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Review

Mitochondria, calcium and cell death: A deadly triad in neurodegeneration

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ABSTRACT

Mitochondrial Ca^{2+} accumulation is a tightly controlled process, in turn regulating functions as diverse as aerobic metabolism and induction of cell death. The link between Ca^{2+} (dys)regulation, mitochondria and cellular derangement is particularly evident in neurodegenerative disorders, in which genetic models and environmental factors allowed to identify common traits in the pathogenic routes. We will here summarize: i) the current view of mechanisms and functions of mitochondrial Ca^{2+} homeostasis, ii) the basic principles of organelle Ca^{2+} transport, iii) the role of Ca^{2+} in neuronal cell death, and iv) the new information on the pathogenesis of Alzheimer's, Huntington's and Parkinson's diseases, highlighting the role of Ca^{2+} and mitochondria.

1. Mitochondria in calcium signalling

The notion that mitochondria are active players in cellular calcium homeostasis dates back to the demonstration of the chemiosmotic theory, based on the concept of a major proton electrochemical gradient that could drive the rapid accumulation of cations across the ion-impermeant mitochondrial inner membrane. Such a notion was corroborated by the direct measurement of Ca^{2+} uptake by isolated mitochondria, and the functional, albeit not molecular, elucidation of the transporters [1].

However, the experiments that in the 80s drove massive interest into calcium as a ubiquitous second messenger also led to the gradual decline in the attention on mitochondrial Ca^{2+} homeostasis. On the one hand, it appeared clear that the endoplasmic reticulum (ER), through resident Ca^{2+} channels (those sensitive to inositol 1,4,5 trisphosphate, IP_3R, and to the plant alkaloid ryanodine, RyR) acts as the intracellular pool of Ca^{2+} mobilized upon cell stimulation. On the other, the development of accurate fluorescent indicators for the measurement of Ca^{2+} concentration in living cells showed that cytosolic Ca^{2+} concentration fluctuates between approx. 0.1 μM at rest to 2–3 μM at the peak of the rise elicited by the opening of plasma membrane or ER Ca^{2+} channels. Under those conditions, the low affinity of the mitochondrial Ca^{2+} transporters should allow little Ca^{2+} uptake into the organelle. Thus, the prevalent notion was that mitochondrial Ca^{2+} accumulation is negligible in physiological conditions, and could become relevant only upon massive Ca^{2+} overload, that could take place in severe cellular dysfunction.

This situation was completely reversed when tools were developed that allowed the selective measurement of mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) in living cells. Targeting to mitochondria the Ca^{2+}-sensitive photoprotein aequorin [2] demonstrated that a rapid $[Ca^{2+}]_m$ peak, reaching values well above those of the bulk cytosol, parallels the $[Ca^{2+}]$ rise evoked in the cytoplasm by cell stimulation [3]. Similar conclusions could be reached also with fluorescent indicators, such as the positively charged Ca^{2+} indicator rhod 2 (that accumulates within the organelle) [4] and the more recently developed GFP-based fluorescent indicators [5]. With the latter probes, endowed with a much stronger signal than the photoprotein, single-cell imaging of organelle Ca^{2+} could be carried out [6]. With these tools in hands, not only the notion was confirmed that mitochondria promptly respond to cytosolic $[Ca^{2+}]$ rises, but also that the $[Ca^{2+}]_c$ oscillations, the typical response to agonists of many cell types, are paralleled by rapid spiking of $[Ca^{2+}]_m$, thus providing a frequency-mediated signal specifically decoded within the mitochondria, as clearly shown in hepatocytes [7], cardiomyocytes [8], and HeLa cells [9]. The apparent discrepancy between the promptness and amplitude of the mitochondrial Ca^{2+} response and the low affinity of

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the organelle transporters was reconciled by the demonstration that mitochondria are in close contact with the source of the cytosolic Ca^{2+} rise (i.e. the ER via IP_3Rs and RyRs and the plasma membrane via a wide variety of voltage- and agonist-sensitive Ca^{2+} channels). Thus, upon cell stimulation they are exposed to microdomains of high $[Ca^{2+}]$ that greatly exceed the values measured in the cytosol and well match the affinity of mitochondrial Ca^{2+} transporters [10].

As soon as the concept was established that a Ca^{2+} rise in the cytosol is paralleled by a cycle of mitochondrial Ca^{2+} uptake, and subsequent release (through the pathways that will be briefly described later), the identification of the functional significance of this process became a primary goal. Also in this task, the fine biochemical work carried out in the 70s provided a hypothesis to test: three key metabolic enzymes (the pyruvate, ketoglutarate and isocitrate dehydrogenases) were shown to be activated by Ca^{2+} by different mechanisms: in the case of pyruvate dehydrogenase through a Ca^{2+}-dependent dephosphorylation step, in the latter two cases through the direct binding of Ca^{2+} to the enzyme complex [11]. Recently, also some metabolite transporters were shown to be regulated by Ca^{2+} and participate in the enhancement of aerobic metabolism upon cell stimulation [12]. Thus, an obvious role for mitochondrial Ca^{2+} accumulation could be inferred, i.e. that of rapidly upregulating mitochondrial ATP production in stimulated cells. The possibility of directly monitoring, in parallel, Ca^{2+} and ATP levels within mitochondria and in the cytosol proved that this is indeed the case [13]. Interestingly, this route for controlling mitochondrial metabolic output proved to be affected in mitochondrial genetic disorders. In cybrids harboring the $tRNA_{lys}$ mutation of MERRF (myoclonic epilepsy with ragged-red fibers), mitochondrial Ca^{2+} responses were reduced, and, accordingly, ATP production upon cell stimulation, and the pharmacological correction of the Ca^{2+} alteration also restored the metabolic dysfunction [14,15].

In addition to the function of metabolic coupling, mitochondrial Ca^{2+} accumulation was shown to underlie a role for these organelles in shaping the spatio/temporal patterning of cytosolic Ca^{2+} rises. Mitochondria, distinctly from cytosolic proteins, are highly sophisticated, "tunable" buffers that vary their activity in different phases and functional states of the cell; indeed, their number, shape, distribution [16] and most likely their responsiveness to Ca^{2+} [17] are controlled by converging signalling pathways. This Ca^{2+} buffering activity influences cytosolic Ca^{2+} signals in two conceptually different ways, i.e., *1*) by acting as high-capacity sinks placed on the way of a propagating Ca^{2+} wave and *2*) by clearing Ca^{2+} in restricted microdomains (such as the microenvironment of a Ca^{2+} channel). In the first case, spatial clusters of mitochondria have been demonstrated to isolate functionally distinct domains of polarized cells, namely, a mitochondrial "firewall" was shown to prevent the spread of Ca^{2+} signals from the apical (secretory) region of pancreatic acinar cell from the basolateral region, containing the nucleus [18]. Similarly, neuronal mitochondria have been shown to buffer $[Ca^{2+}]$ increases in defined cellular regions, e.g., the presynaptic motoneuron ending [19]. As to the second case, a thoroughly investigated example is the regulation of Ca^{2+} release through IP_3Rs. In *Xenopus* oocytes, the mitochondria energization state (and thus the capacity to accumulate Ca^{2+}) was shown to modify the propagating Ca^{2+} waves induced by IP_3 [20]. In permeabilized blowfly salivary glands, it was observed that perfusion of IP_3 induced ER $[Ca^{2+}]$ oscillations, the frequency of which increased with the dose of IP_3. Such an effect was observed only upon energization of mitochondria, implying a primary role of these organelles in regulating Ca^{2+} microdomains in the proximity of IP_3Rs and thus the oscillatory pace of stimulated cells [21]. In mammals, this effect has been seen in many cell systems, including hepatocytes, HeLa cells, astrocytes, and BHK cells. As to the cellular consequence, very different effects were observed given the bell-shaped sensitivity of IP_3Rs to Ca^{2+} concentration on the cytosolic side. In astrocytes and hepatocytes, cytosolic excitability appeared enhanced when mitochondrial Ca^{2+} uptake was inhibited,

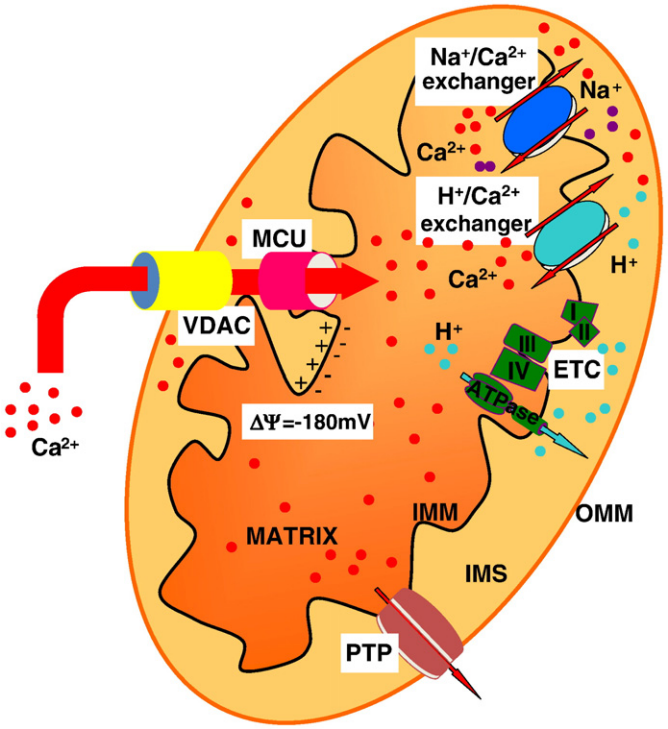

Fig. 1. Schematic map of mitochondrial Ca^{2+} transporters. Mitochondria accumulate Ca^{2+} in the matrix via an electrogenic Ca^{2+} uniporter (MCU) that acts to equilibrate Ca^{2+} according the electrochemical gradient generated by the respiratory chain (ETC). Voltage Dependent Anion Channel (VDAC) controls the Ca^{2+} diffusion through the outer mitochondrial membrane (OMM), thus facilitating mitochondrial Ca^{2+} accumulation. As to the efflux pathways, a Na^+/Ca^{2+} and a H^+/Ca^{2+} exchangers have been shown to operate. The permeability transition pore (PTP) opening plays different roles: its brief opening could allow rapid Ca^{2+} release, but its long-lasting openings (potentiated by apoptotic stimuli and Ca^{2+} itself) could trigger cell death process. IMS, intermembrane space; IMM, inner mitochondrial membrane.

indicating that mitochondrial clearance of the Ca^{2+} microdomain reduced the positive Ca^{2+} feedback on the IP_3R and/or buffered substantial Ca^{2+} loads [22]. Conversely, in BHK cells, inhibition of mitochondrial Ca^{2+} uptake resulted in reduction of ER Ca^{2+} release [23], thus indicating that mitochondria prevent the Ca^{2+}-dependent inactivation of the channel.

The interest in the process of mitochondrial Ca^{2+} homeostasis dramatically increased when it became apparent that also cell death is causally linked to organelle Ca^{2+} loading. On the one hand, it was clear that cellular Ca^{2+} overload, such as that caused by hyperstimulation of ionotropic glutamate receptors, leads to Ca^{2+} cycling across the mitochondrial membranes, collapse of the proton gradient and bioenergetic catastrophe, thus leading to cell death by necrosis, as discussed in more detail later in this review. On the other hand, Ca^{2+} proved to sensitize cells to apoptotic challenges, acting on the mitochondrial checkpoint. This notion, subsequently confirmed by the study of other anti- and pro-apoptotic proteins, emerged from the analysis of the effect of Bcl-2 on Ca^{2+} signalling. We refer to other specifically focused reviews for a detailed coverage of this topic [24]. Briefly, we here summarize that Bcl-2, by partially emptying the ER Ca^{2+} store, reduces the release from this organelle and the loading of mitochondria. As a consequence, the efficacy of apoptotic challenges in opening the permeability transition pore (PTP), causing mitochondrial morphological alterations and releasing caspase cofactors, such as cytochrome c, is greatly reduced [25].

2. The basics of mitochondrial Ca^{2+} transport

For entering the mitochondrial matrix, Ca^{2+} needs to cross two lipid bilayers, the outer and inner mitochondrial membranes (Fig. 1).

The outer mitochondrial membrane (OMM) is permeable to ions and small proteins (MW<10 kDa) due to the abundance of a large conductance channel, known as mitochondrial porin or voltage-dependent anion channel (VDAC). It should be noted, however, that the channel appears to be gated in vivo, and permeability is controlled by ATP and other regulatory factors [26]. Ca^{2+} diffusion through the OMM was thus traditionally considered not to be a limiting factor in mitochondrial Ca^{2+} uptake. Recent data showed that the availability and selective placement of VDAC channels at ER/mitochondria contact sites facilitate mitochondrial Ca^{2+} accumulation, in keeping with the idea that the latter process requires the fast and efficient transfer of Ca^{2+} microdomains from the mouth of the Ca^{2+} channels located in neighbouring ER or plasma membranes to the transporters of the ion-impermeant inner membrane (IMM) [27]. The IMM is an ion-impermeable membrane, with a much larger extension of the OMM (and consequent formation of foldings into the internal space, known as cristae). The activity of respiratory chain complexes allows the translocation of H^+ in the space between the two membranes, which consequently generates an electrochemical gradient ($\Delta\mu H$) composed of a chemical (ΔpH) and electrical ($\Delta\psi H$) component. In mitochondria, most of the $\Delta\mu H$ established by the respiratory chain is supposed to be in the form of $\Delta\psi H$ (around −180 mV), which provides a huge driving force for Ca^{2+} entry into the organelle. Indeed, collapse of the $\Delta\psi H$ by protonophores, such as p-[trifluoromethoxyl]-phenyl-hydrazone (FCCP), abolishes mitochondrial Ca^{2+} uptake.

Mitochondrial Ca^{2+} traffic takes place essentially through two pathways: i) an electrogenic pathway, the mitochondrial Ca^{2+} uniporter (MCU), corresponding to the channel activity demonstrated by Clapham et al. [28] that acts to equilibrate Ca^{2+} with its electrochemical gradient, and thus accumulates the cation into the matrix; ii) two exchangers (with H^+ and Na^+, mostly expressed in nonexcitable and excitable cells, respectively), that utilize the electrochemical gradient of the monovalent cations to prevent the attainment of electrical equilibrium (that would imply, for a mitochondrial membrane potential, $\Delta\psi_m$, of −180 mV and a cytosolic Ca^{2+} concentration of 0.1 μM, accumulation of Ca^{2+} into the matrix up to 0.1 M).

Numerous works has given the biochemical properties of these two pathways, and it is possible to summarize as it follows:

i) Given the huge driving force for accumulation, studies in isolated organelles with clamped $\Delta\Psi$ (by establishing K^+ diffusion potentials with valinomycin in non-respiring mitochondria) allowed to estimate a V_{max}>1400 nmol Ca^{2+} (mg protein)$^{-1}$ min^{-1} and an apparent K_m<10 μM in sucrose-based media [29]. Also in isolated mitochondria, a number of inhibitors were identified. Ruthenium compounds (typically Ruthenium Red, RR), represent a class of non-competitive inhibitors, but unfortunately their poor permeability across cellular membranes has made it of little use in studies in intact cells. A second class of inhibitors is divalent cations that are themselves transported by the uniporter (e.g. Sr^{2+}, Mn^{2+}, Ba^{2+} and lanthanides).

ii) As to the efflux pathways, studies in isolated organelles allowed to estimate their properties. The mitochondrial $Na+/Ca^{2+}$ exchanger (mNCX) has a V_{max} ranging between 2.6 (liver) and 18 nmol Ca^{2+} (mg protein)$^{-1}$ min^{-1} (heart). The dependence on Na^+ is sigmoidal, with typical K_m values of about 8–10 mM Na^+. Ca^{2+} efflux is inhibited by Sr^{2+}, Ba^{2+}, Mg^{2+} or Mn^{2+}, and by a variety of compounds of pharmacological interest such as diltiazem, verapamil and other blockers of the voltage-dependent calcium channels, and more specifically by CGP37157 [30]. As to the H^+/Ca^{2+} exchanger (mHCX), (1) it saturates at Ca^{2+} loads of 25 nmol (mg protein)$^{-1}$; (2) its V_{max} is not influenced by the concentration of inorganic phosphate and does not exceed a rate of 1.2 nmol Ca^{2+} (mg protein)$^{-1}$ min^{-1}; and (3) it extrudes Ca^{2+} against a gradient that is much higher than thermodynamically permissible for an electroneutral H^+/Ca^{2+} exchanger [31]. Thus, either Ca^{2+} efflux occurs via a nH^+/Ca^{2+} exchanger with n>2, or it has an active component. Accordingly, Ca^{2+} efflux is inhibited rather than stimulated by small depolarizations [32].

2.1. The permeability transition pore

This high-conductance channel (PTP) mediating mitochondrial swelling, postulated on the basis of experimental evidence dating back more than 40 years [33], has attracted enormous interest in the last decade, when its role in mitochondrial dysfunction and mitochondria-dependent cell routes has become clear. It is a high-conductance, non-selective channel that exhibits a prominent dependence on matrix Ca^{2+} and is inhibited by Cyclosporin A (CsA). Reversible PTP openings have been resolved both in individual isolated mitochondria [34] and in intact cells [35]. PTP could play a role in various conditions. Long-lasting openings, triggered by apoptotic challenges and potentiated by Ca^{2+}-mediated cellular signals [36] cause morphological transitions of mitochondria underlying the release of caspase cofactors into the cytosol, and the initiation of the cell death process. Conversely, given the large size and lack of selectivity of the PTP (providing charge compensation within the channel and allowing maximal Ca^{2+} flux at zero potential) brief PTP openings could allow at least in principle rapid Ca^{2+} release from the organelle [37].

3. Calcium routes to neurodegeneration

It was hypothesized already in the 70s that prolonged stimulation of N-methyl-D-aspartate (NMDA) ionotropic glutamate receptors can induce massive cell death in the brain (excitotoxicity), by causing Ca^{2+} and Na^+ overload in post-synaptic neurons [38]. Excitotoxicity plays a central role in promoting cell death during neuronal ischemia and substantial work has been placed in clarifying the different phases of the process, and their reversibility, and the potential pharmacological targets. It is now accepted that a primary Ca^{2+} increase occurs, as a consequence of direct entry through NMDA receptors, but also following depolarization, and hence opening of voltage-gated Ca^{2+} channels, release from internal stores and reversal of the plasma membrane Na^+/Ca^{2+} exchanger (NCX). Cell death, however, does not depend on this initial Ca^{2+} rise, but rather invariably follows a delayed massive accumulation of Ca^{2+}, occurring a few hours after the toxic challenge and representing a no-return transition into the death process. Recent work has highlighted events occurring in the interphase between the two Ca^{2+} rises, and suggested a progression route into the secondary Ca^{2+} overload and delayed cell death. Specifically, Bano et al. [39] showed that calpain-mediated cleavage on NCX is a critical step for allowing the delayed Ca^{2+} deregulation: inhibition of NCX cleavage protects from excitotoxic challenges, and conversely downregulation of the exchanger sensitizes to sub-lethal stimuli. As to mitochondria, direct measurement of matrix Ca^{2+} showed that the impairment of their Ca^{2+} uptake capacity is downstream of the delayed Ca^{2+} dysregulation, and not the cause of it. Thus, the delayed Ca^{2+} increase is not due to the sudden discharge from the mitochondrial buffer that still retains the capacity to accumulate Ca^{2+}. Rather, the delayed, massive influx of Ca^{2+} into the cell leads to organelle Ca^{2+} overload, collapse of the electrochemical proton gradient and bioenergetic catastrophe, leading to necrotic cell death [40].

Interestingly, such a commitment mechanism appears to closely match an emerging paradigm clarified in a number of apoptotic routes. Indeed, we and other showed that mitochondrial involvement in apoptosis (morphological transitions and release of caspase

cofactors) often utilizes Ca^{2+} as a key sensitizing factor. In agreement with this notion, Bcl-2 reduces the state of filling of intracellular Ca^{2+} stores, thereby reducing mitochondrial loading upon physiological and pathological challenges and protecting cells from apoptotic death [25]. The pro-apoptotic protein Bax antagonizes this signalling effect, by augmenting the state of filling of intracellular Ca^{2+} stores [41]. In addition, closely mimicking the data described above in excitotoxicity, early cleavage of plasma membrane Ca^{2+} pumps (PMCA) by caspases was shown to greatly enhance the efficacy of apoptotic challenges in neurons [39] and hepatocytes [42]. In this context, one can envision a common route to neurodegeneration in neurons, in which Ca^{2+} plays an important regulatory role and eventually, by affecting the mitochondrial checkpoint, renders cell death obligatory. Thus, while the fate into a rapid necrotic outcome or a more controlled apoptotic elimination will depend on the intensity of the insult and on the bioenergetic balance of the cell, the observation of common signalling themes and molecular targets highlights the extensive crosstalk between the different death pathways and the potential strategies for pharmacological intervention.

4. Looking into complex models: the pathogenesis of neurodegenerative disorders

4.1. Alzheimer's disease (AD)

Alzheimer's disease (AD) is a devastating neurological disorder clinically characterized by impairment of cognitive function and changes in behavior and personality. Morphological hallmarks of the pathology are cortical and hippocampal atrophy, accumulation of abnormal fibers in neuronal cell bodies (neurofibrillary tangles composed of a hyperphosphorylated form of the microtubular protein tau), and the presence, in the extracellular space, of senile plaques, the major component of which is the peptide β-amyloid (Aβ) in two most frequent forms, Aβ40 and Aβ42. These derive from the transmembrane protein APP (Amyloid Precursor Protein), that can be alternatively processed by three different enzymes, named α, β, and γ secretases. The combined action of β and γ secretases leads to the formation of a soluble fragment (sAPPβ) and of Aβ, together with its cytosolic counterpart AICD (APP Intra-Cellular Domain) [43].

Although the majority of AD cases are sporadic, a significant fraction of AD is inherited in a dominant pattern. Mutations in the genes encoding for APP and for Presenilin-1 and -2 (PS1 and PS2), two proteins belonging to the γ-secretase enzymatic complex, have been linked to the familial form of AD (FAD; see [44] for a recent review). Since the majority of FAD mutations have been found to increase the Aβ42/Aβ40 ratio, the initial hypothesis was that the disease was dependent on the enhanced fibrillization of the more amyloidogenic Aβ42 [45].

Although this concept has never been questioned, about a decade ago various experimental observations suggested that an alteration in intracellular Ca^{2+} homeostasis could also contribute to the development of FAD, and more in general to the pathogenesis of AD. Indeed, PSs mutations were shown to alter the expression, or the sensitivity, of ER Ca^{2+} release channels (RyR and IP_3R) in different cell models [46–49] and in neurons from Tg AD mice [50,51], leading to the "Ca^{2+} overload" hypothesis [45,52], i.e. the idea that exaggerated ER Ca^{2+} release affects cellular targets such as mitochondria, favouring cellular demise (Fig. 2). The Ca^{2+} overload mechanism has however remained largely mysterious until it was found that wt PSs, but not the FAD mutants, can form Ca^{2+} permeable leak channels in the ER [53,54], thus providing a clear case for enhanced Ca^{2+} release in the pathological model. Although the data are straightforward, and support a very appealing model, the following work in other laboratories was not entirely consistent with the first formulation of

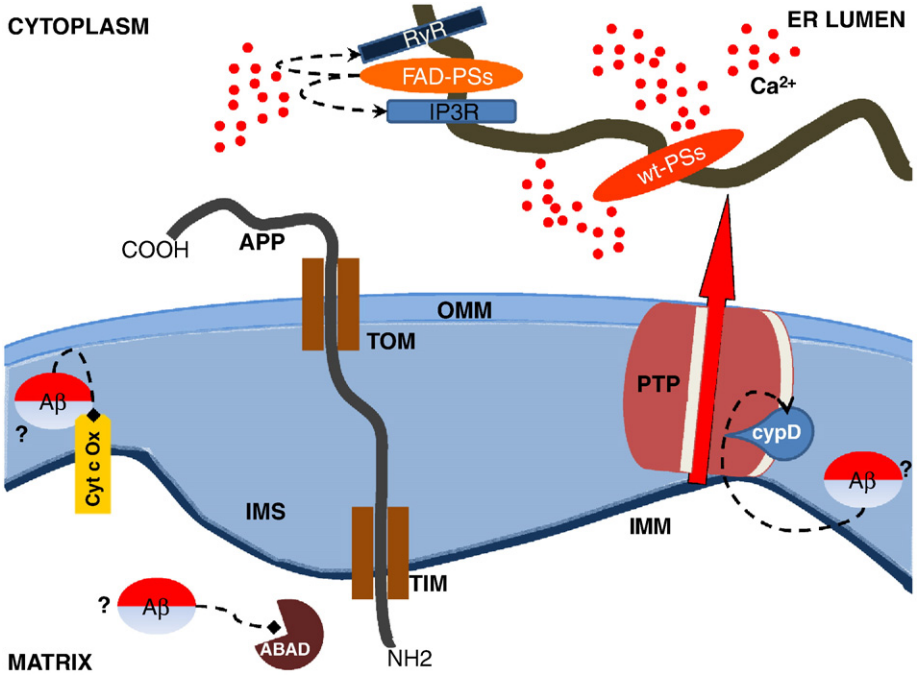

Fig. 2. Presenilins (PSs), Amyloid Precursor Protein (APP) and peptide β−amyloid (Aβ) can affect mitochondrial functionality by different means. FAD-linked PSs mutations may alter the expression/sensitivity of ER Ca^{2+} release channels (RyR and IP_3R) leading to an exaggerated ER Ca^{2+} release that in turn may cause abnormal mitochondrial Ca^{2+} uptake. wt PSs, but not the FAD mutants, were reported to form Ca^{2+} permeable leak channels in the ER providing a clear explanation for the enhanced Ca^{2+} release found in different AD models. APP can interact with the mitochondrial TIM/TOM protein import complex. The presence of an acidic domain within APP sequence may be responsible for an incomplete mitochondrial translocation that, in turn, inhibited the entry of nuclear-encoded mitochondrial proteins. Mitochondrial Aβ accumulation has been correlated with impaired enzymatic activities of cytochrome *c* oxidase (Cyt c-OX) and inhibition of mitochondrial Aβ-binding alcohol dehydrogenase (ABAD), leading to mitochondrial oxidative damage. Intra-mitochondrial Aβ was demonstrated to directly interact with cyclophilin D (CypD), the PTP component that binds CsA and renders the channel more sensitive to Ca^{2+}, making AD mitochondria more sensitive to PTP opening. The origin of intra-mitochondrial Aβ peptides is however unclear. ER, endoplasmic reticulum; OMM outer mitochondrial membrane. IMS, intermembrane space; IMM, inner mitochondrial membrane.

the hypothesis (see for example [55–59]). Specifically, Zatti et al. [60–62] showed that some FAD-linked PS2 mutations caused a reduction, not an increase, in ER/Golgi Ca^{2+} levels. This experimental discrepancy, while not disproving the Ca^{2+} hypothesis, may thus allow two possible conclusions. The first is that the system is likely to be very complex, with additional unidentified regulatory elements, and the use of different cell systems and experimental approaches (e.g. silencing or overexpression, knock-out or knock-in models) may trigger equally different compensatory mechanisms and calcium effects in the cells. Secondly, given that the discrepancies mostly refer to PS2, a speculative, but appealing, possibility is that PS2 and PS1 play distinct roles in ER/Golgi Ca^{2+} handling. In particular, FAD-PS1 mutations, that cause an increase in the ER Ca^{2+}, unavoidably exacerbate cell death. Conversely, PS2 mutants, by favouring low ER Ca^{2+} levels, might confer relative protection to other routes of cell intoxication, such as Aβ peptides and oxidative damage. This hypothesis would be consistent with the above described role of the ER/mitochondrial Ca^{2+} relationship, and with the clinical observation that FAD-linked PS2 mutations have been associated to milder phenotypes [60–62].

No matter how the synergistic "Ca^{2+} hit" occurs, mitochondrial dysfunction appears an obligatory downstream step in the pathogenesis of AD. Decreased cytochrome *c* oxidase activity, increased free-radical generation leading to oxidative stress and reduced energy metabolism was described in the brain of AD patients before Aβ plaques formation [63–67]. Moreover, electron microscopy analysis of mitochondria in various regions of AD brain revealed significant morphological organelle alterations, such as reduced size of mitochondrial cristae [68].

As to the mechanism, converging evidence points to a role for Aβ peptides and the PTP and, possibly, to a Ca^{2+}-sensitization step. Endogenous as well as ectopically expressed wt or FAD-linked Swedish APP have been found to localize to mitochondria in different cell types [69–71]. In isolated mitochondria, Aβ peptides were shown to inhibit mitochondrial respiration [72,73] and, in the presence of Ca^{2+}, cause the opening of PTP. The involvement of PTP was further reinforced by the analysis of mouse models in which cyclophilin D, (CypD), the PTP component that binds CsA and renders the channel more sensitive to Ca^{2+}, was knocked out [74,75]. Interestingly, this genetic alteration substantially improves the cognitive abilities of a mouse model of AD and alleviates Aβ-mediated reduction of long-term potentiation [76]. Moreover, intra-mitochondrial Aβ was demonstrated to directly interact with CypD, thus providing a molecular basis for this pathogenic mechanism [76]. Other putative damaging effects of Aβ were reported. In PC12 cells, Aβ blocked the entry of nuclear-encoded proteins into mitochondria causing decreased mitochondrial membrane potential, increased ROS production, oxygen glucose deprivation and altered mitochondrial morphology [77]. In another study, Aβ increased neuronal ROS production, activated mitochondrial fission proteins Drp1 and Fis1 and caused mitochondrial fragmentation [78]. Finally, in the mitochondrial matrix Aβ peptides were shown to interact with and inhibit the activity of mitochondrial Aβ-binding alcohol dehydrogenase (ABAD), leading to mitochondrial oxidative damage, increased carbonylation of mitochondrial proteins and impaired activity of respiratory complexes. Some of these alterations were found to occur in AD mouse models before the development of senile plaques, suggesting that mitochondrial dysfunction is an early event in the pathogenesis of the disease [79].

Thus calcium and mitochondria may represent the common theme linking the various reported aspects of the pathogenesis of AD. Several aspects remain however to be solved, and we wish to point out two crucial ones. The first is a clear understanding of the dysregulation of calcium signalling, as discussed above. The second one refers to the origin of intra-mitochondrial Aβ, if a primary role is attributed to this molecule. Experiments of limited trypsin proteolysis and chemical cross-linking showed that APP interacts with the mitochondrial TIM/TOM protein import complex and its orientation is such that the N-terminal resides inside the mitochondria while the large C-terminal part of the protein faces the cytosol [69,71]. Thus, although the presence within mitochondria of γ-secretase constituents and functional γ-secretase activity has been reported [80], it is unlikely that Aβ is being produced on the matrix side. Moreover, the mitochondrial presence of functional β-secretase, essential for the generation of the C99 peptide, substrate for γ-secretase, has not been reported yet. As to direct permeation from the ER, the Golgi complex or secretory vesicles to the cytosol, and then across the outer and the inner mitochondrial membranes (the latter notoriously highly impermeable to most solutes, including ions), this audacious possibility awaits experimental confirmation.

4.2. Huntington's disease (HD)

Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by motor, cognitive and psychiatric symptoms including depression, personality changes, weight loss, dementia and motor disturbances. The latter are characterized by uncontrolled movements (chorea), developing in the terminal stage into severe akinesia. The disease is inherited in an autosomal dominant fashion and the mutation responsible for the disease was identified as a CAG-triplet expansion in exon 1 of *huntingtin* gene. The CAG sequence codes for glutamine and in HD, an expansion of the polyglutamine (polyQ) stretch beyond 35 glutamines results in pathogenicity and causes the selective death of the GABAergic spiny neurons of the striatum. The length of the polyQ stretch inversely correlates with the age of onset symptoms. Mutant Huntingtin (polyQ-Htt) forms intracellular aggregated, particularly in cell nuclei, and, to a lesser extent, in the cytoplasm, neurites, and terminals [81]. The aggregates have both been suggested to be toxic as well as neuroprotective to the cells. The exact function of Htt is still unknown: it is considered a scaffolding protein mediating protein–protein interactions and to play a role in processes as diverse as axonal transport, regulation of transcription, exocytosis, Ca^{2+} homeostasis, bioenergetic metabolism and prevention of apoptosis [82]. Dysfunctions in any one of these processes could be involved in the etiology of HD. Moreover, a series of seminal, independent observations have highlighted a possible role for Ca^{2+} and mitochondria also in this neurodegenerative disorder (Fig. 3). The first experimental evidence linking neurodegeneration in HD to mitochondrial impairment was the observation that treatment of mice with 3-nitropropionic acid (3-NPA), an inhibitor of complex II of the respiratory chain, induces a degeneration of striatal neurons in vivo, recapitulating HD pathogenesis [83]. In addition, the activities of the respiratory complexes II, III and IV were shown to be reduced in HD [84]. Then, Panov et al. showed that mutant but not wild type Htt directly impairs mitochondrial function. They also proposed that mutant Htt localize on mitochondrial membranes in the neurons of mutant mice, suggesting that a direct relationship may occur between the observed defects and the disease [85]. Finally, Bezprozvanny et al. demonstrated that Htt forms a ternary complex with Htt-associated protein-1A (HAP-1A) and type 1 IP_3Rs. In this complex, polyQ-Htt, but not the wild type Htt, facilitates Ca^{2+} release from the ER and renders neurons more sensitive to Ca^{2+}-mediated cellular dysfunction [86]. As to the mechanistic link, mitochondrial Ca^{2+} overload appeared the decisive commitment step [87]. In keeping with this view, Choo et al. showed that mutated, but not wild type, Htt induces PTP opening in isolated mitochondria [88], and we recently demonstrated a facilitated opening of PTP in permeabilized polyQ-Htt expressing cells [89]. Thus, also in this case PTP appears to be the final commitment step in a number of cellular stress conditions, with Ca^{2+} acting as a potent sensitizing factor [89]. As to the stress signals converging on mitochondria, logical candidates are ROS, putatively involved in age-related disorders and in conditions of mitochondrial impairment. Interestingly, the coordinated genomic program mediated by

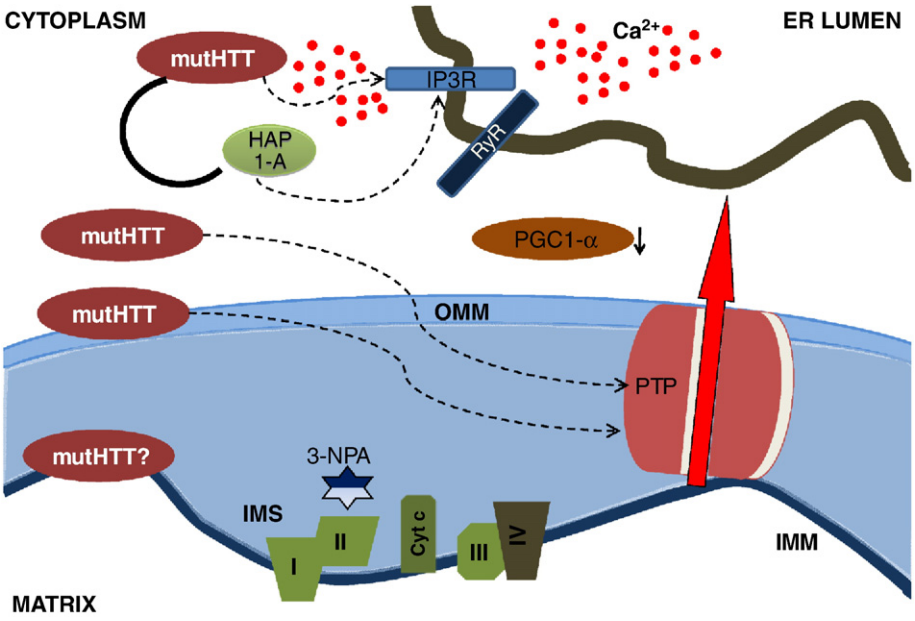

Fig. 3. Mutant Huntingtin (mutHtt) impairs mitochondrial function by transcriprional and non-trascriptional mechanisms. The transcriptional effects are mediated by nuclear translocation of mutHtt. One important consequence of the regulation of gene transcription is the downregulation of PGC1α, and thus the reduced expression of nuclear-encoded mitochondrial proteins involved in the respiratory chains and in the oxidative-stress defense. MutHtt also associates with the outer mitochondrial membrane (OMM) directly affecting the PTP opening susceptibility and making striatal neurons more vulnerable to excitotoxic stimuli. MutHtt reduces complex II activity and the treatment with the complex II inhibitor 3-nitropropionic acid (3-NPA) induces striatal neurodegeneration in vitro and in vivo. The association of mutHtt with HAP-1A and with the IP3R type I facilitates ER Ca^{2+} release, thus making mitochondria more susceptible to Ca^{2+} overload. ER, endoplasmic reticulum; IMS, intermembrane space; IMM, inner mitochondrial membrane.

Peroxisome proliferator-activated receptor-coactivator (PGC-1α), a transcription factor that upregulates ROS-scavenging systems upon oxidative stress [90], appears dysfunctional in HD striata [91], and also in cellular models of the disease [92]. Indeed, in both cases the level of the PGC-1α transcript is downregulated, and, at least in part, the scavenging enzymes are accordingly reduced. This observation appears very appealing, as recent work has highlighted PGC-1α transcriptional regulation as a promising drug target [93].

Another important way in which mitochondrial function could be impaired in HD is through abnormal axonal trafficking of mitochondria to and from the synapse. PolyQ-Htt has been proposed to inhibit axonal transport through several mechanisms and, recently, it was shown that fragments of Htt associate with mitochondria thus interfering with their microtubule-associated transport [94]. Overall, the data so far obtained appear very coherent, and highlight a straightforward pathogenic route, based on the triggering of a calcium-dependent mitochondrial dysfunction. What remains to be assessed is how the putative damaging effects of polyQ-Htt are coordinately regulated. Are mitochondrial respiratory deficiencies and calcium-mediated organelle dysfunction two independent effects of mutated Htt, or are they causally linked? Or does the latter gradually induce the former, in a vicious cycle of ROS production and genetic damage that can be further exacerbated by toxic challenges affecting the brain? And in this picture, why isn't PGC-1α and its anti-oxidant program upregulated? Relative to the respiratory deficiencies, recent work on cultured striatal neurons transfected with a polyQ-Htt showed downregulation of complex II components at the protein, but not at the mRNA, level [95]. Complex II deficiency appears thus due to a post-transcriptional (proteolytic) effect. Whether a calcium-sensitive protease (e.g. calpains) is involved, as was reported in other non genetic models of neurodegeneration [39], has yet to be demonstrated.

4.3. Parkinson's disease (PD)

Parkinson's disease (PD) is a chronic progressive neurodegenerative disorder clinically characterized by motor impairments involving resting tremor, progressive rigidity, bradykinesia and postural instability. PD pathology is characterized most prominently by loss of dopaminergic neurons in the substantia nigra and formation of intraneuronal protein aggregates called Lewy body.

Mitochondrial involvement in PD has been an established notion for many years, since the recognition of the mechanism of action of MPTP (1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine). This compound is formed during production of synthetic heroine, and its metabolite MPP+ is a mitochondrial toxin, that blocks complex I of the respiratory chain. In drug abusers, it causes a form of PD that is clinically indistinguishable from the sporadic variety. Further studies corroborated this observation. Biochemical analysis of PD samples revealed the presence of a mild, systemic defect of complex I, and chronic exposure to the most classical complex I inhibitor (rotenone) accurately recapitulated the pathological, biochemical, and behavioral features of PD. The mechanism of complex I inhibition toxicity probably involves oxidative stress, caused by the block of the respiratory chain, but the selective vulnerability of dopaminergic neurons still remain elusive to explain [96].

The recent identification of a cohort of genes involved in the familial forms of PD further corroborates the notion of mitochondrial involvement, as apparently unrelated proteins seem to share this organelle as a common theme, and possibly point to a signalling role for Ca^{2+} (Fig. 4). Specifically, mutations were reported in genes encoding for α-synuclein, DJ-1, Leucin Rich-Repeated Kinase (LRRK2), ubiquitin C-terminal hydrolase L1 (UCHL1), phosphatase-tensin homologue (PTEN)-induced kinase 1 (PINK1), and parkin, as well as within the mtDNA (for a recent review, see [97]). Mutations in the gene encoding the mitochondrial serine protease HtrA2 have also been linked to PD in several families.

α-synuclein mutations are linked to autosomal dominant familiar PD. Inclusions immunopositive for α-synuclein are found in Levy's body, raising to the possibility that the toxicity of the protein is due to an abnormal form of aggregation or fibril formation, in analogy on what happens in AD. In a recent study [98] the different levels of α-synuclein oligomerization have been linked to cell death. In particular,

an heterogeneous mixture of small oligomers of α-synuclein can lead to Ca^{2+} dysregulation, probably via a pore-forming mechanism. Calpain, a major Ca^{2+}-activated protease, can cleave α-synuclein, producing a truncated form more prone to aggregate, thus leading to formation of protofibrils [99]. Overall, these observations suggest that a vicious cycle of Ca^{2+} dysregulation (coordinately activated by the oligomers and, possibly, excitotoxic stimulation) and fibril deposition eventually leads to severe Ca^{2+} overload, to the point of mitochondrial permeability transition and commitment to neuronal cell death.

Parkin has been identified as a ubiquitin-protein ligase (E3) that acts along with the ubiquitin-conjugating enzymes (E2s) in selectivity of ubiquitination and recognition of substrates [100]. Inactivation of parkin leads to reduction in UPS-mediated degradation of its substrates, among which a glycosylated form of α-synuclein. Interestingly, *Drosophila* parkin null mutants exhibited defects in mitochondrial function with signs of increased oxidative stress, muscle degeneration and male sterility. Reduced levels of mitochondrial proteins involved in mitochondrial oxidative phosphorylation were also reported in parkin-knock-out mice, which exhibited normal brain morphology, but increased striatal extracellular dopamine levels [101]. Overexpression of parkin in cultured cells prevents mitochondrial swelling and stress-induced apoptosis. The protein appears localized in the mitochondrial matrix, where it enhances mitochondrial gene transcription and biogenesis in proliferating cells, but the exact mechanism for the protective function of parkin in mitochondria is unknown [102]. Recently, parkin has been shown to promote the selective clearance of damaged mitochondria through the mitophagic process [103] further reinforcing the concept that it has a neuroprotective role.

PINK1 is highly conserved protein, containing a catalytic serine-threonine kinase domain, ubiquitously expressed in the human brain. It is unambiguously localized to mitochondrial membranes, and its overexpression protects cells from mitochondrial depolarization and apoptosis induced by the proteosomal inhibitor MG132, and from mitochondrially-induced apoptosis triggered by staurosporine [104]. Loss of function mutations are responsible for male sterility, and muscle and dopaminergic neuronal degeneration in *Drosophila* [105]. Overexpression of parkin was shown to rescue the mitochondria dysfunction caused by PINK1 deficiency (but did not rescue the increased sensitivity of PINK1 mutant flies to apoptosis), suggesting that the two proteins could cooperate (probably regulating the balance between mitochondrial fission and fusion, [106]) in preserving mitochondrial integrity in various stress conditions [107]. Interestingly, mutant PINK1 has been shown to exacerbate mitochondrial alterations (disturbing the mitochondrial Ca^{2+} fluxes) promoted by mutant α-synuclein, thus suggesting a cooperative role of these two proteins [108].

LRRK2 is a recently found gene, mutated in familiar late-onset PD. Its product is a kinase [109] and a significant fraction of the protein is associated with mitochondria [110]. Pathogenic PD mutations are linked to activation of the intrinsic apoptotic pathway, with cytochrome c released into the cytosol and activation of caspase-3 [111].

Finally, little is known of the function of the DJ-1 gene product. DJ-1 expression is particularly abundant in brain region where it is largely cytoplasmic except for a pool localized in mitochondria. In particular, DJ-1 was reported to be located in the mitochondrial intermembrane space and in the matrix, while very little, if any, is associated with outer or inner mitochondrial membranes. This finding suggests that DJ-1 may act within mitochondria but its precise neuroprotective mechanism remains obscure (for a review, see [112]). Recent findings suggest that DJ-1 is an atypical peroxiredoxin-like peroxidase [113] indicating that it may play a role in reducing mitochondrial oxidative stress.

These data highlight mitochondria involvement in PD. Obviously, an intriguing possibility is that mitochondrial Ca^{2+} homeostasis, and thus the sensitivity to Ca^{2+}-mediated challenges, are directly or indirectly controlled by these proteins. Work on this topic is under way in several laboratories, including ours, and it is easy to predict that also in this model of neurodegeneration the concerted action of

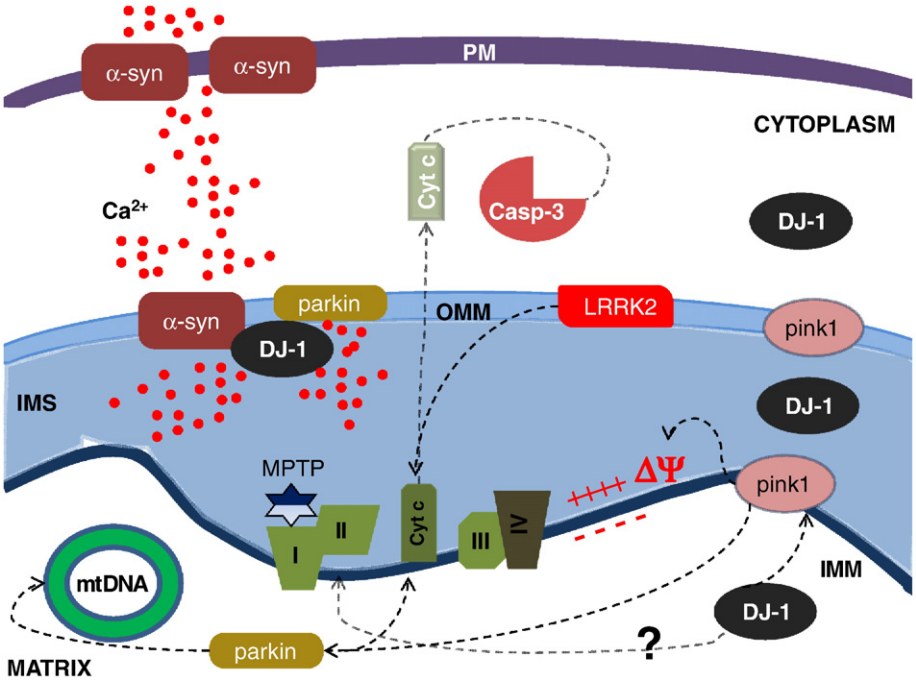

Fig. 4. Mutations in familial PD-linked genes encoding α-synuclein (α-syn), parkin, pink1, DJ-1 and LRRK2 cause mitochondrial dysfunctions through common intersecting pathways. Mutant α-syn promotes Ca^{2+} influx and mitochondrial Ca^{2+} overload and mutant parkin exacerbates this effect. DJ-1, PINK1 and Parkin may act in series on the same protein targets: genetic data suggest that pink1 is upstream of parkin, and that all of them exert a protective role preserving mitochondrial functions, morphology and preventing mitochondrial-induced apoptosis. LRRK2 mutations induce apoptotic death through cytochrome c release and activation of caspase-3. Complex I activity is reduced in PD and its inhibition by MPTP or rotenone causes dopaminergic degeneration. Mutations in mtDNA-encoded complex I subunits also cause PD. PM, plasma membrane; OMM outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane.

Ca^{2+} and toxic challenges on mitochondria will prove to represent the mechanism leading through time to neuronal dysfunction. Indeed, preliminary evidence from our laboratory, demonstrating an alteration in mitochondrial Ca^{2+} homeostasis in a PD model, supports this possibility. Specifically, by using recombinant aequorin to evaluate Ca^{2+} fluxes [3] we observed an alteration of mitochondrial Ca^{2+} signals in SH-SY5Y cells overexpressing wild type or mutated (G2019S and R1441C) LRRK2, associated with PD (Celsi in preparation).

Overall, the picture emerging from the study of the pathogenesis of the neurodegenerative disorders appears terribly complex, with many uncertainties and gaps to fill. Nevertheless, the common role of mitochondria dysfunction (metabolic, morphologic or dynamic) appears very clear, and provides not only a leading theme for further studies, but also a promising pharmacological target in these devastating diseases.

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