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In vivo brain imaging of mitochondrial Ca^{2+} in neurodegenerative diseases with multiphoton microscopy

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Abstract

Mitochondria are involved in a large number of essential roles related to neuronal function. Ca^{2+} handling by mitochondria is critical for many of these functions, including energy production and cellular fate. Conversely, mitochondrial Ca^{2+} mishandling has been related to a variety of neurodegenerative diseases. Investigating mitochondrial Ca^{2+} dynamics is essential for advancing our understanding of the role of intracellular mitochondrial Ca^{2+} signals in physiology and pathology. Improved Ca^{2+} indicators, and the ability to target them to different cells and compartments, have emerged as useful tools for analysis of Ca^{2+} signals in living organisms. Combined with state-of-the-art techniques such as multiphoton microscopy, they allow for the study of mitochondrial Ca^{2+} dynamics *in vivo* in mouse models of the disease. Here, we provide an overview of the Ca^{2+} transporters/ion channels in mitochondrial membranes, and the involvement of mitochondrial Ca^{2+} in neurodegenerative diseases followed by a summary of the main tools available to evaluate mitochondrial Ca^{2+} dynamics *in vivo* using the aforementioned technique.

Keywords

Mitochondria; calcium; multiphoton microscopy; Alzheimer's disease; fluorescent proteins; GECIs

1. Introduction

Mitochondria are crucial and highly dynamic organelles known for providing energy to the cell in the form of adenosine triphosphate (ATP). They also regulate a multitude of cellular functions, including Ca^{2+} buffering, reactive oxygen species (ROS) production, cellular

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Declaration of interests

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metabolism and apoptotic cell death [1]. ATP production is vital for maintenance of the proper neuronal function, because these cells have limited glycolytic capacity and do not have a backup system to provide energy as is possible in other cells [2]. Besides being energy suppliers of the cell, mitochondria play a central role in regulating intracellular Ca^{2+} signaling. By handling Ca^{2+}, mitochondria control important physiological processes such as the synthesis of hormones, neurotransmitter metabolism or cardiac activity. However, excessive Ca^{2+} levels within the mitochondrial matrix, i.e., mitochondrial Ca^{2+} overload, could lead to an increase in the generation of ROS and the resulting apoptotic cell death that takes place during ischemic episodes and in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) [3].

Traditionally, most studies about the role of mitochondria in neurodegenerative diseases have been performed on cell lines, primary cells extracted from animal models, or in *ex vivo* samples from postmortem human tissue. Recently, the development of more compelling methods, like multiphoton microscopy and genetically targeted indicators, to evaluate mitochondrial dynamics, have ameliorated the study of the organelle's dysfunction in the living brain. Understanding mitochondrial Ca^{2+} dynamics is critical to further understand the role of mitochondria in normal physiology but also in a pathological state. Here, we first briefly review the mitochondrial structure and the Ca^{2+} channels that participate in mitochondrial Ca^{2+} homeostasis. We then address the involvement of mitochondrial Ca^{2+} dysregulation in neurodegenerative diseases. Finally, we summarize recent progress in mitochondrial Ca^{2+} imaging using *in vivo* multiphoton microscopy and optical Ca^{2+} indicators targeted to mitochondria, followed by an example of using Yellow Cameleon (YC) to evaluate mitochondrial Ca^{2+} *in vivo* using this technique and a transgenic mouse model of AD.

2. Brief review of mitochondrial structure

Mitochondria are dynamic entities that extend from the soma to neurites, forming a network along the neuron. They undergo replication, fission and fusion. Depending on the metabolic demand, they migrate along axons and dendrites in anterograde or retrograde directions [4], become stationary in regions where the metabolic demand is higher, and move again depending on physiological changes within the cell. Because of this, they must adapt to any physiological or environmental change that occurs in the neurons [5]. Mitochondria are quasi-independent from the rest of the cell: they have their own DNA, different from the nuclear DNA, and thus synthesize some of their own proteins, which play vital roles in regulating cellular bioenergetics. The rest of their proteins are encoded by nuclear DNA and targeted to mitochondria via different transport mechanisms. Inherited mitochondrial DNA mutations are responsible for the etiology of several human diseases [6, 7]. The different mitochondrial functions are possible due to their compartmentalized organization. Two differentiated membranes are part of the mitochondria, the outer mitochondrial membrane (OMM) -which forms the outer-most border- and the inner mitochondrial membrane (IMM) -which encapsulates the mitochondrial matrix space. IMM has a much larger extension than the OMM due to its cristae (foldings in its internal space), specialized in the oxidative phosphorylation (OXPHOS) [8]. The space between OMM and IMM - the intermembrane space - contains enzymes, such as creatine kinase, and cytochromes, such as cytochrome c.

The OMM is permeable to ions and small molecules, due to the abundant expression of large channels on its surface, known as porins or voltage dependent anion channels (VDAC). VDAC was the first mitochondrial channel described [9]. Substrates needed to produce ATP such as pyruvate or ADP, and other metabolites such as Ca^{2+}, Na^{+}, and K^{+} go through this channel from the cytosol and across the OMM. The ATP produced in mitochondria can then reversely traverse the VDAC and reach the cytosol. The IMM on the other hand, is a non-permeable membrane, and only those solutes with specific transporters can cross it. It contains ion channels and transporters, like the mitochondrial Ca^{2+} uniporter (MCU) complex or the Na^{+}/Ca^{2+} exchanger (NCLX), and mitochondrial enzyme systems like the electron transport chain (ETC). ETC is the molecular machinery used for energy production. It is comprised of complexes I-IV located in the IMM. The energy released by these complexes is used to pump H^{+} from the mitochondrial matrix into the intermembrane space, generating an electrochemical gradient ($\Delta\mu H$) across the IMM. The return of H^{+} into the mitochondrial matrix following the gradient established is carried out by the F_1-F_0 ATP synthase (complex V), which drives phosphorylation of ADP to make ATP [10]. This huge potential difference provides the driving force for cytosolic Ca^{2+} to accumulate in the matrix via the MCU. The main mitochondrial Ca^{2+} transporters and channels are described in the following section and represented in Figure 1.

3. Mitochondrial Ca^{2+} homeostasis: transporters and channels

In the 60s and 70s, studies conducted in isolated mitochondria revealed that they were able to take up high amounts of Ca^{2+} [11]. Since then, it has been widely accepted that mitochondrial Ca^{2+} homeostasis plays an important role in physiology and pathophysiology.

3.1 Mitochondrial Ca^{2+} influx: The Mitochondrial Ca^{2+} Uniporter Complex

The MCU complex is the main route for Ca^{2+} uptake into the mitochondrial matrix, although a rapid uptake mode (Ram) for short Ca^{2+} pulses activated by lower external Ca^{2+} concentration has also been described [12].

In 2011, the groups of Mootha and Rizzuto independently identified the protein CCDC109A as necessary and sufficient for mitochondrial Ca^{2+} uptake *in vitro* and *in vivo*, which matched the features of the MCU [13, 14]. In the following years, numerous studies characterizing the uniporter arose. The MCU complex is a macromolecular complex of proteins and includes the pore forming and regulatory subunits. The MCU is the ion conducting pore. It is ubiquitously expressed throughout varying organisms and human tissues. MCU monomers oligomerize, likely as a tetramer, to form the pore [13]. Two other proteins participate in pore activity: the MCUb and the essential MCU regulator (EMRE). The MCUb shares 50% of similarity with the MCU and hetero-oligomerizes with it [15]. It does not conduct Ca^{2+} on its own, but its presence inhibits mitochondrial Ca^{2+} uptake. EMRE in an auxiliary protein that keeps the regulatory subunits mitochondrial Ca^{2+} uptake 1 (MICU1) and MICU2 (see below) attached to the MCU complex [16].

Mitochondrial Ca^{2+} levels in the matrix are similar to those of the cytoplasm (~100 nM) under resting conditions. The MCU is therefore inactive despite the driving force of the mitochondrial membrane potential ($\Delta\Psi m$) that is produced by the ETC. Upon stimulation,

the MCU activates and transports Ca^{2+} into the mitochondrial matrix instantaneously. This response is regulated by the MICU family, located in the intermembrane space. MICU1 was the first component of the uniporter complex to be reported [17], a few months before the identification of the MCU and after the completion of the protein inventory MitoCarta. MICU1 and MICU2, which constitute an obligate heterodimer [18], act as MCU Ca^{2+} sensors using their two Ca^{2+}-binding EF-hand motifs, which confer the Ca^{2+} sensitivity. MICU1 and 2 are considered the gatekeepers of the MCU, as the combination of both proteins regulates the uniporter and prevents Ca^{2+} uptake at low extramitochondrial Ca^{2+} concentrations [19, 20]. MICU1 participates when the extramitochondrial Ca^{2+} concentration is high, activating the open channel state. At low concentrations, however, MICU2 is the main player, leading to minimal accumulation of Ca^{2+} within mitochondria [18], and preventing mitochondrial Ca^{2+} overload at resting conditions. MICU3, a paralogue of MICU1 and MICU2, is mainly expressed in the central nervous system (CNS) and enhances mitochondrial Ca^{2+} uptake in neurons [21]. Together, these regulators allow a short response rate to low extracellular Ca^{2+} (resting conditions) and a large capacity at high Ca^{2+} levels. This phenomenon explains the Ca^{2+} buffering function of mitochondria. In addition, the mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1) [22], which is necessary for the MCU-mediated mitochondrial Ca^{2+} uptake, and the small calcium-binding mitochondrial carrier protein (SCaMC, also known as SLC25A23) [23], which interacts with MCU and MICU1, also participate in the regulation of the complex.

3.2 Mitochondrial Ca^{2+} efflux: NCLX and LETM1

Ca^{2+} efflux from the mitochondrial matrix depends on two main mechanisms: an electrogenic exchange of Na^+/Ca^{2+} (3 or 4 Na^+ ions per Ca^{2+}) encoded by the *NCLX* gene, and an ubiquitous H^+/Ca^{2+} exchange (likely an electroneutral process of 2 H^+ per Ca^{2+}).

The Na^+-dependent Ca^{2+} release exchanger (NCLX) was first discovered by Ernesto Carafoli in 1974 in cardiac mitochondria [24]. In 2010, Sekler's group identified the Na^+/Ca^{2+} exchanger, expressed only in internal membranes and enriched in the mitochondrial fraction, specifically in the IMM [25]. It was named the Na^+/Ca^{2+} Li^+ permeable exchanger (NCLX), being characterized by its exchanging of Ca^{2+} with Na^+ and Li^+, the only member of its family to do so. The Ca^{2+} extrusion is coupled with the influx of Na^+ into the mitochondrial matrix from the cytosol, which can be effectively replaced by Li^+, and hence its name. This is different from other members of the Na^+/Ca^{2+} exchangers, which do not transport Li^+. This process is allosterically regulated by $\Delta\Psi m$ and by the channel's phosphorylation [26]. Future investigations are needed to determine other regulatory factors and their regulation process.

The Leucine zipper and EF-hand containing transmembrane protein 1 (Letm1) is required for maintaining mitochondrial morphology and cristae structures [27]. Additionally, it has been suggested as a Ca^{2+} extrusion mechanism when Ca^{2+} levels are elevated in the mitochondrial matrix [28]. This protein was proposed by Clapham to catalyze the exchange $Ca^{2+}/2H^+$ [29]. However, other researchers have proposed Letm1 to be part of the K^+/H^+ antiporter [30], rather than being involved in mitochondrial Ca^{2+} extrusion [31], and its molecular nature is still debated.

3.3 Mitochondrial-associated ER membranes

Mitochondrial Ca^{2+} levels are tightly regulated by the ER via mitochondrial-associated ER membranes (MAMs). MAMs are contact points between juxtaposed ER and OMM. They are enriched with VDAC and the MCU on the mitochondrial side, and with inositol trisphosphate receptor (IP_3R) on the ER. The structure is coupled by tethers such as the chaperone protein Grp75 [32] or mitofusin2 [33]. The most important roles of MAMs include intracellular trafficking of mitochondria and ER, energy metabolism, protein folding and cell autophagy [34–36]. In addition, they mediate Ca^{2+} transfer from the ER to mitochondria [37, 38]. These contact sites allow for the formation of Ca^{2+} hotspots that meet the low affinity threshold of MCU to take up Ca^{2+}. The mitochondrial Ca^{2+} uptake from the ER allows for regulation of mitochondrial oxidative metabolism and ATP production [39]. However, excessive mitochondrial Ca^{2+} uptake compromises mitochondrial function, triggering the transient collapse of the mitochondrial membrane potential and leading to apoptosis or necrosis [40, 41]. ER-mitochondria association also regulate macroautophagy, and blocking Ca^{2+} transfer from the ER to mitochondria stimulates autophagy in response to the cell's impaired bioenergetics [42]. Relatedly, mitochondria tethering to the plasma membrane and the Golgi compartment have also been observed [43, 44].

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

Neurodegenerative diseases are a large group of heterogeneous disorders characterized by the selective death of neuronal subtypes. Mitochondrial dysfunction contributes to many neurodegenerative diseases, such as AD or PD [3]. When the brain demands high amounts of energy, the number of mitochondria increases mainly at synapses. Accumulation of misfolded or damaged proteins within neurons is a hallmark of most age-related neurodegenerative diseases. Protein accumulation inside mitochondria can lead to mitochondrial dysfunction and eventually threaten neuronal survival. An immediate consequence of dysfunctional mitochondria is the increase of ROS production. This which promotes oxidative damage to DNA, RNA, proteins and lipids, and eventually leads to cell death. Additionally, many studies suggest that alteration of Ca^{2+} homeostasis is a hallmark of many of these pathologies [45]. In the sections below, we review the main aspects of mitochondrial Ca^{2+} dysregulation in some of the most prevalent neurodegenerative diseases.

4.1 Mitochondria and their role in cell death

Besides ATP production, mitochondria along with mitochondrial Ca^{2+} are needed for initiating apoptotic cell death. An excess of Ca^{2+} taken up by mitochondria, due to impairment of mitochondrial Ca^{2+} influx or efflux, can lead to mitochondrial Ca^{2+} dysregulation and overload. This may cause mitochondrial swelling, respiration impairment, enhanced ROS production and trigger mitochondrial permeabilization and cell death. This process requires opening of the mitochondrial permeability transition pore (mPTP), which is believed to be the initial trigger for apoptosis and necrosis [46]. After mitochondrial permeabilization, different proapoptotic factors are released into the cytoplasm through the OMM. These include cytochrome c, apoptosis inducing factor (AIF), endonuclease (endo) G, and Smac/diablo, each one with different downstream targets. In addition to this, caspases get activated.

Mitochondria are the main intracellular source of ROS. ROS are reactive molecules with an unpaired electron, which can have detrimental effects to the cells. The main ROS produced by mitochondria is the anion superoxide (O^{2-}), although other ROS are produced, such as hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-), nitric oxide (NO) and peroxynitrite ($ONOO^-$). ROS are mainly produced in the mitochondrial matrix, as byproducts of the mitochondrial ETC. In normal conditions, antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase act as free radical scavengers and eliminate the excess of ROS. Oxidative stress takes place when there is an imbalance in the generation and detoxification of ROS. This leads to oxidation and damage of proteins, DNA and lipids. Whereas transiently elevated ROS can have physiological functions, such as cell signaling or promoting protein synthesis [47], chronically elevated mitochondrial ROS production can lead to cellular dysfunction. Since ROS exposure can lead to further intracellular ROS production, these molecules can have detrimental downstream effects such a mtDNA mutation. ROS is particularly damaging to already susceptible neurons, since those neurons require high energy and have large amounts of mitochondria. Upon oxidative stress-induced accumulation of ROS in neurons, proteins and toxic wastes are deposited in the brain, impairing brain homeostasis [48]. Combined with increased ROS production, diminished production of antioxidant agents can further induce cell death and neurodegeneration.

4.2 Mitochondrial Ca^{2+} dysregulation and AD

AD is the most prevalent neurodegenerative disorder. It is commonly associated with age, and leads to progressive cognitive impairment and dementia. Unfortunately, the direct cause of AD is still unknown. Most AD cases are sporadic, with a late onset, and the symptoms appearing after the age of 60. A small fraction of cases - less than 1% - are genetically inherited and characterized by an early onset. Three genes, the amyloid precursor protein (*APP*) and presenilin-1 and 2 (*PSEN1* and *PSEN2*) have been identified as responsible for the familial form of AD (FAD). The main hallmarks of AD are (i) amyloid plaques, formed after increased accumulation of extraneuronal amyloid beta (Aβ) peptide, which assemble from monomeric and oligomeric intermediates, (ii) intraneurofibrillary tangles (NFTs), composed of hyperphosphorylated and misfolded tau, and (iii) severe neuronal loss that leads to memory impairment. Several hypotheses have been postulated to explain the origin of the disease. Since all genetic mutations that lead to FAD are involved in Aβ processing, the “amyloid cascade hypothesis” was proposed [49]. This hypothesis suggests that the origin of AD pathogenesis results from Aβ overproduction and/or the failure of Aβ clearance mechanisms. However, alternative hypotheses have recently gained momentum due to the lack of correlation between Aβ deposition and cognitive decline, and the negative outcomes of many clinical trials targeting Aβ [50, 51]. Among the most relevant of these are the “cholinergic hypothesis” [52], the “tau propagation hypothesis” [53], the “inflammatory hypothesis” [54] and the “glymphatic system hypothesis” [55]. The “Ca^{2+} hypothesis of AD” postulates that activation of the amyloidogenic pathway causes remodeling of the neuronal Ca^{2+} signaling pathway, altering Ca^{2+} homeostasis and impairing mechanisms involved in learning and memory [56]. Disrupted Ca^{2+} homeostasis has been found both in cells from sporadic and familial cases of AD [57–59]. Mitochondrial Ca^{2+} dyshomeostasis

has also been related to AD, due to the neurotoxic effects of Aβ, tau, and other risk factors associated to AD (recently reviewed in [60]).

Both Aβ and tau have been found in mitochondria from human AD brain tissue and transgenic mouse models of AD [61, 62], and when they are exogenously applied to cells [63]. Moreover, some studies indicate that Aβ accumulation in mitochondria starts before the deposition of extracellular plaques [64]. Their import into mitochondria requires the participation of the translocase of the OMM (TOM) and IMM (TIM) import channels [65, 66]. Once in the mitochondrial matrix, they interact with specific intra-mitochondrial proteins, leading to degeneration of the organelle [62, 67].

Aβ itself impairs mitochondrial morphology and function. Using intravital multiphoton imaging, our group has previously shown that the structure and function of mitochondria are impaired in the living brain of transgenic mouse models of AD expressing amyloid deposits [68]. In the regions close to Aβ plaques, decreased mitochondrial number, reduced mitochondrial membrane potential and increased oxidative stress were observed. Altered mitochondria morphology and distribution have also been observed in neurons from AD patients [69] and *in vitro* after Aβ, APP or PS1 expression [70, 71].

Aβ oligomers have been shown to induce mitochondrial Ca^{2+} uptake and overload in primary cells *in vitro* [72], particularly in primary cells aged long-term *in vitro* [73], trigger Ca^{2+} transfer from ER to mitochondria [74], and enhance the Ca^{2+} amount transferred from the ER to mitochondria following an stimulus to release Ca^{2+} from ER (like acetylcholine or caffeine) [75]. Aβ-driven mitochondrial Ca^{2+} overload leads to mPTP activation, cytochrome c release and cell death via apoptosis or necrosis [72]. The use of mitochondrial uncouplers, such as FCCP or CCCP (cyanide-4-(trifluoromethoxy)phenylhydrazone and carbonyl cyanide chlorophenylhydrazone respectively), or NSAIDs (non-steroidal anti-inflammatory drugs), used at low concentrations, induces a mild mitochondrial depolarization, inhibiting mitochondrial Ca^{2+} overload elicited by Aβ without leading to an increase in cytosolic Ca^{2+} and preventing those events. This suggests that mitochondria itself plays a key role in the cell death induced by Aβ, and supports a different mechanism of neuroprotection for NSAIDs via mitochondrial depolarization and that is not related to their anti-inflammatory effect [59, 73]. Using multiphoton microscopy *in vivo* in a mouse model of cerebral amyloidosis (APP:PS1), our group recently demonstrated that a significative amount of mitochondria exhibit high levels of Ca^{2+} and mitochondrial Ca^{2+} overload in neurons compared to wild-type (Wt) controls, but only after plaque deposition [76, 77]. Furthermore, topical application of Aβ oligomers -naturally secreted soluble Aβ oligomers, known as conditioned media- onto the naïve Wt brain *in vivo* also increased the mitochondrial Ca^{2+} concentration. This effect could be prevented by using the MCU inhibitor Ru360 [76]. This result implied that an intact MCU is required for Aβ driven mitochondrial Ca^{2+} uptake. In addition, neurons with elevated mitochondrial Ca^{2+} in their neuronal soma died after a short period of time, thus linking mitochondrial Ca^{2+} overload with neuronal cell death *in vivo*.

Tau is a monomeric protein that stabilizes microtubules, allowing the axonal transport of organelles. In AD, tau becomes abnormally hyperphosphorylated and misfolded, detaching

from microtubules and aggregating into neurofibrillary tangles. When this takes place, mitochondrial transport and therefore the cell's energy supply get compromised, eventually leading to neurodegeneration [78]. It has been shown that, in addition to Aβ, pathological tau also disrupts mitochondrial Ca^{2+} homeostasis. In cells overexpressing tau and cells exposed to tau aggregates, mitochondrial Ca^{2+} buffering is defective [79, 80]. Cortical neurons exposed to extracellular tau and AD patient induced pluripotent stem cell (iPSC)-derived neurons expressing a tau mutation exhibit higher basal mitochondrial Ca^{2+} levels [81], likely due to the inhibition of NCLX by tau.

Additionally, RNA-seq and microarray analyses from human AD and control brains [82–84] of the expression of the genes involved in the mitochondrial Ca^{2+} transport were shown to be altered in AD. Expression of the genes involved in mitochondrial Ca^{2+} influx (the MCU complex) was downregulated, whereas the expression of the gene encoding the channel involved in mitochondrial Ca^{2+} efflux (NCLX) was upregulated [76]. In our opinion, this might suggest a compensatory effort to avoid mitochondrial Ca^{2+} overload in the human AD brain. However, using a different technique, others have found decreased expression of NCLX in human AD brains and in the 3xTg-AD mouse (which develops both plaques and neurofibrillary tangles), which could also explain the mitochondrial Ca^{2+} overload observed *in vivo* [85].

MAMs-associated proteins are also dysregulated in AD [86], and their expression has been reported to be upregulated in post-mortem AD brains, in mouse models of the disease and in primary neurons exposed to Aβ and tau [86]. In addition, it has been proposed that there is an optimal distance between ER and mitochondria, and if this optimal distance is disturbed, this can lead to higher levels of Ca^{2+} transfer from ER to mitochondria [75], which can trigger apoptosis. Increased MAM activity and ER-mitochondria contact points have been observed in human fibroblasts from AD patients [87], and in primary neurons exposed to Aβ and tau [62, 88].

4.3 Mitochondrial Ca^{2+} dysregulation and Parkinson's disease

PD is a neurological locomotory disorder that involves rigidity, bradykinesia and a resting tremor. It is considered the most common movement disorder and the second most common neurodegenerative disorder after AD. The main histopathological hallmarks of PD are Lewy body inclusions containing α-synuclein, a small lipid-binding protein, and loss of pigmented dopaminergic neurons in a small and defined area of the brain involved in motor control called *substantia nigra pars compacta* [89]. The phenotypes of both sporadic and familiar forms of PD exhibit the same symptoms, suggesting that they might share common underlying mechanisms. PD belongs to a larger group of neurodegenerative diseases, known as synucleinopathies. On the cellular level, mitochondrial dysfunction and oxidative stress have been described in many studies investigating PD pathology [90–92]. In addition, chronic inflammation, aberrant protein folding and aggregation, and genetic and environmental factors have all been implicated in the etiology of PD. Familial hereditary PD involves mutated genes that negatively impact mitochondrial physiology, supporting the idea that mitochondrial signaling and homeostasis are implicated in PD pathology [93]. PD-risk genes include loss-of-function mutations in PTEN-induced putative kinase 1 (*PINK1*),

which is involved in the neuronal stress-response pathway, Parkin, and Parkinsonism associated deglycase (*PARK7*, best known as DJ-1).

In PD, global Ca^{2+} dysregulation impairs neuronal signaling and damages mitochondria, resulting in cell death [94]. L-type voltage gated Ca^{2+} channels (VGCCs) are autonomously active, leading to increased extracellular Ca^{2+} influx and elevated cytosolic Ca^{2+} [95]. α-synuclein itself increases cytosolic Ca^{2+} by forming pores in the plasma membrane that allow Ca^{2+} entry into the cytosol [96, 97]. In addition, the increase of intracellular Ca^{2+} levels may promote α-synuclein aggregation, leading to a vicious cycle which further increases intracellular Ca^{2+} levels. α-synuclein has been found in mitochondria [98, 99] as well as in MAMs [100]. Defects in mitochondrial dynamics (i.e. fission, fusion and transport) and quality control are major contributors in PD pathology. Relatedly, it has been found that several PD-associated proteins (including α-synuclein, PINK1, DJ-1, Parkin and leucine-rich repeat kinase 2 (LRRK2)), which mutations are the most common causes of familial and sporadic forms of PD [101], directly participate in the regulation of mitochondrial dynamics and oxidative stress [102–104].

Although increased Ca^{2+} influx at the plasma membrane significantly contributes to the pathogenesis of PD, the organelles also exhibit Ca^{2+} dysregulation in this pathology. Particularly, mitochondrial Ca^{2+} overload and dysfunction have been observed in PINK1-deficient neurons [105, 106]. These neurons are sensitive to dopamine, which induce mitochondrial Ca^{2+} overload, triggering mPTP opening and eventual cell death [105]. This effect is thought to be a result of the impairment of mitochondrial Ca^{2+} extrusion. Mitochondrial Ca^{2+} efflux is also inhibited in cells from PD patients bearing PINK1 mutations [107]. In the absence of PINK1, NCLX activity is severely impaired, leading to mitochondrial Ca^{2+} overload. Pharmacological or genetic activation of NCLX corrected these defects, rescuing the mitochondrial phenotypes observed in PD models [107]. This pointed to NCLX as a novel therapeutic strategy in PD. Additionally, inhibition of mitochondrial Ca^{2+} uptake decreased the oxidative stress observed in dopaminergic neurons, suggesting that oxidative stress could also be a consequence of mitochondrial Ca^{2+} overload in PD [108]. Knocking out MCU and inhibiting Ca^{2+} influx into the mitochondrial matrix rescues dopaminergic neuronal loss in PINK1 mutant cells in zebrafish models of PD [109].

The link between Ca^{2+} dyshomeostasis and mitochondrial dysfunction in PD progression is further supported by the fact that both α-synuclein and DJ-1 interact with MAMs via Grp75 [110]. This interaction promotes MAMs assembly and function by controlling ER-mitochondria Ca^{2+} transfer and lipid homeostasis [100, 111, 112]. α-synuclein disrupts these ER-mitochondria contacts, impairing Ca^{2+} exchange between the two organelles and affecting the PD phenotype [113]. This suggests that MAMs homeostasis can also play a role in the pathogenesis of PD. Together, these studies imply that proper mitochondrial Ca^{2+} homeostasis is crucial for the correct homeostasis of dopaminergic neurons, and that anti-PD treatments could include compounds that prevent mitochondrial Ca^{2+} overload.

4.4 Mitochondrial Ca^{2+} dysregulation and Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is hallmarked by the progressive loss of upper or lower motor neurons in the spinal cord and brain, leading to

fatal paralysis and death within 5 years. Following the initial dysfunction, neurodegeneration induced by inflammatory agents and eventual loss of trophic support take place. Most cases are sporadic, with only 10% being familial. Several mutations in proteins were identified to be causally related to familial forms of ALS. Mutations in the Cu/Zn-superoxide dismutase (*SOD1*) gene – encoding the antioxidant enzyme SOD – are among the most frequent, accounting for the 20% of the familial cases [114]. Other mutations include *TAR DNA binding protein* (*TARDBP*, TDP-43), Fused in sarcoma (*FUS*) and *C9orf72* [115]. Oxidative stress is one of the major contributors to ALS pathology. Mutations in SOD1 lead to enhanced ROS production [116]. Accumulation of damaged proteins also takes place in the CNS of ALS patients, which interferes with neuronal transport of mitochondria in the axons, reducing ATP production and impairing the synaptic transmission by altering Ca^{2+} signaling [117]. Furthermore, mitochondrial degeneration in motor neurons has been proposed to trigger ALS onset in familial mouse models of the disease expressing mutant SOD1 [118]. Oxidative stress, proteasome dysfunction and excitotoxicity have also been proposed at the onset of the sporadic disease. Despite this, its definitive etiology remains elusive [119–121].

Vulnerable neurons in ALS, such as spinal and brain stem neurons, have very low Ca^{2+} buffering capacity. They exhibit reduced levels of Ca^{2+} buffering proteins, like parvalbumin and calbindin amongst others [122]. Low cytosolic Ca^{2+} buffering capacity is therefore a primary risk factor for motor neuron decline, whereas enhancing Ca^{2+} buffering capacity protects motor neurons from degeneration [123, 124]. These excessive Ca^{2+} levels due to diminished Ca^{2+} buffering capacity lead to mitochondrial degeneration in motor neurons. Further, these neurons are enriched in Ca^{2+} permeable glutamate AMPA receptors. On top of this, the presence of mutant SOD1 increases AMPA receptor permeability to Ca^{2+}, thus increasing Ca^{2+} levels even more [125]. Following stimulation, they mediate glutamate-excitotoxicity, resulting in neuronal degeneration and cell loss [126, 127].

Mitochondrial dysfunction is also involved in the pathology of ALS, particularly in ALS subtypes associated with SOD1 mutation and in sporadic cases of the disease. This includes the generation of free radical species, changes in mitochondrial morphology, and activity of its complexes, especially in the upper and lower motor neurons, frontal cortex and spinal cord [128–130]. Defects in mitochondrial function, including mitochondrial swelling and vacuolization, have been found both in sporadic and familial forms of ALS, through histopathological observations in ALS patients and transgenic mouse models of the disease [130, 131].

Mitochondrial regulation of Ca^{2+} levels is particularly important in motor neurons, where Ca^{2+} uptake by mitochondria is greater than in other cell types. This makes motor neuronal mitochondria more susceptible to Ca^{2+} mediated damage [127, 132]. Mitochondrial Ca^{2+} overload might result from the increased activity and permeability of AMPA receptors in ALS motor neurons [127], which can lead to increased ROS production and oxidative stress. Mutant SOD1 transgenic mouse models show decreased mitochondrial Ca^{2+} buffering capacity in the CNS, even before the disease onset, pointing to an early loss of the Ca^{2+} buffering as a cause of the disease [133]. Furthermore, reduced MCU Ca^{2+} uptake has been observed in motor neurons [134]. Interestingly, mitochondria might be strategically located close to the ER or plasma membranes to effectively buffer Ca^{2+} in the motor neurons in

order to compensate for their lower levels of Ca^{2+} buffering protein expression [135]. Impaired MAM function can lead to reduced mitochondrial Ca^{2+} uptake, and increased cytosolic Ca^{2+} levels after triggering ER Ca^{2+} release [136, 137]. MAM disruption has been reported in mutant SOD1, Sig1R, TDP-43 and FUS-related ALS [134, 136, 137], suggesting that disrupted ER-mitochondria communication might be a common characteristic in ALS, and a primary cause of motor neuron death in the disease.

5. Tools to study mitochondrial Ca^{2+} dynamics *in vivo*

As discussed above, mitochondria are dynamic organelles, with the capacity to move, divide and fuse, to travel along the cell and to take up Ca^{2+}. The research of these events has become an important tool for researchers in the investigation of mitochondrial abnormalities related to neurodegenerative diseases. The study of these events *in vivo* became possible after the development of fluorescence reporters along with live cell microscopy techniques. Just until a few years ago, most of the studies investigating mitochondrial structure and function were developed *in vitro* or with the use of biochemical assays. Unavoidably, with the new techniques of fluorescent imaging technology, such as multiphoton microscopy, most recent research is aimed at applying these measurements *in vivo*. Multiphoton microscopy allows greater tissue penetration and less phototoxicity, making it possible to use the imaging approaches established in isolated cells in intact sections of tissue or the living brain. The selection of the best probe will depend on the question that is being asked. Ideally, the Ca^{2+} indicator should localize specifically into the target, (i.e., mitochondria), and show robust fluorescence, increased signal to noise ratio and fast kinetics. Additionally, its affinity for Ca^{2+} should be suitable for detecting the expecting Ca^{2+} changes in the organelle. Finally, it should not be toxic or interfere with the mitochondrial environment. The two main methods for studying mitochondrial Ca^{2+} homeostasis are fluorescent dyes and genetically encoded Ca^{2+} indicators (GECIs). Whereas fluorescent dyes are electrically retained in the mitochondrial matrix, GECIs can be maintained in the mitochondrial intermembrane space or in the external surface of the OMM. A variety of reporters to measure free mitochondrial Ca^{2+} levels are listed in Table 1.

5.1 Chemically engineered Ca^{2+} indicators: Fluorescent dyes

The use of fluorophores as a dye to target organelles is a broad technique used for investigators. These molecules change their fluorescence properties upon binding to Ca^{2+}. The uptake of these fluorescent dyes into mitochondria depends on the negative mitochondrial membrane potential across the inner membrane. The fluorophores, however, are usually lipophilic and positively charged. They are frequently used as markers of mitochondrial localization and membrane potential, but also as an indicator for mitochondrial Ca^{2+} uptake. Fluorescent dyes need to be prepared as acetoxymethyl ester (AM), allowing for their diffusion into the cytoplasm [138]. Once in the cytoplasm, they get hydrolyzed by non-specific but ubiquitous esterases and the Ca^{2+} sensitive moiety is generated [139]. However, accumulation within mitochondria is highly variable and depends on the system and procedure used. A potential drawback is the toxicity of the AM hydrolyzed products (acetate and formaldehyde), but these effects have been largely minimized [140].

In the nineties, Rhod-2 was first introduced [141], as a Ca^{2+}-dependent molecule for longer wavelength Ca^{2+} imaging [142]. Since then, it has been observed that Rhod-2 accumulates in mitochondria and has been used in several studies assessing mitochondrial Ca^{2+} of cultured cells and slice preparations, such as glia [143] or neuronal cells [144–146]. Several rhod derivates are commercially available, including Rhod-2, Rhod-5N, X-rhod-1, X-rhod-5F, X-rhod-FF and X-rhod-5N, each with different excitation or emission wavelengths. They are mainly used as cell-permeant AM esters, presenting a delocalized positive charge, and they accumulate within the mitochondrial matrix, where they are hydrolyzed and therefore trapped [141, 147]. However, it has been demonstrated that these constructs are no truly specific for targeting mitochondria, since they can sometimes be found in the cytosol or extramitochondrial localizations, causing inaccuracy during long term duration experiments [148]. One of the other main drawbacks is that they are intensity-based Ca^{2+} sensors, and unlikely other ratiometric Ca^{2+} sensors such as fura-2 or indo-1, quantitative measurements of mitochondrial Ca^{2+} may be inaccurate. When imaging *in vivo,* other problems may include the potential toxicity in the AM hydrolysis as well as the surgical method to deliver the dye to the tissue of interest itself [140].

5.2 Genetically encoded Ca^{2+} indicators: Fluorescent and bioluminescent proteins

In the early 1990s, the green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria* was discovered and cloned [149]. The advances in genetic manipulation allowed for targeting proteins selectively to different cellular locations including organelles. This has allowed for the study of the mitochondria as well as the dynamics of specific mitochondrial proteins. Aside from just GFP, a broader toolkit of fluorescent proteins (FP) for *in vivo* imaging has been produced [150, 151]. Several molecular parameters within mitochondria can be assessed by using FPs. They include mitochondrial membrane potential, pH, Ca^{2+} (GECIs) or Cl^{-} levels. GECIs show reduced toxicity. They display a varying range of affinities for Ca^{2+} (different Kd), and can be targeted selectively to different locations including mitochondria [152–154]. Their main drawbacks are the requirement of genetic manipulation, the sensitivity that some of them have to mitochondrial pH, and the effects that the mitochondrial matrix environment as opposed to the cytoplasm can have on their affinity for Ca^{2+}. GECIs can be either bioluminescent (Aequorin-based GECIs) or fluorescent (GFP-based GECIs).

Bioluminescent GECIs derive from the photoprotein aequorin, which is isolated from the *Aequorea Victoria* jellyfish [155]. Aequorins were the first mitochondria targeted GECI [156], called mtAEQ. It was composed of the mitochondrial targeting sequence (cytochrome c oxidase polypeptide VIII (COX8)) and the native aequorin. The use of mtAEQ helped to provide the first evidence that mitochondria were able to accumulate Ca^{2+} in living cells [157]. When aequorin binds to Ca^{2+} in the presence of the external cofactor coelenterazine, a photon of light is produced. One of its main limitations is that once the reaction takes place, the indicator is consumed. The main advantages of using aequorin, however, is the lack of excitation illumination, making the measurements autofluorescence and phototoxicity free, and completely free of background noise. A drawback of the aequorin-based indicators is their low brightness relative to other GECIs [158]. Due to their low photon emission rates, obtaining subcellular resolution is very challenging. Additionally,

they do not allow for long-term imaging, since aequorin gets consumed during the course of the reaction [159].

Fluorescent GECIs were developed in the nineties [160, 161]. They are comprised of a Ca^{2+} sensing polypeptide, which triggers a change in the fluorescence fused protein. There are two types of fluorescent GECIs: Föster resonance energy transfer (FRET)-based GECIs and single-wavelength fluorescent GECIs.

FRET-based GECIs use the energy transfer potential of two fluorophores with partially overlapping excitation/emission spectra. This way, the donor fluorophore transfers non-radiative energy to allow fluorescence of the second fluorescent acceptor. Both molecules are usually linked via a Ca^{2+}-binding domain [162], since FRET only occurs when both proteins are in close proximity. The first and most well-known family of FRET-based Ca^{2+} indicators are cameleons. These are formed by a blue and yellow fluorescent protein linked by calmodulin fused to a kinase-binding peptide (M13) [160, 161]. Cameleons are ratiometric sensors, and therefore they provide an approximate absolute free Ca^{2+} concentration. In addition, motion artifacts and differences in sensor expression can be eliminated, as this is an important advantage while performing imaging in a living breathing animal [163–165]. The main drawbacks of these reporters are their lower signal-to-noise ratio, decreased brightness and slower kinetics than their single-fluorophore GECI counterparts [166]. Cameleons, however, have been extensively improved since the first generations, and the new versions show decreased sensitivity to pH, higher fluorescence intensity and less photobleaching [167].

On the other hand, single-wavelength fluorescent GECIs show large changes in fluorescence intensity. They were developed after finding that insertion of proteins into GFP still gives it the ability to fluoresce [168]. The first single-wavelength GECI developed was the camgaroo family [169], followed by pericams [170]. The most known example of single-fluorophore GECIs is the GCaMP family. As for other GECIs, the first generations of GCaMP exhibited low brightness, poor expression, and temperature and pH sensitivity [171]. However, the most recent versions (GCaMP5, GCaMP6 and GCaMP7) have improved brightness, dynamic range and Ca^{2+} affinity [172–174]. GCaMPs rely of the circular permutation of GFP, fused to a M13 and calmodulin-binding domains [175]. When Ca^{2+} binds to the calmodulin, it results in the increase of fluorescence intensity. Compared to FRET-based indicators, single-wavelength fluorescent GECIs exhibit a generally greater dynamic range, mostly due to their lower basal fluorescence, and are less likely to photobleach in long-term assays. However, as they are based in fluorescence intensity, they do not provide measures of free absolute Ca^{2+} concentration [176].

A new family of GECIs based on the red fluorescent protein (RFP) named GECO was developed a few years ago [177, 178]. They are intesiometric and exhibit improved signal-to-noise ratios. GECOs have been targeted to measure Ca^{2+} in different organelles or mitochondrial compartments simultaneously. GECOs emission fluorescence falls in longer wavelengths, allowing for multiplexing and Ca^{2+} imaging in different compartments (or cell populations) at the same time. Additionally, red-shifted indicators allow for greater tissue penetration, reduced phototoxicity and less scattering, clear advantages for *in vivo* imaging

[179, 180]. These indicators include RCaMP, jRCaMP1a, jRCaMP1b and jRGECO1a [181, 182], and exhibit different affinities for Ca^{2+} with different dynamic ranges. Recently, low affinity indicators targeted to ER and mitochondria have been developed for the measurement of free Ca^{2+} levels in these organelles, which are known as CEPIA, with CEPIAmt being specific to mitochondria [183].

Our group has recently targeted Yellow Cameleon 3.6 [184] to mitochondria. We introduced the mitochondrial targeting sequence COX8A to the N terminus, duplicated in tandem in order to enhance the specificity of its location [185]. 2mtYC3.6 was subcloned into an associated adenovirus (AAV) hsyn (the synapsin promoter for neurons), for a final product of AAV.hSyn.2mtYC3.6. The expression cassette contained the following components: (1) human synapsin 1 gene promoter, (2) 2mt (mitochondrial targeting sequence), (3) YC3.6, (4) WPRE and (5) Simian virus 40 (SV40) [76]. This virus was injected in the somatosensory cortex of mice, and a cranial window was performed in the skull (Figure 2). Multiphoton microscopy and this reporter allowed us to evaluate the free Ca^{2+} concentration in neuronal mitochondria and to compare the Tg mouse (APP:PS1, a mouse model of AD) and their Wt controls *in vivo* [76]. An example of the images obtained can be observed in Figure 3. The use of this ratiometric indicator targeted to mitochondria allowed us to observe in the living brain the different mitochondrial Ca^{2+} levels *in vivo* in different compartments (somas and neurites) and ages (pre- and post-plaque), and follow neurons longitudinally with elevated Ca^{2+} concentration [76]. Using this technique, we directly observed and demonstrated elevated Ca^{2+} levels in neuronal mitochondria in the Tg mouse *in vivo* (see results above).

6. Conclusion and future perspectives

Neurodegenerative diseases are likely multifactorial and can arise from mutation of different genes. However, Ca^{2+} dyshomeostasis and particularly mitochondrial Ca^{2+} impairment seem to be a common characteristic. Neurodegenerative diseases have been long associated with oxidative stress and impaired respiration. Mitochondria are critical for the maintenance of appropriate Ca^{2+} levels, and mitochondrial Ca^{2+} overload might be the cause that leads to mitochondrial dysfunction, oxidative stress and cell death. Altered mitochondrial Ca^{2+} homeostasis might result from a dysfunctional MCU complex (increased influx), altered mitochondrial Ca^{2+} extrusion (impaired NCLX) mechanisms, altered capacity of Ca^{2+} buffer or increased Ca^{2+} transfer from ER to mitochondria (MAMs). This might result in greater mitochondrial and cytosolic Ca^{2+} levels. Interventions aimed at reducing mitochondrial Ca^{2+} uptake or to enhance mitochondrial Ca^{2+} release could be effective for the treatment of these diseases. However, it might also represent a great challenge. Therefore, more research efforts are needed to elucidate the role of mitochondrial Ca^{2+} dyshomeostasis in this type of pathogenesis. Ca^{2+} imaging has been instrumental for the study of neurodegenerative diseases in animal models, mostly *in vitro*, in cell cultures or in brain slices. The combination of Ca^{2+} indicators and novel optical imaging techniques with cellular resolution that emerged in the recent years have allowed a better characterization of mitochondrial Ca^{2+} dynamics, and the patho-physiological mechanisms involved in the neurodegenerative diseases. Therefore, Ca^{2+} imaging is a powerful approach to perform functional studies in neuronal populations in the healthy and diseased brain *in vivo*. Recent advances in optical techniques, such as multiphoton microscopy, are more adequate for the observation of

different cell types or protein aggregates in the cortex of the mouse brain (such as in AD). However, due to depth-penetration limitations, this technique is not suitable for the exploration of deeper brain regions (as in the case of PD). *In vivo* two photon imaging experiments are still limited to the superficial layers of the neocortex, cerebellum and olfactory bulb. The evaluation of deeper brain structures requires different technologies, such as two-photon imaging coupled to Gradient Index (GRIN) lenses, which provides optical interface to the brain, allowing for endoscopy imaging of deep brain regions [186, 187]. Other techniques include single fiber volumetric recordings [188] or fiber-bundle-bases confocal imaging [189, 190]. The development of these other techniques will give access to the *in vivo* study of diseases in which deeper brain regions are impaired. Understanding the complexity of mitochondrial Ca^{2+} dynamics *in vivo* will help understanding the role of mitochondrial Ca^{2+} dysfunction in these and other neurodegenerative diseases. To date, the MCU subunit of the mitochondrial uniporter complex is one of the best studied. Further research is required to ascertain the therapeutic candidacy of the other subunits of the uniporter complex, of NCLX and the MAMs. Overexpressing and knock-out (KO) mouse models, and virally-mediated gene transfer approaches will be necessary to validate the findings, as well as to evaluate their clinical relevance. With these results in hand and with the knowledge on the molecular mechanisms linking disrupted mitochondrial Ca^{2+} homeostasis to neurodegenerative diseases broadening, more specific therapeutic agents targeting these channels will be developed, providing hope for the treatment of these diseases.

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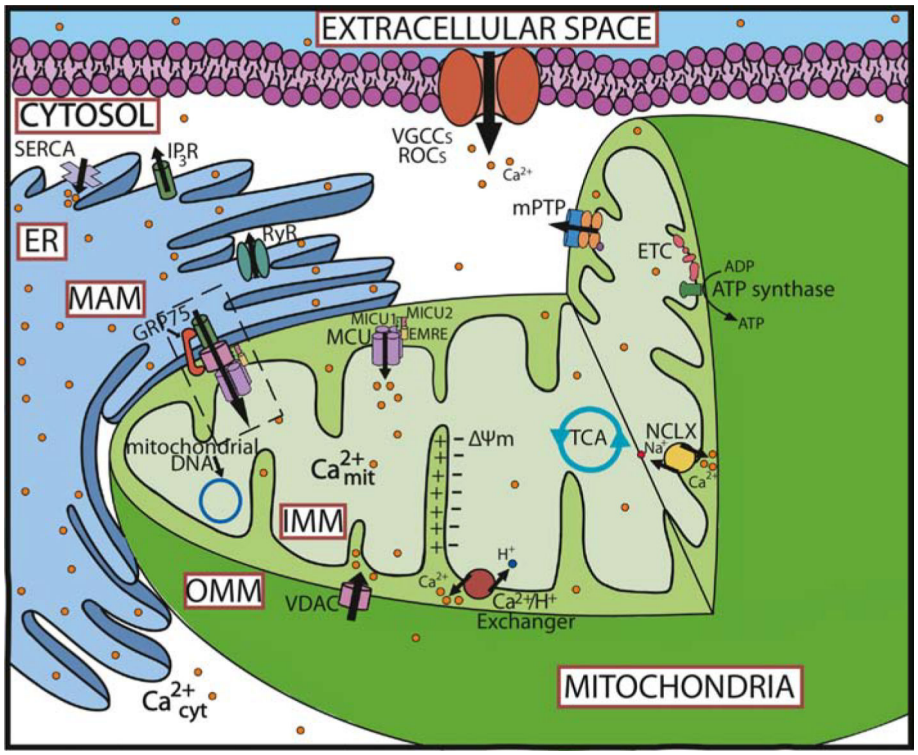

Fig. 1.

Main components of the mitochondrial Ca^{2+} homeostasis in neurons The main components of Ca^{2+} regulation in the cell are part of the plasma membrane (VGCCs and ROCs among other channels and pumps), endoplasmic reticulum, and mitochondria. In the mitochondria, the MCU complex is the part of the main Ca^{2+} influx pathway. VDAC contributes to this movement by allowing the flow of ions through the OMM. Ca^{2+} efflux is predominantly regulated by the NCLX and the H^+/Ca^{2+} exchangers. In addition to this, the mPTP can also vent Ca^{2+} ions out of the cell. Mitochondria-ER communication is moderated through the MAMs (comprised of the MCU complex, VDAC, and IP_3R among other proteins and tethers), and allow the flow of Ca^{2+} ions into the mitochondria from the ER for further regulation. In the ER, Ca^{2+} release is regulated through the RyR and IP_3R pathways, while SERCA is involved in its extrusion from the cytosol. Abbreviations: ΔΨm (mitochondrial membrane potential), ER (endoplasmic reticulum), ETC (electron transport chain), GRP75 (glucose-regulated protein 75), H^+/Ca^{2+} (H^+/Ca^{2+} exchanger), IMM (inner mitochondrial membrane), IP_3R (inositol trisphosphate receptor), MAM (mitochondrial associated membranes), MCU (mitochondrial Ca^{2+} uniporter), MICU (mitochondrial Ca^{2+} uptake proteins), (mtDNA (mitochondrial DNA), NCLX (Na^+/Ca^{2+} Li^+ permeable exchanger), mPTP (mitochondrial permeability transition pore), ROCs (receptor-operated Ca^{2+} channels), RyR (ryanodine receptor), SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase), TCA (tricarboxylic acid), VDAC (voltage dependent anion channel), VGCCs (voltage gated Ca^{2+} channels).

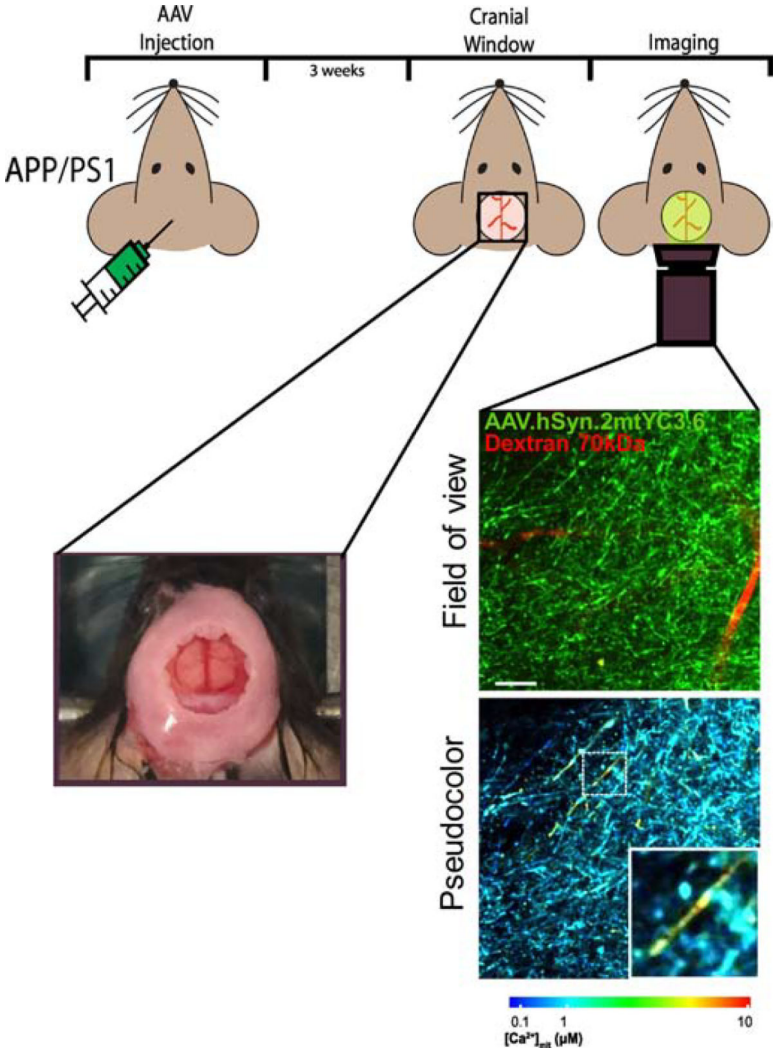

Figure 2. Schematic of the study of mitochondrial function *in vivo*

The use of fluorophores in conjunction with *in vivo* imaging to evaluate intracellular function can be summarize as such: (1) a fluorescent indicator is injected into the cortex of the mouse model, (2) and after 3 weeks viral expression should be sufficient for imaging. (3) At this point a cranial widow is surgically implanted and (4) *in vivo* imaging with multiphoton microscopy may commence. Left image represents a cranial window in the mouse skull. Field of view shows the reporter in neuronal mitochondria (green) and the blood vessels labeled with fluorescent Dextran Texas Red (red). Pseudocolor images represent the color coded (according to the lower bar) mitochondrial Ca^{2+} concentrations for the corresponding neurites. Warm colors represent high Ca^{2+}, whereas blue colors represent low Ca^{2+} concentration. Scale bar represents 15 μm.

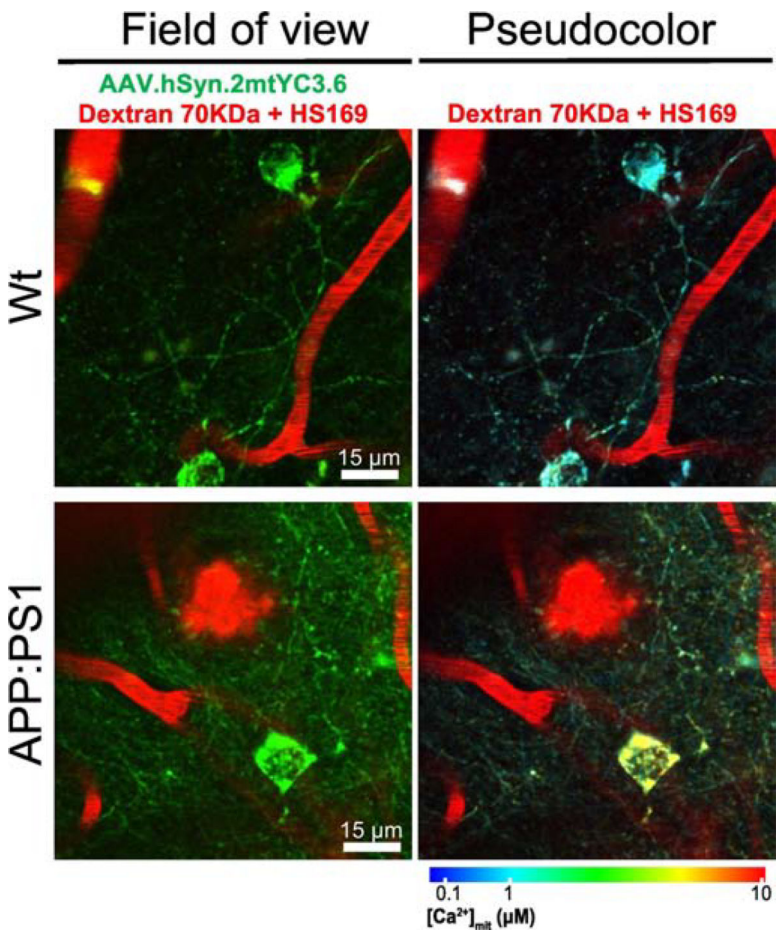

Figure 3. ***In vivo*** **multiphoton microscopy images of mitochondrial Ca^{2+} in neurons in Wt and APP/PS1 mice.**

APP/PS1 transgenic (Tg) mice and wild-type (Wt) mice were injected with the mitochondrial Ca^{2+} reporter AAV.hSyn.2mtYC3.6 and a cranial window was implanted. Mitochondrial Ca^{2+} was assessed with multiphoton microscopy. Pictures represent Wt (top) and APP/PS1 Tg mice (bottom). Field of view shows the reporter in neuronal mitochondria (green), the blood vessels labeled with fluorescent Dextran Texas Red (red) and amyloid plaques labeled with the dye HS169 (red, [198]). Pseudocolor images represent the color coded (according to the lower bar) mitochondrial Ca^{2+} concentrations for the corresponding neurons. Warm colors represent high Ca^{2+}, whereas blue colors represent low Ca^{2+} concentration. Scale bar represents 15 µm.

Table 1

Main reporters to measure free mitochondrial Ca^{2+} concentration *in vitro* and *in vivo.*

Name	Intensiometric/ratiometric	Excitation/emission (nm) single photon	Kd for Ca^{2+}	Ref.
Fluorescent dyes				
Rhod2	Intens	Ex: 540; Em: 580	320 μM	[141]
Rhod-5N	Intens	Ex: 551; Em: 576	0.5 mM	[191]
Rhod-FF	Intens	Ex; 550; Em: 580	19 μM	[192]
Fluorescent proteins				
mtGCaMP5G	Intens	Ex; 497; Em: 515	460 nM	[193]
mtGCaMP6s	Intens	Ex; 497; Em: 515	144 nM	[193]
4mtD3cpv	FRET	Ex: 433; Eml: 483; Em2: 534	760 nM	[194]
2mtYC3.6	FRET	Ex: 433; Eml: 483; Em2: 534	4.21 μM	[76]
mito-GEM- GECOl	Ratio	Ex: 377; Eml: 447; Em2: 520	340 nM	[177]
mito-R-GECOl	Intens	Ex; 562; Em: 624	482 nM	[177]
Mito-CAR- GECO	Intens	Ex; 560; Em: 609	490 nM	[195]
mito-LAR- GECOl.2	Intens	Ex; 561; Em: 620	12 μM	[196]
CEPIA2mt	Intens	Ex; 488; Em: 508	160 nM	[183]
CEPIA3mt	Intens	Ex: 488; Em: 508	11 μM	[183]
CEPLA4mt	Intens	Ex: 488; Em: 508	56 μM	[183]
R-CEPIA3mt	Intens	Ex: 565; Em: 641	3.7 μM	[197]
R-CEPIA4mt	Intens	Ex: 565; Em: 641	26.9 μM	[197]
R-Pericam mt	Ratio	Exl: 415; Ex2: 494; Em: 517	11 μM	[170]
2mt8PR	FRET	Exl: 415; Ex2: 494; Em: 517	1.7 μM	[185]