Mitochondrial localization of NCXs: Balancing calcium and energy homeostasis

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Abstract

It is well established that mitochondria are the main source of ATP production within cells. However, mitochondria have other remarkable functions, serving as important modulators of cellular Ca^{2+} signaling, and it is now generally recognized that control over Ca^{2+} homeostasis is intrinsically interwoven with mitochondrial abilities to adjust and tune ATP production. In this review, we describe the mechanisms that mitochondria use to balance Ca^{2+} homeostasis maintenance and cell energy metabolism. In recent years, the knowledge on the molecular machinery mediating Ca^{2+} influx/efflux has been improved and, albeit still open to further investigations, several lines of evidence converge on the hypothesis that plasma membrane Na^{+}/Ca^{2+} exchanger (NCX) isoforms are also expressed at the mitochondrial level, where they contribute to the Ca^{2+} and Na^{+} homeostasis maintenance. In particular, the connection between mitochondrial NCX activity and metabolic substrates utilization is further discussed here. We also briefly focus on the alterations of both mitochondrial Ca^{2+} handling and cellular bioenergetics in neurodegenerative diseases, such as Parkinson's and Alzheimer's disease.

Key words: Bioenergetics, Calcium, Glutamate, Mitochondria, Na^{+}/Ca^{2+} exchange.

1. Introduction

As the site of cellular respiration and oxidative phosphorylation, mitochondria are the main source of ATP production within a cell, and it is now well recognized that the bioenergetic functions supported by mitochondria are generally controlled by Ca^{2+} ions.

From a historical perspective, mitochondria have long been considered organelles whose only function was to buffer cytosolic Ca^{2+}. However, since the 1960s the role of mere "Ca^{2+} sinks" has started to be reconsidered. In particular, between the 1970s and '80s, physiological concentrations of Ca^{2+} were found to regulate several mitochondrial dehydrogenases [1-4], causing an increased supply of reducing equivalents to drive respiratory chain activity and, consequently, ATP synthesis [5]. These findings lead to the introduction of a scheme called "parallel activation model": an increase in cell energy demand in response to different stimuli (e.g. nutrients, hormones, neurotransmitters, etc.) produces higher cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$). This is relayed to the mitochondrial matrix, causing an increase in mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_m$), which in turn stimulates energy production to sustain the higher energy demand [6]. Overall, both Ca^{2+} and ATP have been demonstrated to be essential modulators of several cellular processes, in both health and disease [7-12]. Therefore, over the last years the regulation of mitochondrial Ca^{2+} homeostasis and its relationship with cell bioenergetics has received increasing attention. In this review, we will specifically focus on the Na^+/Ca^{2+} exchanger (NCX) – one of the master regulators of cell Ca^{2+} cycling – and discuss recent observations that point to a key role of the NCX Ca^{2+} balancing activity in controlling cell energy metabolism and mitochondrial Ca^{2+} homeostasis.

2. Na^+-dependent Ca^{2+} transport: not a single actor playing on the stage of mitochondrial membranes

Ca^{2+} ion flows across the cell membrane and organelles are vital components of the signaling networks that control the overall cell activities. One of the main regulators of the Ca^{2+} homeostasis is the Na^+/Ca^{2+} exchanger (NCX). NCX catalyzes the bidirectional and rheogenic exchange of 3 Na^+ and 1 Ca^{2+} ions across the plasma membrane, operating either in Ca^{2+}-efflux/Na^+-influx mode (forward mode) or Ca^{2+}-influx/Na^+-efflux mode (reverse mode) [13]. NCX belongs to a multigene family (*Slc8a1-3)* encoding three different isoforms, namely NCX1, NCX2, and NCX3. Exchanger isoforms display a tissue-specific distribution:

NCX1 is widely distributed among tissues, NCX2 is mainly found in the brain, and NCX3 expression is essentially restricted in the brain and skeletal muscles [14]. NCX primarily works in the forward mode, i.e. it removes Ca^{2+} from the cell, thereby contributing to clamp intracellular Ca^{2+} at low viable levels. Deviations from physiological Ca^{2+} range can be also prevented by the buffering capacity of mitochondria: Ca^{2+} accumulation within the matrix is primarily controlled at the inner mitochondrial membrane (IMM) by the mitochondrial Ca^{2+} uniporter (MCU) [15] and thermodynamically favored by the negative membrane potential ($\Delta\psi_m$) generated by the respiratory chain [16, 17]. This activity is balanced by mechanisms that mediate Ca^{2+} extrusion. In this regard, a Na^+-dependent exchange mechanism controlling Ca^{2+} efflux has been long determined also in mitochondria [16, 18-20], however over the years, the molecular identity of this exchanger has remained elusive. From a historical perspective, the first hypothesis on the existence of a mitochondrial Ca^{2+} efflux system dates back to 1960. Accordingly, in 1974 Carafoli et al. [21] provided evidence documenting that in isolated cardiac mitochondria, a Na^+ -dependent Ca^{2+} efflux pathway was active and reasonably implicated in balancing the sustained Ca^{2+} uptake operated by mitochondria. In particular, they showed that K^+, Rb^+ and Mg^{2+} were unable to evoke an efficient mitochondrial Ca^{2+} release, which was instead evoked by Na^+, and surprisingly also by Li^+, the only monovalent cation able to substitute Na^+ in supporting mitochondrial Ca^{2+} efflux [21-24]. This functional characterization paved the way for the subsequent attempts to perform a molecular characterization of this exchanger. In 1992, Garlid and colleagues identified, from the heart beef mitochondria, a 110-KDa protein catalyzing Na^+/Ca^{2+} and Li^+/Ca^{2+} activities, suggesting a Na^+/Li^+ -evoked mitochondrial Ca^{2+} efflux, thus supporting previous findings by Carafoli et al. [21]. Although promising, these results were not followed up by a clear molecular identification, which Palty and colleagues achieved only two decades later. In line with the previously reported experimental observations, in 2010 they identified NCLX (shorthand for $Na^+/Ca^{2+}/Li^+$ exchanger) as an essential component of the Na^+-dependent Ca^{2+} exchanger in mitochondria [19, 27]. Encoded by the *Slc8b1* gene, NCLX localizes into the IMM and catalyzes Li^+/Ca^{2+} exchange as well as Na^+/Ca^{2+} exchange at similar rates, recapitulating one of the distinctive characteristics that was originally noted for mitochondrial Na^+/Ca^{2+} exchange activity [19, 21]. Although substantial effort has been dedicated in searching for the physiopathological relevance of NCLX [25-29], the possibility that members from the NCX transporters also contribute to the mitochondrial Na^+/Ca^{2+} exchange has been in parallel explored. In particular, independent

lines of evidence coming from our and other laboratories converge and support the existence of an endogenous pool of plasma membrane NCX that specifically localizes within mitochondria from diverse cell types, where it contributes to mitochondrial Ca^{2+} handling and ATP production, both in physiological and pathological settings [30-36]. Interestingly enough, a contribution of NCX transporters to mitochondrial Ca^{2+}-clearing mechanisms has been also hypothesized to compensate the lack of NCLX expression that characterizes some subsets of nematode species [34, 35]. With the reasonable assumption that NCX also localize within mitochondria, we may then ask what is the relative contribution of NCX and NCLX to the Na^{+}/Ca^{2+} exchange activity across mitochondrial membranes. According to available information, there may be two complementary and not exclusive models that require appropriate validation. As already anticipated by Abramov [20] one possibility to explore is that NCLX and NCX transporters expressed within IMM can form hetero/homomeric complexes [37] and, thereby, in principle co-operate: blocking activity or expression of one gene product would interfere with the activity of the other. In this case, the loss of function phenotypes observed when NCLX or NCX is blocked/reduced/silenced would simply follow disruption of functional interaction between NCLX and other NCXs. As a matter of fact, functional multimeric complexes have been described for pure NCX1 [37, 38] or for partially truncated NCLX constructs [39]. On the other hand, data set from Scorziello et al., providing evidence for a specific localization of NCX3 on the outer mitochondrial membrane (OMM) [33], open to another scenario: NCLX on the IMM and NCX on the OMM will work in tandem to allow Ca^{2+} efflux from the mitochondrial matrix to cytoplasmic space. This latter suggestion would fit with the recently proposed function of OMM as a more regulated Ca^{2+} barrier than hitherto recognized [16].

The stoichiometry and voltage dependency of mitochondrial Na^{+}/Ca^{2+} exchange have been debated since the its seminal description made by Carafoli [40, 41]. Most data set support a non-electroneutral coupling ratio for mitochondrial Na^{+}/Ca^{2+} exchange [39, 42, 43], as in general reported for the NCX mode. Moreover, depolarization or hyperpolarization shifts in $\Delta\psi_m$ triggered by Na^{+} influx/Ca^{2+} efflux or Ca^{2+} influx/Na^{+} efflux modes are more readily resolved when charge compensation from the electron transport chain is prevented. [43, 44]. Overall, we can assume that the Na^{+}/Ca^{2+} exchange taking place in mitochondria is a rheogenic process involving both NCLX and NCX. Likewise, these transporters exhibit similar ionic

regulation of their activity, with specific reference to the K^+-mediated potentiation and the inhibition mediated by Ni^{2+}, Mg^{2+}, Ba^{2+} and La^{3+} [45-47].

Structural and functional studies on NCX and NCLX interestingly revealed other overlapping and distinctive features. They all are modelled to have two hydrophobic regions of trans-membrane segments separated by a hydrophilic loop [48]. The hydrophobic trans-membrane regions contain conserved motifs, the α-1 and α-2 ions transporting regions, which represent the catalytic core specifically involved in ions translocation [49]. The peculiar difference in transported ions selectivity between NCX and NCLX is largely due to specific variations in ion-coordinating residues involved in exchange processes [49-51]. While NCX isoforms are allosterically regulated by Ca^{2+} through its binding to the Ca^{2+} binding domains CBD1 and CBD2, NCLX has a much smaller regulatory domain which virtually lacks CBD domains. Therefore it is possible that Ca^{2+} act as an indirect modulator (i.e by affecting Calpain-mediated cleavage, Ψ_m changes or MCU activity) rather that as an allosteric factor [52]. Similarly, both NCX and NCLX activity can be strongly regulated by H^+ [52, 53]. Several lines of evidence support the notion that conventional kinases like PKA can stimulate NCLX function [54-56], whereas conflicting results were generated when this issue was explored for NCX. Finally, even if it is known that lipid molecules can profoundly affect function and localization of NCX surface fraction [57-59], equivalent studies addressing these issues for mitochondrial NCX and NCLX have not been performed yet.

2.1 Mitochondrial localization of plasma membrane NCX isoforms, NCX1, NCX2 and NCX3 in the rat CNS

Our research group explored the cellular and subcellular distribution of NCX isoforms in the rat CNS by using immunohistochemistry and electron microscopy techniques [18, 30, 31].

With the use of isoform-specific antibodies, a strong staining for all the three different nuclear-coded NCX isoforms has been observed in the inner mitochondrial membrane of every tested brain area (with the highest expression profiles observed in the cortex and hippocampus), both in neurons and in astrocytes [18, 30, 31]. The plasma membrane NCX isoform mostly shown in brain mitochondria is NCX2, which is equally present in astrocytic and neuronal mitochondria. NCX3 displays a preferential expression at the neuronal level, whereas NCX1 is shown both in astrocytes and in neurons, with the highest levels in the hippocampus [18, 31]. Electron microscopy investigations allowed the resolution of the subcellular localization of NCX-

positive mitochondria in neurons and astrocytes [30]. While in astrocytes NCX-positive mitochondria are preferentially located in thick proximal processes, in neurons most NCX-positive mitochondria are preferentially located in dendrites and neuronal soma, rather than in synaptic terminals [18, 30]. In particular, mitochondria displaying NCX1 immunoreactivity are mostly located in distal dendrites, whereas NCX2 and NCX3 immunoreactivity can be more consistently found in all neuronal compartments. Western blot experiments performed on mitochondrial membrane protein extracts from rat hippocampal and cortical homogenates confirm these findings [18, 30].

Overall, three main speculations can be made based on the NCX expression profiles presented above: 1) the post-synaptic localization of NCXs suggests a potential role in buffering Ca^{2+} ions entering upon post-synaptic receptors activation [18, 30]; 2) glial mitochondria are close to Ca^{2+} influx sources, thus possibly participating in perimembranous $[Ca^{2+}]_c$ buffering during astrocytic Ca^{2+} signals, which may result from either spontaneous or evoked activity [30]; 3) as previously discussed in this review, Ca^{2+} signaling plays a well-recognized role in regulating cell metabolism: considering that significant NCXs immunoreactivity was most probably related to the glutamatergic synapse and that glutamate is also a key metabolic substrate, a connection between NCXs-Ca^{2+}-glutamate and cell metabolism may exist. The following section will analyze the main findings on the role of mitochondrial NCX in a metabolic setting where glutamate is the main source of energy.

2.2 Mitochondrial and plasma membrane Na^+/Ca^{2+} exchange activity inhibitors

It has been studied for several years whether pharmacological approach could be appropriate for the discrimination of plasma membrane and mitochondrial NCX activity [60], however deeper investigations are needed to better clarify this critical issue. Before the development of pharmacological tools, divalent and trivalent cations (Ni^{2+}, Cd^{2+}, and La^{3+}) and organic compounds have been used for a long time to inhibit NCX [13, 61]. However, their unspecific action and cross reactivity with other ion channels and transporters paved the way for the development of new drugs more specifically addressed to target the exchanger. In 1996, the isothiourea derivative (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methane sulfonate (KB-R7943) was identified as a selective NCX inhibitor in cardiac cells [62, 63]. After an initial interest in this compound, its use has been limited in reason of the unspecific action observed on ion channels, receptors and

transporters [64-66]. In 2001, a newly synthesized compound, 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400), showed to act like a potent inhibitor of plasma membrane NCX with minimum effects on other transporters and ion channels [64]. The identification of a Na^+/Ca^{2+} exchange system working also at the mitochondrial level increased the interest in searching for effective and selective inhibitors to study the Na^+/Ca^{2+} exchange activity within these organelles. Interestingly, early studies indicated the ability of some benzothiazepine analogues (diltiazem and clonazepam) to inhibit the mitochondrial Na^+/Ca^{2+} exchange activity [67, 68]. However, subsequent evidence clearly demonstrated a lack of specificity, which therefore prevented their use [69, 70]. Later on, another benzothiazepine analogue, CGP-37157 was found to specifically inhibit the activity of the cardiac mitochondrial Na^+/Ca^{2+} exchanger without affecting L-type Ca^{2+} channels, Na^+-K^+ ATPase and Ca^{2+} ATPase transporters at relevant degree [71]. Other research groups confirmed the pharmacological properties of CGP-37157 also in cells from other tissues [72, 73]. In line with these studies, Palty et al. demonstrated a profound inhibition of NCLX exerted by CGP-37157 at the concentration of 10 μM, which was initially considered safe towards the plasma membrane NCX activity inhibition [19]. Other studies raised some concerns regarding CGP-37157 selectivity, at least in some cell types [74, 75], suggesting caution in its use to study the mitochondrial Na^+/Ca^{2+} exchange activity. The finding that NCX plasma membrane isoform contribute to the mitochondrial Na^+/Ca^{2+} exchange further complicated the possibility to discriminate their specific activity within both physiological and pathological settings (i.e. synaptic transmission and/or neurodegenerative diseases), making this issue not fully resolved. In principle, it is possible to hypothesize that such discrimination may be accomplished by using a low concentration of CGP-37157 (3 μM), which should not significantly inhibit NCLX [19], but at the same time should ensure a significant inhibition of NCX [36]. Nevertheless, also in these conditions it is not possible to rule out an effect of CGP-37157 on NCLX, thus its contribution should be pointed out by using a specific mRNA silencing approach. In parallel, the use of well-known specific plasma membrane NCX inhibitors (i.e. SEA0400 [60, 76]) could reasonably allow to highlight the specific contribution of these isoforms. Overall, more efforts are needed to develop new compounds with the ability to selectively inhibit NCX within the intramitochondrial milieu.

3. Mitochondrial NCXs: linking Ca^{2+} homeostasis to metabolic substrates use.

3.1 Glutamate: a neurotransmitter and a key compound in cell energy metabolism. Mitochondrial localization of Na^+-dependent glutamate transporters

First identified in the 1930s, glutamate was immediately recognized as a key compound in cell energy metabolism due to its high concentrations found both in cytosolic and mitochondrial compartments [77, 78]. Although glutamate is now well recognized as the main excitatory neurotransmitter of the mammalian CNS [79], its role within metabolic processes cannot be overlooked. Inside mitochondria, glutamate is converted into α-ketoglutarate by deaminating or transaminating enzymes [80-82]; then, it can enter the tricarboxylic acid (TCA) cycle as an anaplerotic substrate. This is of paramount importance, especially when a depletion of TCA cycle intermediates may typically take place (i.e. hypoxia): glutamate supplementation is thought to contribute to cell recovery after reoxygenation has occurred [83, 84] (the metabolic role of glutamate in ischemic settings, with special regard to its relationship with mitochondrial exchanger activity, will be discussed in more detail by a different review in this issue).

But what is the connection between mitochondrial NCX expression and glutamate metabolic use? It is generally accepted that glutamate access to the mitochondrial matrix is mediated by the activity of aspartate-glutamate carriers (AGCs) [85]. AGCs are nuclear-coded proteins, which supply the aspartate synthesized within the mitochondrial matrix to cytosol in exchange for the cytosolic glutamate plus a proton [86]. These transporters play a pivotal role in the transport of NADH reducing equivalents from cytosol to the mitochondrial matrix as components of the malate-aspartate shuttle (MAS) [86-88]. In 2004, Ralphe and coworkers reported that transporters other than AGCs can mediate glutamate access into the mitochondrial matrix. They identified Excitatory Amino Acid Transporter type 1 (EAAT1; whose rodent homolog is called GLAST, GLutamate–ASpartate Transporter [78]) in myocardial mitochondria as part of the MAS [89]. EAATs, whose family encompasses five members (EAAT1-5), are the primary regulators of extracellular glutamate concentrations in the CNS [78]. EAATs are Na^+-dependent transporters: the transport of glutamate is coupled to the co-transport of three Na^+ ions and the countertransport of one K^+ ion [90, 91]. Later on, our data lent support and provided new ground to the original findings of Ralphe and coworkers [89]. In particular, we found that EAAT1, as well as EAAT2 and EAAT3 (whose rodent homologs are respectively called GLT-1 – Glutamate Transporter 1 – and EAAC1 – Excitatory Amino Acid Carrier 1 [78]) were also

expressed in the mitochondrial membrane of both rat cerebral cortex and hippocampus [32]. On the same line, immunoblot studies confirmed EAAC1 expression both in mitochondria isolated from brain and cardiac tissues [32]. The existence of EAAT mitochondrial pools in neuronal and glial cells, and the Na^+-dependency of their activity caught our attention. Since EAATs transport glutamate using the favorable Na^+ gradient and increases in Na^+ levels are thereby expected in the highly restricted functional domain of mitochondrial matrix, a mechanism able to restore the Na^+ gradient after glutamate entry is required. NCXs were at that time attractive candidates to investigate for two main reasons. First, we had evidence of NCX mitochondrial localization [30, 31] and a functional interaction between NCX and EAAT transporters was already described on the surface membrane of rodent astrocytes [92-94]. Second, the influence of Na^+/Ca^{2+} exchange activity on mitochondrial bioenergetic functions [5, 95] provided additional background to our working hypothesis: to maintain the Na^+ driving gradient for EAAT-dependent glutamate uptake, NCX would inevitably import Ca^{2+} into mitochondrial matrix, which in turn can stimulate ATP synthesis [32, 96] (see following paragraphs for further details). Coimmunoprecipitation and immunofluorescence analysis, performed on neuronal, glial and cardiac mitochondrial extracts, [32] provided that: 1) NCX and EAAT coassemble in all the examined structures; 2) in general, this multimolecular complex specifically involves two isoforms: NCX1 and EAAT3 (EAAC1). Although the direct physical interaction between NCX and EAAT was not experimentally demonstrated yet, their functional relationship in mediating the metabolic use of glutamate was reported [32, 78].

3.2 Mitochondrial colocalization of NCX and Na^+-dependent glutamate transporters. Metabolic relevance.

The brain is one of the most metabolically active organs in the body. These high energy requirements are mainly due to the maintenance and restoration of ion gradients dissipated by signaling processes, including postsynaptic and action potentials, uptake and recycling of neurotransmitters. Over the last years, the renewed interest in the role of metabolism in supporting brain function has provided new insights on the coupling of neurotransmitter cycling to neuroenergetics. One of the most interesting findings is related to glutamate. Contrary to the previous view of separate metabolic and neurotransmitter glutamate pools [97], magnetic resonance spectroscopy *in vivo* studies have shown that the total pool of tissue glutamate detected seems to be in rapid communication (timescale of seconds to minutes), indicating that the flow of

neurotransmitter glutamate release and recycling is a major metabolic pathway [98, 99], and that glutamate activities as neurotransmitter and as energetic substrate cannot be distinguished [99, 100]. When applied to isolated mitochondria in physiological conditions, glutamate can significantly increase the intracellular ATP content [32]. This response is typically observed in mitochondria isolated from structures where the macromolecular complex formed by NCX and EAAT was identified [32]. As an anaplerotic substrate, glutamate can replenish TCA cycle intermediates and, thereby, be used to enhance ATP production. However, it seems unlikely that such an increase may solely rely on glutamate; the mechanism underlying glutamate entry into mitochondria through EAAT implies that variations in $[Ca^{2+}]_m$ may also play a role. When glutamate gets access to the mitochondrial matrix through EAAT, Na^+ enters with glutamate, giving rise to a slight, but significant, increase in intramitochondrial Na^+ concentrations ($[Na^+]_m$). This temporary imbalance in the $[Na^+]_m$ stimulates the exchanger to work in the reverse mode, triggering a virtuous cycle that ends up in a slight increase in $[Ca^{2+}]_m$. This mechanism has been assumed based on a number of experimental findings [32]. First of all, the hypothesis of a functional interplay between NCX and EAAT comes from the observation that the specific pharmacological inhibition of either NCX or EAAT leads to a complete blockade of glutamate-induced ATP production. A glutamate-dependent mitochondrial Na^+ response (evaluated as CoroNa red fluorescence increase) is clearly observed when glutamate is acutely applied to SH-SY5Y neuroblastoma cells and to C6 glioma cells. A Ca^{2+} response (evaluated as Rhod-2 fluorescence increase) is observed as well.

Overall, the mitochondrial colocalization of NCX and EAAT highlights the existence of a mechanism, where the exchanger balances the Na^+ homeostasis after the glutamate uptake, modulating mitochondrial Ca^{2+} levels too. This stimulates the activity of the mitochondrial dehydrogenases [101], driving the glutamate utilization and, finally, enhancing the synthesis of ATP (Figure 1).

One of the most interesting issues concerning NCX-EAAT interaction is that it occurs with high specificity among the existing isoforms. Immunoprecipitation studies performed on hippocampal and cortical mitochondrial extracts show a strong NCX1 immunoreactivity in the EAAC1 antibody precipitates and, in line with these results, EAAC1 is pulled down by NCX1 antibody on reverse immunoprecipitation [32]. The fact that EAAC1 and NCX1 coassemble in neuronal and glial mitochondria is also strengthened by confocal microscopy experiments. These show their consistent colocalization in immunofluorescence studies

performed on isolated mitochondria spotted on glass microslides [32]. The exact role of the privileged interaction between NCX1 and EAAC1/EAAT3 still remains an unsolved issue. However, based on the available findings, the most reasonable hypothesis is that NCX1, rather than NCX2 and NCX3, has a more specific role in modulating Ca^{2+} levels within a specific metabolic pathway (i.e. glutamate use). The different distribution of the three isoforms within the subcellular comportments is in line with this hypothesis, and it may reflect the possible different functions performed by heterogeneous mitochondria populations.

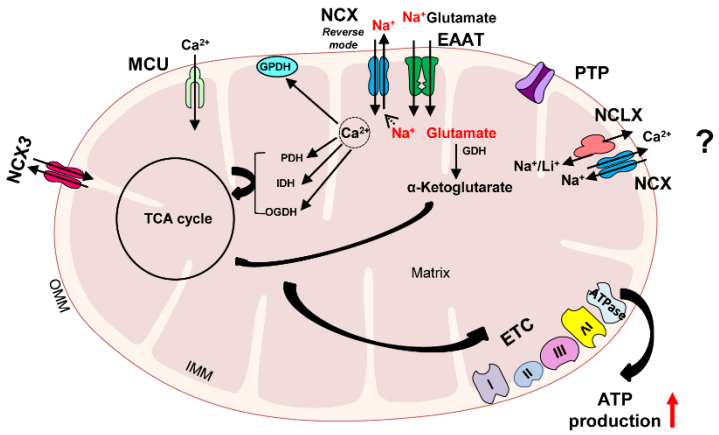

Figure 1. Mitochondrial Ca^{2+} homeostasis and ATP synthesis in the presence of the metabolic compound glutamate. While NCX and EAAT coexist within a multimolecular complex in the inner mitochondrial membrane (IMM), NCX3 has been localized in the outer mitochondrial membrane (OMM), where it probably plays a unique role in controlling the mitochondrial Ca^{2+} transport. By using the favorable Na^+ gradient, EAAT transports glutamate into the cell. This in turn elicits an increase in intracellular $[Na^+]_i$, thereby activating NCX reverse mode. Consequently, $[Ca^{2+}]_i$ increase. Such an increase can be buffered by mitochondria, stimulating Ca^{2+}-dependent dehydrogenases. Once in the cytoplasm, glutamate can enter the mitochondria through EAAT. While restoring the Na^+ gradient, NCX reverse mode activity induces an increase in $[Ca^{2+}]_m$, further increasing dehydrogenases activity and enhancing ATP production by using glutamate as metabolic fuel.

NCX = Na^+/Ca^{2+} exchanger; EAAT = Excitatory Amino Acid Transporter; MCU = Mitochondrial Ca^2 uniporter; NCLX = Li^+/Ca^{2+}, Na^+/Ca^{2+} exchanger; PTP = Permeability transition pore; ETC = Electron transport chain; GPDH = FAD-glycerol phosphate dehydrogenase; PDH = Pyruvate dehydrogenase

complex; IDH = NAD isocitrate dehydrogenase; OGDH = Oxoglutarate dehydrogenase; OMM = Outer mitochondrial membrane; IMM = Inner mitochondrial membrane.

4. Mitochondrial Ca^{2+} handling and neurodegenerative diseases

An increase in $[Ca^{2+}]_m$ is typically a double-edged sword: while a transient and limited rise can stimulate ATP production, an excessive matrix load due to imbalance of mitochondrial Ca^{2+} uptake/extrusion negatively impacts on cellular functions and sensitizes cells towards death stimuli. Subtle or more evident alterations in Ca^{2+} cycling, which naturally and progressively occur in the background with aging, can be further and pathologically promoted by environmental insults and/or genetic factors. In particular, derangements in Ca^{2+} levels have the potential to amplify mitochondrial dysfunction, energetic failure and, consequently, promote cell death, which all are pathological signatures of several neurodegenerative diseases (Figure 2). For instance, numerous (if not all) alterations in cell's Ca^{2+} signaling toolkit that have been described in Parkinson's disease (PD) [26, 54, 102, 103] ultimately impact on Ca^{2+} buffer capacity and bioenergetic functions of mitochondria [104, 105]. In this scenario, the mitochondrial Na^+/Ca^{2+} exchange activity emerges as a promising yet tricking function to target. On one hand, in cell models that recapitulate genetic dysfunctions linked to PD, Ca^{2+} extrusion via NCLX is suppressed so that strategies that restore NCLX-mediated Ca^{2+} efflux rescue mitochondrial functions and viability [54, 55]. On the other hand, acute exposure to α-synuclein plus rotenone (toxins known to promote PD-like phenotype; [36, 106, 107]) promote mitochondrial Ca^{2+} accumulation that is prevented by limiting mitochondrial Ca^{2+} uptake either by CGP-37157 or by silencing NCX1 expression [36].

Perturbations in intracellular Ca^{2+} homeostasis and mitochondrial failure are also at the core of the degenerative processes of Alzheimer's disease (AD) [12, 108], which is characterized by two main hallmark lesions: deposition of amyloid beta (Aβ) and neurofibrillary tangles (mainly consisting of the hyperphosphorylated form of tau protein) [109]. Aβ oligomers can form Ca^{2+}-permeable channels in membranes [110], thereby altering cytosolic and consequently mitochondrial Ca^{2+} homeostasis, similarly to what has been reported for α-synuclein. Further increases in intracellular Ca^{2+} levels originate from Aβ and tau interaction with ligand-gated or voltage-gated Ca^{2+}-influx channels [111]. During AD progression, Ca^{2+} alterations become progressively severe and proceed with mitochondria dysfunctions [112], which culminate

in ATP production failure, oxidative stress and deadly impairment of mitochondrial Ca^{2+} buffer capacity [113-115]. However, it is still a matter of debate whether mitochondrial Ca^{2+} homeostasis dysregulation is a causative or a consequential event of the bioenergetic failure [116]. Nevertheless, it is undoubted that both contribute to the disease pathway and its progression. On one hand, different lines of evidence converge on the assumption that Aβ directly impairs mitochondrial function and the oxidative phosphorylation system. In this regard, it has been demonstrated that Aβ accumulates into mitochondria, decreasing the activity of complexes III and IV of the respiratory chain [117, 118]. This leads to a failure in restoring the bioenergetic homeostasis, a decrease in oxygen consumption, and a drop in intracellular ATP levels. Such a decrease in mitochondrial function causes the Aβ to trigger a vicious cycle, in which an elevation in cytosolic Ca^{2+} levels, oxidative stress, and decreased ATP synthesis induce a further Ca^{2+} overload and an even higher oxidative stress [119, 120]. On the other hand, it is well established that brain metabolism (i.e. glucose metabolism) is significantly impaired in AD patients, and that this alteration may precede the clinical onset of AD, contributing to its etiology [121]. Several lines of evidence suggest that a drop in intracellular ATP levels can drive the Amyloid Precursor Protein processing towards the amyloidogenic pathway rather than the non-amyloidogenic one, leading to Aβ accumulation [122-124]. Different laboratories have already documented that the Na^{+}/Ca^{2+} exchange activity is compromised and potentially promotes remodeling of the Ca^{2+} cycling in AD [125-128]. In particular, available findings converge on a reduction of both the expression and activity of NCX [126, 128]. In this scenario, the question naturally arose as to whether alterations in NCX functions could also impact on metabolic dysfunctions in AD. Jadiya and colleagues have recently provided important pieces to this puzzle [27]: 1) NCLX expression decreases in the frontal cortex of AD, a finding that is matched in 3xTg-AD mice (a murine transgenic model of AD); 2) in 3xTg-AD mice impaired Na^{+}/Ca^{2+} exchange activity precedes neuropathology; 3) deletion of NCLX in 3xTg-AD mice worsens memory decline and AD lesions, which can all be improved by genetic rescue of neuronal NCLX. Does NCX play any role in this scenario? We believe there is enough background to explore this question.

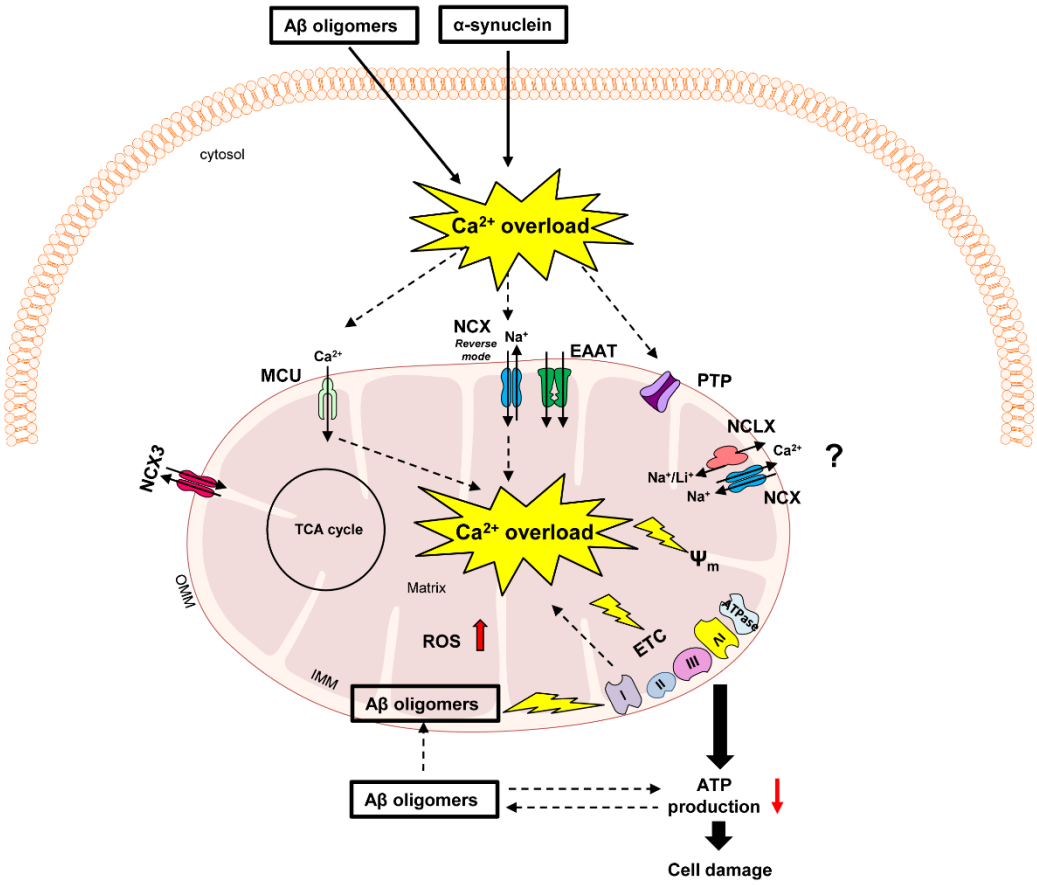

Figure 2. Mitochondrial Ca^{2+} homeostasis: bioenergetics and cell death. In neurodegenerative diseases, Ca^{2+} homeostasis dysregulation may cause bioenergetics failure leading to cell death. For instance, Aβ oligomers or α-synuclein can form Ca^{2+}-permeable channels in the membranes, thereby altering cytosolic and mitochondrial Ca^{2+} homeostasis, leading to ATP production impairment. On the other hand, several lines of evidence suggest that a drop in intracellular ATP levels can be an upstream event of Aβ accumulation.

ROS = Reactive oxygen species; Ψ_m = Mitochondrial membrane potential; OMM = Outer mitochondrial membrane; IMM = Inner mitochondrial membrane.

5. Conclusions

Mitochondria are remarkably autonomous and dynamic cellular organelles that couple cellular metabolism with Ca^{2+} transport processes, therefore influencing the overall cellular network. During the last sixty years several issues related to mitochondria have been clarified; however, their Ca^{2+} handling machinery is extremely intricate, and, at the same time, so fascinating that it still encourages investigators to further understand underlying mechanisms. The finding that NCX plasma membrane isoforms contribute to

Na^+/Ca^{2+} homeostasis in mitochondria add additional layers of complexity to our current knowledge and open new avenues to explore. In this regard, the privileged interaction between NCX1 and EAAC1/EAAT3 has contributed to a better understanding of the metabolic use of glutamate as a source of energy, opening up new insights on the role of this interaction both in health and disease. How the different NCX isoforms could be translocated to the inner mitochondrial membrane is still an unresolved question. In general, proteins encoded by nuclear DNA and addressed to mitochondria own specific mitochondrial targeting sequences that interact with highly specialized transport systems [129, 130]: to the best of our knowledge no specific mitochondria targeting sequences have been identified in any of the cloned NCX isoforms. However, mitochondrial protein sorting is turning out to be a pathway more complex than expected, and for several precursor proteins the import machineries have not been identified yet [130]. Canonical transient receptor potential 3 (TRPC3) channels are another example of plasma membrane channels which localize into the inner mitochondrial membrane, and whose mechanism of mitochondrial integration has not been elucidated so far [131, 132].

As it will be further discussed in a specific review of this special issue, the metabolic role of NCX1-EAAC1/EAAT3 interaction has been investigated in ischemic settings. As a future direction, it will be very interesting to evaluate the contribution of these transporters in other diseases affecting Ca^{2+} handling and metabolic pathways, including Parkinson's and Alzheimer's disease.

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Competing interests

The authors declare no competing interests.

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