaptic activity, which is their most crucial activity. Thus, neurons have developed extensive and intricate Ca<sup>2+</sup> signaling pathways to cope with the requirement. Neuronal Ca<sup>2+</sup> signals are mainly generated by the influx through the voltage dependent or ligand activated Ca<sup>2+</sup> channels, such as ionotropic glutamate receptors (VOCs and ROCs) [1]. The release of Ca<sup>2+</sup> from intracellular stores (i.e. the endo/sarcoplasmic reticulum, ER/SR) by the opening of the inositol 1,4,5 trisphosphate (InsP<sub>3</sub>R) and the ryanodine (RyR) receptors contributes to the transmission of Ca<sup>2+</sup> signals [2]. The strict control of intracellular Ca<sup>2+</sup> concentration is operated by Ca<sup>2+</sup> binding proteins and by energy demanding Ca<sup>2+</sup> transport

proteins. The plasma membrane Ca<sup>2+</sup> ATPase (PMCA) and the plasma membrane Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX) extrude Ca<sup>2+</sup> into the extracellular space (however, the NCX can also operate in the reverse mode). The Ca<sup>2+</sup> pumps of the intracellular organelles, i.e., the ER/SR Ca<sup>2+</sup>ATPase (SERCA) and the secretory pathway Golgi Ca<sup>2+</sup> ATPase (SPCA) pumps, accumulate Ca<sup>2+</sup> in the intracellular stores [3]. Mitochondria also contribute to the spatiotemporal tuning of the cytosolic Ca<sup>2+</sup> concentration thanks to Ca<sup>2+</sup> uptake and release systems. The transport of Ca<sup>2+</sup> by these systems controls how much Ca<sup>2+</sup> enters the cell, the Ca<sup>2+</sup> concentration in cytoplasmic microdomains, the frequency of oscillatory cytosolic Ca<sup>2+</sup> signals and the rate of propagation of a Ca<sup>2+</sup> signal. In turn, mitochondria use their Ca<sup>2+</sup> transporting activity to modulate the rate of ATP synthesis in a number of ways, i.e., by activating Krebs cycle (TCA) dehydrogenases, by promoting the supply of oxidizable substrates and by regulating the activity of the ATP synthase [4,5].

The electron transport chain (ETC), i.e., the molecular machinery for energy production, is organized in five protein complexes located in the inner mitochondrial membrane. Three of these complexes (I, III, IV) pump protons (H<sup>+</sup>) across the inner membrane, thus establishing the electrochemical gradient which is then used

\* Corresponding author at: Department of Comparative Biomedicine and Food Science, University of Padova, Viale G. Colombo, 3, 35131 Padova, Italy.  
Tel.: +39 049 8276150; fax: +39 049 8276125.

E-mail address: [marisa.brini@unipd.it](mailto:marisa.brini@unipd.it) (M. Brini).

by complex V, the ATP synthase, to produce ATP. During their activity, electrons are transported from the reduced substrates, that accepted reducing equivalents from the TCA cycle (NADH and FADH<sub>2</sub>), to the oxygen which is converted to H<sub>2</sub>O. The electron transport, especially in complex I and III, also generates the free radical superoxide (O<sub>2</sub><sup>-•</sup>), most of which is converted to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by the manganese superoxide dismutase (MnSOD). H<sub>2</sub>O<sub>2</sub> is in turn converted to water by glutathione peroxidase and catalase. However, it can also generate the highly reactive hydroxyl radical (OH<sup>•</sup>) by reacting with Fe<sup>2+</sup> or Cu<sup>2+</sup>. Another highly reactive species, the peroxynitrite (ONOO<sup>-</sup>), is produced by the reaction of O<sub>2</sub><sup>-•</sup> with nitric oxide, and free radicals may be generated by the activity of the outer mitochondrial membrane (OMM) monoamine oxidases (MAOA and MAOB), enzymes involved in the metabolism of amines like serotonin, norepinephrine and dopamine. All these oxygen reactive species can induce membrane lipid peroxidation (damaging protein and DNA) or nitration of mitochondrial proteins at tyrosine residues, with consequent loss of function. However, it must be underlined that O<sub>2</sub><sup>-•</sup> and H<sub>2</sub>O<sub>2</sub> also serve important signaling functions in physiological processes (for a comprehensive review see Schon and Przedborski [6]).

The ETC, by generating the electrochemical gradient and, in turn, a difference of membrane potential across the inner mitochondrial membrane, also sustains Ca<sup>2+</sup> transfer into the mitochondrial matrix which occurs downhill the gradient through a low affinity uniporter. Two antiporters, the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger and the H<sup>+</sup>/Ca<sup>2+</sup> exchanger, move Ca<sup>2+</sup> out of the matrix, allowing the return of mitochondrial Ca<sup>2+</sup> to basal values after cell stimulation [7]. Thus, impaired abilities of neurons to maintain appropriate cellular energy levels, and increased generation of mitochondrial reactive oxygen species (ROS) may affect Ca<sup>2+</sup> signaling.

The functional properties of mitochondrial Ca<sup>2+</sup> transporters have been extensively characterized, and very recently some of them, i.e. the mitochondrial Ca<sup>2+</sup> uniporter (MCU), as well as one of its regulators (MICU1), and the Na<sup>+</sup>/Ca<sup>2+</sup> antiporter (NCLX) have also been molecularly identified [8–11]. Fig. 1 summarizes the main players in mitochondrial Ca<sup>2+</sup> transport.

Mitochondrial Ca<sup>2+</sup> accumulation depends on the proton electrochemical gradient that drives its rapid accumulation through MCU that, upon cell stimulation, becomes exposed to microdomains of high Ca<sup>2+</sup> concentration generated by the opening of the Ca<sup>2+</sup> channels in the membrane of the ER. The Ca<sup>2+</sup> microdomains are sensed by the MCU thanks to the close contact of mitochondria with the ER Ca<sup>2+</sup> channels and match its low Ca<sup>2+</sup> affinity [12]. The tethers linking the ER and mitochondria have been visualized [13] and, more recently, mitofusin 2 has been demonstrated to be an essential component of these structures [14]. The concept of mitochondria tethering has been extended also to the plasma membrane and the Golgi compartment [15,16].

In addition to the Ca<sup>2+</sup> transporting systems on the inner mitochondrial membrane (IMM), recent data have revealed an important role of the OMM permeability. Attention has been drawn to the potential role of a large-conductance channel, commonly referred to as the mitochondrial permeability transition pore (mPTP), in modulating mitochondrial Ca<sup>2+</sup>. As to the pathophysiological role, mPTP is most likely involved in the swelling and fragmentation of the mitochondrial network that underlie the release of caspase cofactors from mitochondria. Its molecular identity is still unknown, but it appears to be a multiprotein complex activated by various pathophysiological conditions (e.g. Ca<sup>2+</sup> increases in the mitochondrial matrix and oxidation of critical cysteines). The putatively essential components/regulators are the voltage dependent anion channel (VDAC) of the OMM, the adenine nucleotide transporter (ANT) of the IMM and cyclophilin D [17]. Interestingly, it has been shown that VDAC channels clustered at the ER/mitochondrial contact sites to play a key role in

the rapid transfer of the high Ca<sup>2+</sup> microdomain from the surface of mitochondria to the intermembrane space to which the MCU is exposed [18]. VDAC shuttles between open and closed states (with reference to metabolite transport) and in the closed state shows a higher permeability to Ca<sup>2+</sup>. Remarkably, Ca<sup>2+</sup> itself appears to control the conductance of VDAC, thus suggesting that, during the Ca<sup>2+</sup> signal, the OMM barrier is dynamically reduced and mitochondrial Ca<sup>2+</sup> uptake facilitated.

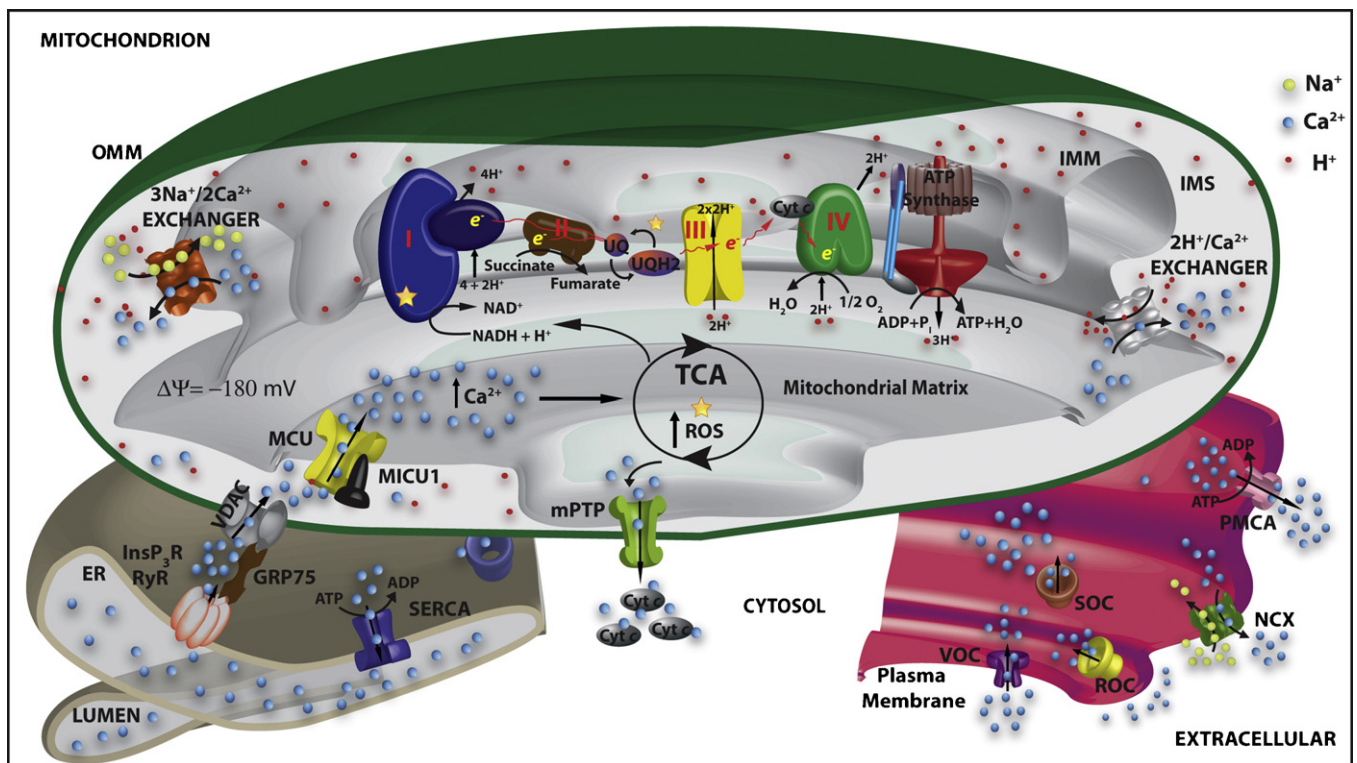
## 2. Mitochondrial Ca<sup>2+</sup> dysregulation and affected processes

Moderate increases in mitochondrial Ca<sup>2+</sup> concentration are necessary and sufficient to adjust ATP production to cell demand, but mitochondrial Ca<sup>2+</sup> overload (MCO) unequivocally leads to disruption of mitochondrial membrane integrity, permeability transition, irreversible oxidative damage and loss of ATP production, finally emerging in cell death in different pathological conditions. MCO can result essentially from three mechanisms: (i) increased mitochondrial Ca<sup>2+</sup> uptake, following release from the ER and Ca<sup>2+</sup> influx from the extracellular space; (ii) reduced Ca<sup>2+</sup> extrusion through the mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; and (iii) changes of mitochondrial Ca<sup>2+</sup> buffering. However, before MCO culminates in cell death, mitochondrial Ca<sup>2+</sup> dysregulation originates in a plethora of disturbances that cells try to control by modulating mitochondrial Ca<sup>2+</sup> related activities. In the following sections we will discuss them with respect to their regulation by Ca<sup>2+</sup>, bearing in mind that they represent the mitochondrial quality control system. Their impairment is a hallmark of the initial phases of neurodegeneration.

### 2.1. Mitochondrial trafficking and fusion/fission

The dynamic properties of mitochondria are critical to all cells but may be particularly important in neurons, due to their unique morphology. Synaptic mitochondria clear Ca<sup>2+</sup> from the cytosol either by directly taking it up or by providing ATP for the Ca<sup>2+</sup> extrusion (PMCA and NCX) and uptake (SERCA) systems. Thus, mitochondrial movements and fusion/fission processes are required, not only during cell division to properly distribute mitochondria to daughter cells, but also to transport mitochondria to their potential sites of action. At the molecular level, mitochondrial dynamics are mediated by three GTPases, namely mitofusins (Mfn1/2), optic atrophy1 (OPA1) and dynamin related protein 1 (Drp1), which are responsible for the mitochondrial fusion/fission process, and by the kinesin and dynein motors that, through the action of the adaptor protein Milton and the atypical GTPase Miro, mediate the traveling of mitochondria along cytoskeletal tracks. These two pathways are strictly connected since the balance between mitochondrial fission and fusion governs the shape and the number of mitochondria, but also their function and distribution. Intriguingly, Ca<sup>2+</sup> acts as the common molecular switch for both processes [19,20].

Mfns are integral OMM proteins and mediate mitochondrial fusion, together with OPA1. OPA1 is located in the intermembrane space, and, by interacting with the IMM, promotes its fusion and cristae remodeling. The process requires a proton gradient, thus mitochondria that are metabolically compromised are prevented from fusing. Mitochondrial fission is governed by the action of Drp1, which is recruited to mitochondria by several post-translational modifications such as phosphorylation and sumoylation. Ca<sup>2+</sup> controls Drp1-dependent fission through calcineurin and CaM-kinase activities, but also by a Miro-dependent Ca<sup>2+</sup> induced mechanism that enhances fusion at resting Ca<sup>2+</sup> concentration, and promotes fragmentation at high Ca<sup>2+</sup> levels [21], thus linking the motility process to fusion/fission. As to Mfns, no direct link with Ca<sup>2+</sup> regulation



**Fig. 1.** The main players in mitochondrial  $\text{Ca}^{2+}$  transport.  $\text{Ca}^{2+}$  flows into the cytoplasm upon cell stimulation and the opening of the inositol trisphosphate receptors ( $\text{InsP}_3\text{R}$ ), the ryanodine receptors ( $\text{RyR}$ ) at the ER/SR membranes and/or of the plasma membrane associated voltage (VOC), receptor (ROC) and store (SOC) operated calcium channels. The generation of localized high  $\text{Ca}^{2+}$  concentration microdomains drives  $\text{Ca}^{2+}$  into the mitochondrial matrix via the mitochondria  $\text{Ca}^{2+}$  uniporter (MCU), this action being potentiated by the voltage-dependent anion channel (VDAC) that, through GRP75, enhanced the ER  $\text{Ca}^{2+}$  transfer. MICU1 is a MCU associated protein that acts as MCU regulator. The efflux mechanism depends on the activity of the  $\text{H}^+/\text{Ca}^{2+}$  and the  $\text{Na}^+/\text{Ca}^{2+}$  exchangers. Increased mitochondrial  $\text{Ca}^{2+}$  concentration stimulates TCA cycle enzymes generating NADH and increasing ATP synthesis and ROS production. Sustained increases in mitochondrial  $\text{Ca}^{2+}$  concentration sensitize mitochondria to permeability transition pore (mPTP) opening with consequent release of cytochrome c (cyt c) and induction of apoptosis. Cytosolic  $\text{Ca}^{2+}$  clearance depends on the activity of the plasma membrane  $\text{Ca}^{2+}$  ATPase (PMCA), of the plasma membrane  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) and of the ER/SR  $\text{Ca}^{2+}$  ATPase (SERCA). ER, Endoplasmic reticulum; OMM, outer mitochondrial membrane; IMS, intermembrane mitochondrial space; IMM, inner mitochondrial membrane.

has been proposed. However, it must be taken into account that Mfn2 has been shown to favor ER-mitochondria tethering, implying that it could modulate the ER-mitochondria  $\text{Ca}^{2+}$  transfer through this physical coupling [14]. As to the motility process, Miro proteins are anchored to the OMM, interact with Milton and kinesin motors and, thanks to the presence of two  $\text{Ca}^{2+}$  binding EF-motifs in the cytosolic domain, they confer  $\text{Ca}^{2+}$  sensitivity to mitochondrial trafficking.

## 2.2. Mitochondrial $\text{Ca}^{2+}$ buffering

Recent work by different groups has clarified that the role of mitochondrial  $\text{Ca}^{2+}$  uptake is not limited to the control of organelle function, but also has a direct impact on the  $\text{Ca}^{2+}$  signals evoked by agonist stimulation in the cytosol. Seminal observation in studies by Friel and Tsien [22] in bullfrog sympathetic neurons showed that a FCCP-sensitive store may influence the degree of activation of intracellular  $\text{Ca}^{2+}$ -dependent processes. Two mechanisms are responsible for this effect. The first operates in the microdomains where mitochondria and  $\text{Ca}^{2+}$  channels come in close contact. Here, the efficiency of mitochondrial  $\text{Ca}^{2+}$  accumulation accounts for the rapid clearing of the high  $\text{Ca}^{2+}$  concentration at the mouth of the release channels, and thus reduces the (positive or negative) feedback effect of the cation on the channel itself [15,23]. Thus, this close co-positioning on one hand allows the MCU to rapidly import large amounts of  $\text{Ca}^{2+}$  into the lumen of the organelle (and thus to provide cellular ATP to fuel energy-requiring processes) and, on the other hand, to modulate  $\text{Ca}^{2+}$  channels activity. The strategic distribution of mitochondria in the different cell compartments is critical

in the regulation and may differ among cells types. In HeLa and GH3 cells, the activation of capacitative  $\text{Ca}^{2+}$  entry mechanism (CCE) results in no differences in mitochondrial  $\text{Ca}^{2+}$  uptake between the organelles population close to the plasma membrane and in that more deeply located, suggesting that mitochondria are excluded from the regions where the store operated  $\text{Ca}^{2+}$  channels (SOCs) are located and/or activated [24]. Indeed, electron microscopy analysis in RBL-1 mast cells has revealed that very few mitochondria were found beneath the plasma membrane, and that SOCs activation failed to change their pattern of distribution [25]. In T cells, mitochondria migrate instead to cell periphery following  $\text{Ca}^{2+}$  entry, thus acting as  $\text{Ca}^{2+}$  buffers and preventing SOCs inactivation [26].

The second mechanism by which mitochondrial  $\text{Ca}^{2+}$  uptake affects cytosolic  $\text{Ca}^{2+}$  signals has been initially demonstrated in pancreatic acinar cells, but has been also shown in neurons [27]. Pancreatic acinar cells have a defined polarized morphology, and the occurrence of cellular  $\text{Ca}^{2+}$  signals in different cellular locations has different physiological consequences. The spreading of the  $\text{Ca}^{2+}$  signal is prevented by clustered mitochondria that accumulate  $\text{Ca}^{2+}$ , thus acting as a “firewall”. When mitochondrial “buffering” is overwhelmed (e.g. upon intense cell stimulation, or when the  $\text{Ca}^{2+}$  uptake ability of mitochondria is impaired),  $\text{Ca}^{2+}$  can freely diffuse to the rest of the cell, with physiological and pathological consequences. In addition to prevent the propagation of a  $\text{Ca}^{2+}$  signal, mitochondria can also delay its propagation to specific cellular compartments, i.e. to the nucleus, thus having a role in the control of gene expression [28]. For example,  $\text{Ca}^{2+}$  uptake and release from mitochondria generate prolonged cytosolic  $\text{Ca}^{2+}$  elevations that trigger the nuclear import of the transcription factor NFAT

(nuclear factor of activated T-cells), which has a role in synaptic plasticity, axonal growth, and neuronal survival [29]. Furthermore, the localization of mitochondria in cell districts where other  $\text{Ca}^{2+}$  removal systems are scarce or absent, for example in dendrites or axons, represents a key element in the  $\text{Ca}^{2+}$  handling.

### 2.3. Mitophagy: organelle-selective autophagic process

Autophagy degrades cellular components by encapsulating them in a double-membrane structure, the autophagosome, which fuses with lysosomes [30]. In this way, autophagy recycles intracellular components to compensate for nutrient deprivation, but also selectively eliminates organelles or protein aggregates to maintain quality control. Recently, selective mitochondrial autophagy, known as mitophagy, has been proposed as a principal mechanism for damaged mitochondria removal. The selective mitochondria elimination prevents the cytotoxic release of pro-apoptotic molecules and the extension of mitochondrial damage to the entire mitochondrial population. Mitophagy is generally preceded by mitochondrial fission [31], which implies that the regulation of mitochondria shape is an essential aspect of mitochondrial quality control.

Different  $\text{Ca}^{2+}$ -signaling related proteins have been identified in the molecular toolkit of autophagy.  $\text{Ca}^{2+}$  has a dual role, being both pro-autophagic and anti-autophagic in different situations. Autophagy appears directly dependent on the levels of ER  $\text{Ca}^{2+}$  and on the activity of  $\text{InsP}_3\text{R}$ . However, there are opposite views on its action. The antiapoptotic proteins Bcl-2 and Bcl-XL have been reported to mediate the decrease of the ER  $\text{Ca}^{2+}$  levels, and of the  $\text{Ca}^{2+}$  release, thus being protective against apoptotic cell death [32]. These proteins have also been shown to inhibit autophagy by lowering ER  $\text{Ca}^{2+}$  levels [33] and by interacting with both the  $\text{InsP}_3\text{R}$  and the autophagic protein beclin 1. Thus, Bcl-2 proteins may have an inhibitory role on both apoptotic and autophagic pathways. However, the mechanisms which trigger these events are yet to be elucidated. On one hand, it has been shown that the constitutive  $\text{Ca}^{2+}$  transfer from ER to mitochondria through the  $\text{InsP}_3\text{R}$  essential to sustain mitochondrial function and bioenergetics [34]: when this signal was abolished, the increased AMP/ATP ratio promoted AMPK activation and stimulation of autophagy [34]. On the other hand, however, it has been reported that elevation of cytosolic  $\text{Ca}^{2+}$  by agents which deplete the ER  $\text{Ca}^{2+}$  store resulted in increased autophagy, and that the addition of the  $\text{Ca}^{2+}$  buffer BAPTA-AM prevented its induction [35]. An interesting model has been proposed to reconcile the inhibitory and the stimulatory role of  $\text{Ca}^{2+}$  signal in autophagy: in healthy cells, spontaneous  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release from the ER is essential to sustain mitochondria bioenergetics. When this signal is missing, ATP depletion accounts for activation of autophagy. Instead, under stress situation,  $\text{Ca}^{2+}$  signaling is enhanced and elevated cytosolic  $\text{Ca}^{2+}$  stimulates autophagic flux (for a detailed discussion of these aspects see the comprehensive review by Parys and co-workers [36]).

As to mitochondria, several pathways indicate their possible link with the autophagic machinery. General consensus has been reached that two proteins, the mitochondrial serine/threonine kinase PINK1 and the E3 ubiquitin ligase parkin, the mutations of which are the major cause of autosomal recessive familial Parkinson's disease, are key to directing mitochondria to the mitophagy process. They are proposed to function in the same pathway, with PINK1 acting upstream of parkin, and being responsible for parkin recruitment to mitochondria by a kinase-dependent mechanism [37]. Once parkin has reached the OMM, it ubiquitinates a series of targets among which mitofusins, [38–40], Drp1 [41], VDAC1 [40,42], and Bcl-2 [43], suggesting that these changes could be early events in the process of mitophagy. Very recently, it has also been shown that PINK1 phosphorylates Miro, thus promoting its

proteasomal degradation by triggering parkin [44]. This finding is particularly intriguing since the Miro yeast homolog has been shown to regulate the ER-mitochondria contact sites [45].

Thus, PINK1 phosphorylation of substrates may represent a necessary early event that triggers the subsequent action of parkin and of the proteasome. Whether mitochondrial  $\text{Ca}^{2+}$  dysregulation could be relevant in these actions remains an unexplored possibility that has been suggested by the studies on the roles of PINK1/parkin (see below, Section 3.2).

### 2.4. Mitochondrial pathway for apoptotic cell death

Apoptosis (programmed cell death) occurs in all multicellular organisms during normal tissue development, and its dysregulation is responsible for the origin of many pathological conditions neurodegeneration and cancer.

The  $\text{Ca}^{2+}$  link with the apoptotic pathways has been established clearly by the finding that the anti-apoptotic Bcl-2 protein lowers the  $\text{Ca}^{2+}$  ER content and that  $\text{Ca}^{2+}$  can sensitize cells to apoptotic challenges, acting on the mitochondrial checkpoint [32]. The finding that Bcl-2, in addition to being localized in the cytoplasm and at the nuclear envelope, is also associated to the ER and mitochondrial membranes further reinforced the link. Bcl-2 and Bcl-XL, the other anti-apoptotic member of Bcl-2 family, directly interact with the  $\text{InsP}_3\text{R}$  on the ER membrane, and sensitize it to low agonist doses, promoting the leakage of  $\text{Ca}^{2+}$  from ER. These effects are reverted by overexpression of the pro-apoptotic protein Bax [46]. The knock down of Bax and of Bak, another pro-apoptotic Bcl-2 family member protein, reduces the  $\text{Ca}^{2+}$  content of the ER; instead, Bax overexpression increases it [47,48]. Accordingly, double knock-out Bax/Bak mouse embryonic fibroblasts (MEF) are resistant to apoptotic stimuli [49].

The amount of  $\text{Ca}^{2+}$  released from the ER is critical to the transduction of the signal in mitochondria and the modulation of the  $\text{InsP}_3\text{R}$  opening by pro or antiapoptotic proteins is a key element that determines mitochondrial changes in physio/pathological responses. Mitochondria are the depositories of proapoptotic proteins like Smac/DIABLO, Omi/HtrA2, AIF and EndoG which are in equilibrium with the antiapoptotic proteins XIAP, cIAP-1 and cIAP-2, their relationship finely regulating cell death and life balance. Thus, the role of mitochondria and  $\text{Ca}^{2+}$  is an essential determinant:  $\text{Ca}^{2+}$  loads in the matrix sensitize the mPTP to apoptotic stimuli, inducing its opening, mitochondrial changes in morphology, and the release of cytochrome *c* [50] and caspase activation [51,52].

Numerous studies have also established a link between mitochondrial dynamics and the apoptotic pathway. Loss of mitofusins and OPA1 leads to mitochondrial fragmentation and enhances sensitivity to cell death stimuli, while their overexpression leads to increased survival in neurons [53,54]. The molecular basis of this action could be traced back to the cytochrome *c* release and caspase activation induced by mitochondrial fragmentation. Interestingly, however, Bax and Bak proteins have been found to associate with components of the fission/fusion machinery suggesting their mutual cross-talk [55].

## 3. Mitochondrial $\text{Ca}^{2+}$ dysregulation and neurodegenerative diseases: the cause or the consequence?

Neurodegenerative diseases are a large group of heterogeneous disorders characterized by the selective death of neuronal subtypes. A number of studies suggest that the alteration of  $\text{Ca}^{2+}$  homeostasis is a hallmark of these pathologies; in particular,  $\text{Ca}^{2+}$ -dependent mitochondrial dysfunction, defects in morphology and trafficking may be critical to the degeneration of neurons in Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) diseases, in amyotrophic

lateral sclerosis (ALS) and in demyelinating diseases [56–61]. The molecular etiology of AD is causally related to the altered synaptic bioenergetics and function. Cognitive defects in AD patients correlate with the loss of dendritic spines and synapses [62], and alterations in mitochondrial function [61], morphology and dynamics [63–65] are particularly important. As to PD, our understanding of the molecular mechanisms has dramatically improved after the discovery of rare familial forms linked to mutations in LRRK2,  $\alpha$ -synuclein ( $\alpha$ -syn), parkin, DJ-1 and PINK1. Functional studies suggest that these proteins have important roles in regulating the balance between mitochondrial fission/fusion processes [66,67] and in controlling mitochondria motility along microtubules [44]. In HD mitochondrial abnormalities and oxidative damage, defects in the activity of complex II (and III) of the respiratory chain have been observed, and it has been proposed that mutant huntingtin may sensitize mPTP opening in model cells, thus disrupting mitochondrial  $\text{Ca}^{2+}$  homeostasis and increasing the susceptibility to apoptotic stimuli [68,69].

As to ALS, the familial cases are related to mutations in the mitochondrial Cu/Zn superoxide dismutase (SOD1) gene, which result in the alteration of complex II and IV of the respiratory chain, and possibly in the abnormal structure and number of mitochondria in motor neurons and in skeletal muscles [70].

The evidence that mitochondrial abnormalities and oxidative damage play a central role in the pathogenesis of several neurodegenerative diseases is thus well supported. The molecular determinants of the defects, however, are still debated. The possibility that dysregulation of the mitochondrial  $\text{Ca}^{2+}$  could be a common feature of the molecular etiology of these diseases is now gaining momentum. The different neurodegenerative pathologies will be discussed separately in the next sections.

### 3.1. Alzheimer's disease

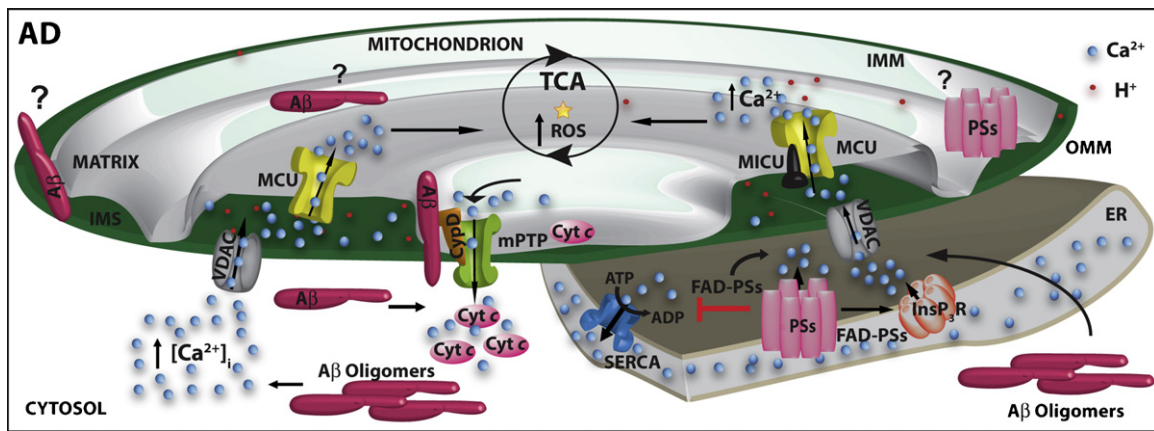
AD is the first cause of dementia in aged people worldwide. It begins with mild, slowly progressing loss of memory and then continued with debilitating symptoms such as complete loss of cognitive abilities and bodily functions, ultimately leading to death. Although AD cases are mostly sporadic, with the symptoms first appearing after age 60, a small fraction of cases (1–2%) is genetically inherited and characterized by an early age of onset (<60). Three genes, namely the amyloid-precursor protein (APP) and the presenilin-1 and 2 genes (PSEN-1 and 2), have been identified as responsible for the Familial forms of Alzheimer's disease (FAD). The study of their gene products has allowed to gain new insights on the pathogenic mechanisms of the condition [71]. Sporadic and familial AD are both characterized by the presence of extracellular amyloid plaques. They are composed of aggregates of amyloid  $\beta$  (A $\beta$ ) peptides derived from the amyloid precursor protein (APP) cleavage by the  $\beta$ - and  $\gamma$ -secretase, and intracellular neurofibrillary tangles, formed by filaments of hyperphosphorylated tau protein [72,73]. Based on the toxicity evoked by the deposition of A $\beta$  aggregates, the amyloidogenic pathway has been proposed as the main pathological event at the basis of the AD pathogenesis [74]. However, the finding that the oligomeric soluble fraction of the A $\beta$  protein [75], rather than the fibrillar and insoluble form, is crucial to the impairment of the cognitive function [76] has led to the possibility of an alternative hypothesis. Disturbances in  $\text{Ca}^{2+}$  signaling have been found in both sporadic [77,78] and familial cases of AD [79]. The development of a  $\text{Ca}^{2+}$  dysregulation hypothesis was supported by the finding that A $\beta$  oligomers can insert in the plasma membrane and form ion conducting channels, thus possibly mediating excitotoxicity by enhancing  $\text{Ca}^{2+}$  influx [80] and eventual neurodegeneration [81]. Fig. 2 summarizes the main findings on this issue. A $\beta$  oligomers have been found to induce massive  $\text{Ca}^{2+}$  transfer between ER and mitochondria [82] and

mitochondrial  $\text{Ca}^{2+}$  overload [83]. Altered mitochondrial morphology and/or distribution have been found in neurons from brains of AD patients [84,85] and in model cells upon A $\beta$  [86], APP [87] and FAD-Presenilin-1 [88] expression. Excessive  $\text{Ca}^{2+}$  taken up into mitochondria increases ROS production, inhibits ATP synthesis, induces the opening of the mPTP, and the release of cytochrome c, triggering the initiation of apoptosis [89,90]. A $\beta$  can also accumulate in mitochondria and interact with specific intra-mitochondrial targets, directly leading to the dysfunction of this organelle [91]. A study on brain mitochondria from an AD animal model has shown that A $\beta$  interacts with Cyclophilin D (CypD) and promotes the opening of the mPTP, thereby causing neuronal injury and a decline in cognitive functions. Accordingly, genetic ablation of CypD renders brain mitochondria more resistant to mPTP opening [92], suggesting it could represent a potential therapeutic target.

The " $\text{Ca}^{2+}$  hypothesis" is also strongly supported by studies on FAD. It has been shown that mutated presenilins (PSs) may contribute to the dysregulation of  $\text{Ca}^{2+}$  homeostasis that, in turn, may be responsible for the mitochondrial impairment observed in AD [86]. PS1 and PS2 are located in different intracellular compartments such as the ER, the Golgi apparatus [93], and mitochondria, where they participate to the formation of active  $\gamma$ -secretase complexes [94,95]. They have been proposed to function as low conductance  $\text{Ca}^{2+}$ -leak channels in the ER membrane, thus contributing to maintain physiological  $\text{Ca}^{2+}$  concentration within the ER. Mutations causing AD have been shown to abrogate this channel activity, and result in enhanced ER  $\text{Ca}^{2+}$  levels and, following cell stimulation, increased cytosolic  $\text{Ca}^{2+}$  transients [96,97]. Nevertheless, a number of studies have instead observed either no alteration or reduced ER  $\text{Ca}^{2+}$  levels in FAD PS-expressing cells [79,98–101]. These studies have proposed that, rather to function as  $\text{Ca}^{2+}$  leak channels, PS are able to modulate  $\text{Ca}^{2+}$  leak by modulating  $\text{InsP}_3\text{Rs}$  and/or RyRs permeability [102] and, possibly, by reducing SERCA activity [103]. FAD mutant PS1 and PS2 have been shown to interact with the  $\text{InsP}_3\text{R}$  and to enhance  $\text{Ca}^{2+}$  release from the ER at low physiological concentrations of agonist [104,105], thus reducing ER  $\text{Ca}^{2+}$  content rather than augmenting it. Interestingly, PSs have been found to be enriched in the ER-mitochondria-associated membranes (MAM) [106] and, it is worth mentioning that the T122R PS2 mutation has been shown to enhance the ER-mitochondria tethering [107]. Thus, two opposite scenarios can be envisaged: the increased ER-mitochondria interaction could potentially result in toxic mitochondrial  $\text{Ca}^{2+}$  overload or, alternatively, it could result in a compensatory phenomenon to ensure proper  $\text{Ca}^{2+}$  signaling to mitochondria when ER  $\text{Ca}^{2+}$  levels are reduced. On the other hand, it must also be taken into account that the enhanced recruitment of mitochondria close to ER could be simply the consequence of dysfunctional  $\text{Ca}^{2+}$  handling by the store, since mitochondria dynamics is tightly regulated by  $\text{Ca}^{2+}$ .

### 3.2. Parkinson's disease

PD is the second most common progressing neurodegenerative disorder. It is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta and by the accumulation of intracellular inclusions called Lewy bodies. Like the AD, most of the cases of PD are sporadic and probably caused by environmental toxins, but about 5–10% of the cases are of genetic origin. Evidence linking mitochondrial dysfunctions and oxidative stress with PD originated from a seminal study on chemically induced Parkinsonism in 1983, in which drug addicts were shown to develop rapid onset PD-like symptoms after injection of heroin contaminated with 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP) [108]. The study demonstrated that the active metabolite of MPTP was a potent inhibitor of complex I of the respiratory chain and a substrate of the dopamine transporter.



**Fig. 2.** Mitochondrial dysfunctions and  $\text{Ca}^{2+}$  homeostasis in AD. Peptide  $\beta$ -amyloid ( $\text{A}\beta$ ) oligomers affect mitochondrial functionality either by increasing cytosolic  $\text{Ca}^{2+}$  concentration through a pore-forming mechanism at the plasma membrane and/or by enhancing ER  $\text{Ca}^{2+}$  release. Possible mitochondrial  $\text{A}\beta$  accumulation impairs mitochondrial energy metabolism, leading to mitochondrial oxidative damage.  $\text{A}\beta$  may sensitize mPTP opening by interacting with cyclophilin D (CypD). FAD-linked mutant presenilins (PSs) may alter the expression/sensitivity of ER  $\text{Ca}^{2+}$  release channels (RyR and  $\text{InsP}_3\text{R}$ ) leading to an exaggerated ER  $\text{Ca}^{2+}$  release and abnormal mitochondrial  $\text{Ca}^{2+}$  uptake. A reduction in SERCA activity has also been described. Wild-type PSs, but not the FAD mutants, were reported to form  $\text{Ca}^{2+}$  permeable leak channels in the ER. PSs have also been found in mitochondria.

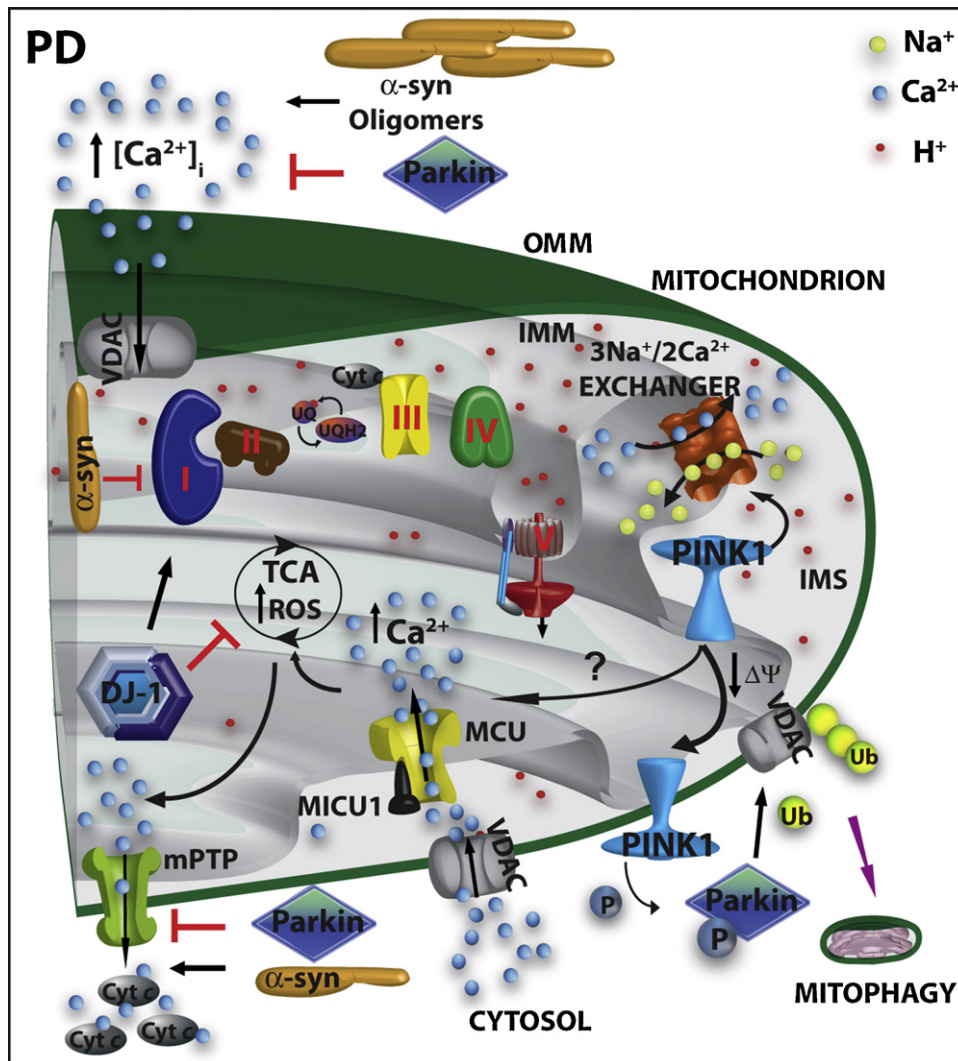
It accumulated in dopaminergic neurons to which it conferred toxicity and eventually produced cell death. Further studies on humans and on animal models exposed to the complex I inhibitors rotenone, paraquat and 6-hydroxydopamine (6-OHDA) have linked mitochondrial exposure to toxins to the degeneration of dopaminergic neurons, dopamine depletion, and increased oxidative stress [109]. The subsequent discovery of hereditary forms of PD caused by dominant and recessive mutations in nuclear genes encoded proteins functionally related to mitochondria has added additional importance to the study of mitochondrial dysfunction in PD [110]. The products of two dominantly inherited genes,  $\alpha$ -synuclein ( $\alpha$ -syn) and LRRK2 (dardarin), and of several autosomal recessive inherited genes, DJ-1, parkin, PINK1, and Omi/HTRA2, have been found to be localized in, and/or to interfere with, mitochondria. The number of reports on this relationship is increasing exponentially: general consensus has now been reached on the role of PINK1/parkin in the regulation of mitophagy (see above), and emerging evidence indicates that also  $\alpha$ -syn and DJ-1 may participate in the pathways of mitochondrial quality control by regulating mitochondrial shape.

Very few papers have considered the possibility that these proteins may also interfere with mitochondrial  $\text{Ca}^{2+}$  signaling. The proposed mechanisms by which this could occur are schematized in Fig. 3.  $\alpha$ -syn has been described to modulate  $\text{Ca}^{2+}$  influx, suggesting that its oligomers could enhance the plasma membrane ion permeability [111] either by becoming directly inserted into the plasma membrane to form a pore [112], or by modulating the open probability of the plasma membrane  $\text{Ca}^{2+}$  channels [113]. However, the link with the PD pathogenesis is still missing, and controversial effects have been reported on the intracellular  $\text{Ca}^{2+}$  increase by PD-related  $\alpha$ -syn mutants [111,113]. Recent studies have shown that  $\alpha$ -syn can associate to mitochondria and that its accumulation increases mitochondrial  $\text{Ca}^{2+}$  levels and, as a consequence, nitric oxide levels, oxidative damage and cytochrome c release [114]. However, these observations are controversial [115] and we have obtained evidence that  $\alpha$ -syn plays a dual opposite role on the modulation of mitochondrial  $\text{Ca}^{2+}$  accumulation depending on its level of expression and its intracellular distribution [116]. We have recently reported that moderate level of  $\alpha$ -syn overexpression enhances mitochondrial  $\text{Ca}^{2+}$  homeostasis by augmenting ER-mitochondria contact sites thus, possibly, sustaining cell bioenergetics. Accordingly,  $\alpha$ -syn “loss of function” impaired mitochondrial  $\text{Ca}^{2+}$  transients and enhanced autophagic process [116]. Interestingly, it has been recently shown that glial cells

obtained from the mesencephalon of mice overexpressing mutated human  $\alpha$ -syn, displayed severe mitochondrial damage, including morphological changes and reduced  $\text{Ca}^{2+}$ -storage capacity [117]: the reduced mitochondrial  $\text{Ca}^{2+}$  buffering capacity could, in turn, lead to an increase in cytosolic  $\text{Ca}^{2+}$  with consequent calpain activation [118].

DJ-1 is a multifunctional protein and, despite its predominant role as an antioxidant [119], it also appears to have a role in maintaining the cytosolic basal  $\text{Ca}^{2+}$  concentration and to permit the depolarization-induced  $\text{Ca}^{2+}$  release from the SR in muscle cells [120]. An interesting link between DJ-1,  $\text{Ca}^{2+}$  handling and mitochondria has been reported by studies of the properties of L-type voltage dependent  $\text{Ca}^{2+}$  channels of dopaminergic neurons of the substantia nigra. These channels are characterized by a specific pore forming subunit (Cav1.3) which confers to the channel the property to be opened at relatively hyperpolarized potentials, allowing them to sustain the pacemaking activity which characterises these neurons [121]. The sustained engagement of these channels during pacemaking activity has an obvious high metabolic cost, and induces a very large  $\text{Ca}^{2+}$  penetration into the neurons. Surmeier and co-workers have elegantly demonstrated that, in dopaminergic neurons, the enhanced  $\text{Ca}^{2+}$  entry created an oxidant stress in mitochondria that thus became specifically vulnerable. Interestingly, the situation was further exacerbated by DJ-1 knockout, implying an essential role of DJ-1 in protecting dopaminergic neurons by  $\text{Ca}^{2+}$ -induced mitochondrial uncoupling and ROS production during physiological pace-making [122].

More compelling evidence for a possible role of mitochondrial  $\text{Ca}^{2+}$  dysfunction in the pathogenesis of PD comes from studies on the mitochondrial kinase PINK1. The first suggestions arose from the finding that the expression of mutant, but not of wt, PINK1 exacerbated the mitochondrial defects observed in a cellular model of PD expressing mutated A53T  $\alpha$ -syn. The defects, i.e., loss of  $\Delta\Psi_m$ , increased mitochondrial size with loss of cristae and reduced ATP levels, were partially recovered by cyclosporine A and, fully rescued by the inhibitor of MCU ruthenium red, thus leading to the suggestion that mitochondrial  $\text{Ca}^{2+}$  uptake was involved [123]. Other studies have expanded the investigation of the role of PINK1 in mitochondrial  $\text{Ca}^{2+}$  metabolism, but the results are controversial. In one case the absence of PINK1 induced mitochondrial  $\text{Ca}^{2+}$  accumulation, possibly as a consequence of the impairment of mitochondrial  $\text{Ca}^{2+}$  efflux through the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger [124]. In another study, PINK1 depletion



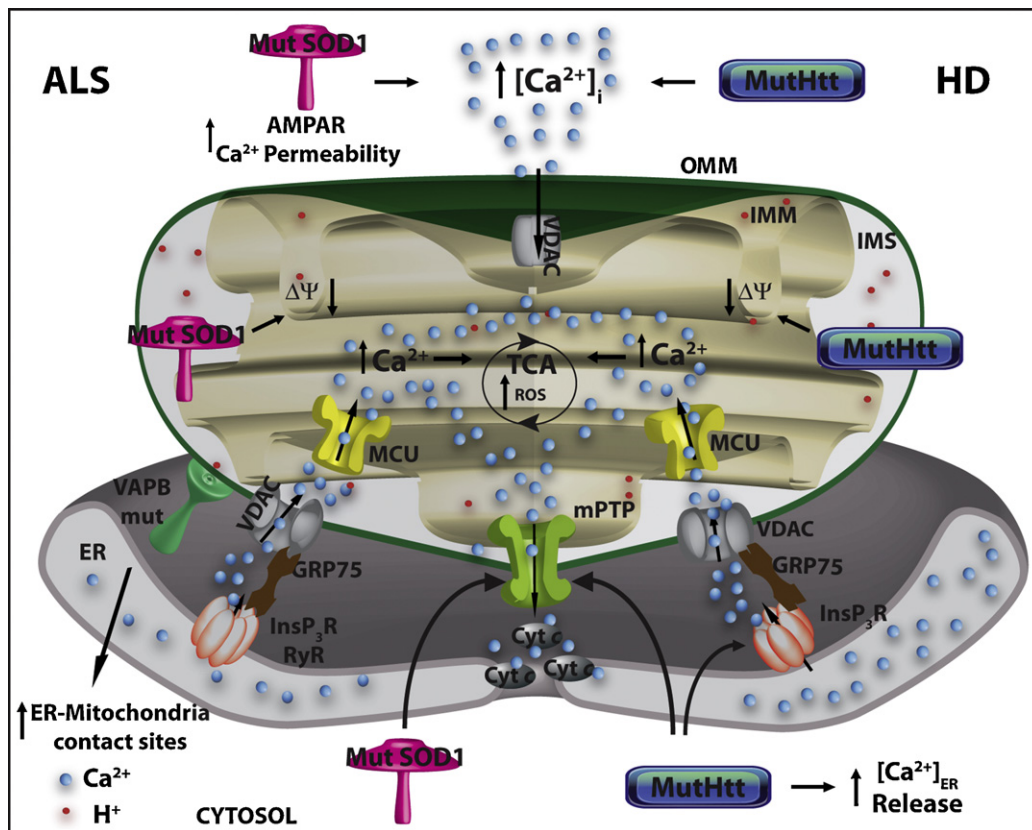
**Fig. 3.** Mitochondrial dysfunctions and  $\text{Ca}^{2+}$  homeostasis in PD.  $\alpha$ -syn monomers impair complex I and complex III activity, while oligomeric  $\alpha$ -syn has been shown to potentiate intracellular  $\text{Ca}^{2+}$  influx through the VOC. DJ-1 scavenges mitochondrial ROS and sustain complex I activity. PINK1, possibly, modulates the activity of the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and/or of the MCU. Additionally, together with parkin, it acts as a sensor to direct damaged mitochondria to the mitophagy process. Parkin attenuates protein misfolding and may protect against apoptotic stimuli, by preventing the opening of mPTP.

impaired mitochondrial  $\text{Ca}^{2+}$  uptake (with no effect on  $\text{Ca}^{2+}$  extrusion) and consequently ATP production [125]. Further studies are evidently necessary to clarify the molecular targets of PINK1 action. However, it is interesting to note that an increased sensitivity of mitochondria to  $\text{Ca}^{2+}$ -induced permeability has been shown to precede dopaminergic defects in PINK1-deficient mice, suggesting that mitochondrial  $\text{Ca}^{2+}$  degeneration could be an early event in the pathogenesis of PD [126].

### 3.3. Huntington's disease

HD is an autosomal-dominant disease characterized by the loss of striatal GABAergic neurons. Major symptoms are motility impairment (chorea), dementia, and other neuropsychiatric defects. The genetic cause is the abnormal expansion of CAG repeats (corresponding to a poly-glutamine stretch (poly-Q)) in exon 1 of the gene coding for Huntingtin protein (Htt). The mutant forms of Htt contain more than 35 repeats and there is a direct correlation between the number of glutamines over 35 and the severity of the disease. Htt is a large protein (348 kDa) mainly localized in the cytosol, but it is also found in the ER, mitochondria, nucleus and Golgi compartment. Its function is presently unclear, although its involvement

has been suggested in cellular pathways like transcription regulation, mitochondrial function, ROS production, apoptosis and organelles trafficking [127,128]. It is, nevertheless, essential to life: its homozygous deletion causes death at the embryonic age in mice, and its heterozygous deletion causes neurodegeneration [129]. The role of the poly-Q expansion at the N-terminal is not known, but it has been reported that its ablation in mice ameliorates motor/behavioral deficits, and extends lifespan [130]. The poly-Q expansion is cleaved off proteolytically, caspase 6 being most likely responsible for the cleavage [131]. The cleaved fragments increase probability of protein misfolding events and show propensity to aggregate and to form fibrils and oligomers [132,133]. However, whether the Htt fragment aggregates play a role in HD pathogenesis, and whether the oligomers (and/or aggregates) are toxic or protective, is still debated [133,134]. The link between  $\text{Ca}^{2+}$  homeostasis and HD has been established in a number of studies, most of them focused on mitochondrial  $\text{Ca}^{2+}$  handling dysfunction. The results have been controversial and no unequivocal conclusion can be drawn. Fig. 4 summarizes the main pathways that have been proposed, and a succinct overview of the data present in the literature will be presented here. As it was found in other neurodegenerative diseases, mitochondria isolated from lymphoblast of HD



**Fig. 4.** Mitochondrial dysfunctions and  $\text{Ca}^{2+}$  homeostasis in ALS and HD. Mutant SOD1 in ALS and mutant Htt in HD enhance intracellular  $\text{Ca}^{2+}$  permeability, impair mitochondrial membrane potential and increase the susceptibility to mitochondrial  $\text{Ca}^{2+}$  overload, thus inducing mPTP opening, the release of cytochrome c (cyt c) and apoptosis. Mutant Htt increases ER  $\text{Ca}^{2+}$  release by acting on the  $\text{InsP}_3\text{R}$ . Mutant VAPD (a protein found associated with mutant forms of ALS) enhances ER-mitochondria tethering, leading to an augmented mitochondrial  $\text{Ca}^{2+}$  uptake. Increased mitochondrial  $\text{Ca}^{2+}$  concentration, in turn, stimulates TCA cycle enzymes generating NADH and increasing ATP synthesis and ROS production.

patients and from the brain of HD mice models have impaired ability to take up  $\text{Ca}^{2+}$ . They are more susceptible to  $\text{Ca}^{2+}$  overload, with the vulnerability proportional to the length of the mutant Htt poly-Q expansion [135,136]. The matrix  $\text{Ca}^{2+}$  overload favors mPTP opening, which has been shown to be prevented by CsA in studies on mitochondria isolated from striatal neurons expressing mutant Htt [137] and from muscle of HD model R6/2 mice [138]. These findings have been confirmed also in cultured cells, e.g., the expression of mutant Htt in mouse immortalized striatal cells made mitochondria unable to handle large  $\text{Ca}^{2+}$  loads, very likely because of induced mPTP opening. The observed decrease of mitochondrial  $\text{Ca}^{2+}$ , appears to be a compensatory attempt to prevent the mitochondrial  $\text{Ca}^{2+}$  stress that would irreversibly damage the organelles and eventually lead to cell death [69]. However, the ablation of cyclophilin D in HD mouse models failed to induce any improvement in the disease progression, despite the increased  $\text{Ca}^{2+}$ -buffering capacity of their mitochondria [139–141]. These controversial findings are possibly related to the different experimental conditions, but also to the loss of the contribution of the ER compartment in experiments performed in isolated mitochondria. In fact, a number of reports have described the involvement of ER  $\text{Ca}^{2+}$  handling in HD cellular models. Htt was shown to influence intracellular  $\text{Ca}^{2+}$  signaling by acting on the  $\text{InsP}_3\text{R}$  activity and expression [69,142–144], and striatal precursors of HD neurons display increased  $\text{Ca}^{2+}$  content in the ER [145] that could compromise mitochondrial  $\text{Ca}^{2+}$  handling. Another aspect of mitochondria in other neurodegenerative diseases is shared by HD: the mitochondrial fission/fusion balance is affected by the presence of mutant Htt in different HD models, possibly as a consequence of the enhanced calcineurin activity, and thus of the calcineurin-dependent Drp1

recruitment to mitochondria. The enhanced mitochondria fragmentation led to enhanced apoptotic sensitivity, thus accounting for an early role of  $\text{Ca}^{2+}$  dysregulation in the death of striatal neurons [145].

#### 3.4. Amyotrophic lateral sclerosis

ALS is a neurodegenerative disease caused by the progressive loss of motor neurons in motor cortex, brain stem and spinal cord [146]. Like other neurodegenerative disease, ALS presents alterations of mitochondrial morphology and function, accumulation of phosphorylated neurofilaments in the axons and somatic accumulation of protein inclusions formed by TDP-43 (TAR DNA binding protein-43) and ubiquitin [147–149]. ALS is a sporadic disease and despite several hypotheses implicating oxidative stress, proteasome dysfunction, glutamatergic excitotoxicity and mitochondria dysfunction, its etiology remains elusive [150]. However, in the last years, several mutations in some proteins were identified to be causally related to familial forms of ALS: among them, those in superoxide dismutase 1 (SOD1) are the most frequent, accounting for the 20% of the familial cases [151]. As previously mentioned, SOD1 is key enzyme in the defense against oxidative stress, as it converts the superoxide anion in hydrogen peroxide. It has been proposed that its mutations could cause a gain of toxic function [147]. At the moment, the best model for the study of the disease is represented by transgenic mice expressing mutant SOD1 [150].

To understand the role of  $\text{Ca}^{2+}$  dysfunction in ALS, it is important to underline that ALS-vulnerable motor neurons, like spinal and brain stem neurons, have very low  $\text{Ca}^{2+}$  buffering capacity as compared to not-vulnerable neurons. This specific characteristic



accounts for the rapid and low cost recovery of  $\text{Ca}^{2+}$  transients that, in physiological condition, is necessary for the rapid relaxation times during high-frequency rhythmic activity. However, under pathological conditions it may generate the appearance of high  $\text{Ca}^{2+}$  concentration microdomains that would account for excitotoxic cell damage [152]. Another interesting characteristic of these neurons is their enrichment in  $\text{Ca}^{2+}$ -permeable glutamate AMPA-receptor channels. Under overstimulation, they mediate glutamate-excitotoxicity, which results in selective motor neuron degeneration and death [153–155]. The  $\text{Ca}^{2+}$  permeability of AMPA receptor is regulated by the GluR2 subunit, and motor neurons express low amount of GluR2 mRNA in comparison with other neurons [156–158]. The GluR2 expression level is regulated by the astrocytes surrounding the motor neurons, and its  $\text{Ca}^{2+}$  permeability is controlled by a mechanism of RNA editing that replaces a positively charged arginine with a neutral glutamine, thus making the channel impermeable to  $\text{Ca}^{2+}$ . Reduced editing results in acute neurodegeneration in mice and, accordingly, defective editing was detected in motor neurons of individuals affected by sporadic ALS [159]. The absence of the GluR2 subunit accelerated motor neurons degeneration in mutant SOD1 mice, whereas the induction of GluR2 expression increased life span of these mice [160,161]. It has also been shown that mutant SOD1 augmented AMPA receptor permeability to  $\text{Ca}^{2+}$  [162].

Experimental evidence shows enhancement of glutamate in motor neurons of patients affected by sporadic and genetic ALS [163,164] possibly due to the loss of the astroglial glutamate transporter GLT1 and thus to a decrease of glutamate uptake by the surrounding astrocytes. However, contrasting findings have shown that the chronic elevation of glutamate by the impairment of its transport has no effect on spinal motor neurons in vivo [165].

In the last years an increasing number of papers pointed out the importance of mitochondrial dysfunctions, in particular of defects in the electron transport chain and in the morphology of the organelles, both in sporadic and familial ALS [166]. Fig. 4 summarizes the main findings. As previously discussed, different mitochondrial populations may be strategically located close to the ER or to the plasma membrane  $\text{Ca}^{2+}$  channels to promptly buffer high  $\text{Ca}^{2+}$  microdomains generated by cell stimulation. This aspect is of particular relevance for neuronal cells that undergo continuous stimulation [167], and especially for motor neurons that have reduced levels of  $\text{Ca}^{2+}$  buffering proteins, like parvalbumin, calbindin, etc. [168,169]. Although not numerous, the studies on mitochondria support a causative role for  $\text{Ca}^{2+}$  overload in neurons from individuals affected by sporadic and genetic ALS. Mitochondria at the synapse of motoneurons of mutant SOD1 mice display a greater membrane potential depolarization after  $\text{Ca}^{2+}$  uptake, in line with the proposal that the  $\text{Ca}^{2+}$ -buffering capacity of these organelles could be compromised [170]. Accordingly, the overexpression of mutant SOD1 in neuroblastoma cells increases the cytosolic  $\text{Ca}^{2+}$  level [171]. Emerging evidence thus supports the MCO hypothesis, in which mPTP opening, cytochrome c release, and activation of the apoptotic cascade are direct consequences. Interestingly, the ablation of cyclophilin D in SOD1 mutant mice delays disease onset, and CsA treatment is beneficial [172,173].

Finally, it is appropriate to mention another interesting possibility that may link mitochondrial  $\text{Ca}^{2+}$  dysfunction to ALS pathogenesis: the vesicle-associated membrane protein (VAMP)-associated protein B (VAPB) has been found to be mutated in familial ALS [174]. VAPB is an ER membrane anchored-protein which exposed its C-terminal domain on the cytosolic side [175] and has been reported to induce ER-stress and consequent  $\text{Ca}^{2+}$ -mediated death in motor neurons [176]. It has then been found that VAPB is enriched in the MAMs, where it interacts with PTPIP51 (a mitochondrial outer membrane protein) and, where it

is necessary to support ER-mitochondria  $\text{Ca}^{2+}$  transfer, since its siRNA-mediated silencing disrupts  $\text{Ca}^{2+}$  signaling between these two organelles [175]. Interestingly, a mutant form of VAPB (P56S), but not the wild-type protein, induces mitochondria clustering, impaired mitochondria  $\text{Ca}^{2+}$  uptake and increased cytosolic  $\text{Ca}^{2+}$  levels. In turn, these alterations result in an impairment of the anterograde axonal transport of mitochondria towards the cell periphery [177].

#### 4. Conclusions

The picture emerging from the study of the pathogenesis of neurodegenerative conditions appears extremely complex and multifaceted. Intriguingly, common elements are shared by most of them making crucial to investigate on these aspects. Defects in the respiratory chain and oxidative stress have been recognized in almost all neurodegenerative disorders. However, reviewing the most recent literature, they do not appear as the primary cause, but rather the result of impaired mitochondria function. Compromised mitochondrial quality control and  $\text{Ca}^{2+}$  handling are instead suggested to be primary events. Therefore, any approach aimed at the modulation of mitochondrial dynamics and/or stimulation of mitochondrial biogenesis may be beneficial for the treatment of the diseases. Targeting mitochondrial  $\text{Ca}^{2+}$  dysregulation may represent a great challenge, at least to delay the insurgence of neurodegenerative symptoms and alleviate their progression. Thus, both in basic and applied research there is growing interest on this topic, further accentuated by the recent identification of key molecules (the NCX, MICU1, a uniporter regulator and MCU, the uniporter itself).

#### Conflict of interest statement

No conflict of interest statement.

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