Trends in Neurosciences

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Review

Mitochondria and Calcium in Alzheimer's Disease: From Cell Signaling to Neuronal Cell Death

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Mitochondrial dysfunction has been implicated in the pathogenesis of almost all neurological diseases, including Alzheimer's disease (AD). Historically, a primary focus in this context has been the link between mitochondrial dynamics and amyloid β toxicity. Recent evidence suggests that dysregulation of mitochondrial calcium homeostasis is also related to tau and other risk factors in AD, although an ongoing challenge in the field is that data collected from different models or experimental settings have not always been consistent. We examine recent literature on mitochondrial dysregulation in AD, with special emphasis on mitochondrial calcium. We include data from *in vitro* systems, genetic animal models, and AD-derived human tissue, and discuss whether mitochondrial calcium transporters should be proposed as therapeutic candidates for the development of neuroprotective drugs against AD.

Highlights

Ca^{2+} levels are tightly regulated in mitochondria. If excessive Ca^{2+} levels are reached within mitochondria, then key mitochondrial functions are impaired, leading to enhanced generation of reactive oxygen species and activation of apoptosis, processes that take place in AD.

The combination of different technologies, such as RNA-seq, targeted fluorescent probes, multiphoton microscopy, and transgenic mouse models of AD, has made it possible to understand the underlying mechanisms of mitochondrial Ca^{2+} dysregulation and its contribution to a more general Ca^{2+} impairment in AD.

Aβ causes cytosolic and mitochondrial Ca^{2+} overload both *in vitro* and *in vivo*. Misfolded and hyperphosphorylated tau protein also disrupts Ca^{2+} homeostasis in mitochondria.

Identifying targets to maintain mitochondrial Ca^{2+} homeostasis and correct mitochondrial function, particularly mitochondrial Ca^{2+} transporters, could offer promising venues for the development of drugs against AD.

Mitochondrial Ca^{2+} Homeostasis and Neurodegeneration

Ca^{2+} is a tightly regulated second messenger that is crucial for normal neuronal function. By binding to proteins, Ca^{2+} modulates different neuronal processes, such as energy production, survival, and death, and plays an important role in learning and memory. Subcellular organelles such as the endoplasmic reticulum (ER), mitochondria, and lysosomes have an important role in regulating Ca^{2+} homeostasis. Mitochondria are crucial intracellular organelles that provide most energy for the cell in the form of ATP (Box 1). Neurons have limited glycolytic capacity and rely on proper mitochondrial ATP production to maintain ionic gradients and generate axonal and synaptic membrane potentials. In addition to supplying energy, mitochondria contribute to the spatiotemporal tuning of the intracellular Ca^{2+} signaling. Proper Ca^{2+} levels in the mitochondrial matrix tightly regulate oxidative phosphorylation (OXPHOS) activity, maintaining the rate of ATP production. However, if mitochondria take up an excess of Ca^{2+} owing to an increase in cytosolic Ca^{2+} or excessive Ca^{2+} transfer from the ER, mitochondrial respiration can be impaired, leading to enhanced production of reactive oxygen species (ROS), reduced ATP production, and impaired mitochondrial membrane permeabilization, possibly with subsequent cell death. This can involve release of proapoptotic cofactors into the cytosol, and apoptosis via caspases or necrosis, depending on the residual ATP concentration and the nature and severity of the insult [1].

Abnormal mitochondrial Ca^{2+} handling has been observed in many neurodegenerative diseases including AD, Parkinson's disease, and Huntington's disease [2]. AD is the most common neurodegenerative disorder and the most frequent cause of dementia. Although aging is its main risk factor, the direct cause(s) of **late-onset sporadic AD** (sAD, see Glossary) remains unknown. Decades of research have built a body of evidence supporting the existence of Ca^{2+} dyshomeostasis and mitochondrial dysfunction in neurons in AD. The use of Ca^{2+} reporters targeted to mitochondria and intravital imaging in transgenic (Tg) mouse models of the disease

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Box 1. Mitochondrial Structure and Function

In neurons, mitochondria form a dynamic network that extends from the neuronal soma to the neurites. Depending on physiological changes in metabolic demand within the neuron, individual mitochondria can migrate along axons and dendrites in retrograde and anterograde directions or become stationary in regions where the metabolic demand is higher. They undergo replication, fission, and fusion. Mitochondria have their own DNA, that is different from the nuclear DNA, and are thus capable of synthesizing some of their own proteins. The mitochondrial electron transport chain (ETC) located at the inner mitochondrial membrane (IMM) is the molecular machinery for ATP production. The functional diversity of mitochondria is possibly due to the extraordinary compartmental organization of this organelle. Two separate membranes enclose the mitochondria: the outer mitochondrial membrane (OMM) and the IMM, and these define the mitochondrial matrix space. The surface of the IMM is much larger than that of the OMM owing to its multiple folds towards the internal space, known as cristae, that are specialized in oxidative phosphorylation (OXPHOS). Both membranes encapsulate the intermembrane space that contains important enzymes such as creatine kinase and cytochromes such as cytochrome *c*. The OMM is permeable to ions and small molecules (<10 kDa) owing to the expression of large channels called porins or voltage-dependent anion channels (VDACs). The ATP produced in mitochondria can reversely traverse VDACs and reach the cytosol. The IMM is an ion-impermeable membrane, which creates a barrier between the cytosol and the mitochondrial matrix, where only those solutes with specific transporters and ion channels can reach. The IMM contains ion channels and transporters, including the mitochondrial Ca^{2+} uniporter (MCU) complex or the Na^{+}/Ca^{2+} exchanger NCLX, and enzyme systems such as the ETC. Mitochondria produce ATP via the tricarboxylic acid (TCA) cycle and OXPHOS. The energy released by the ETC complexes is used to pump H^{+} from the mitochondrial matrix into the intermembrane space, generating an electrochemical gradient ($\Delta\mu H$) consisting of a chemical component (ΔpH) and electrical ($\Delta\Psi m$) component of 150–180 mV (negative with respect to cytosol) across the IMM. This huge potential difference, known as the mitochondrial membrane potential, provides the driving force for the cytosolic Ca^{2+} to accumulate in the mitochondrial matrix via the MCU.

have recently enabled a better understanding of the contribution of mitochondrial dysfunction to Ca^{2+} dyshomeostasis and AD. In addition, identifying targets to maintain Ca^{2+} balance and mitochondrial homeostasis is of high relevance as a potential strategy for preventing or mitigating the pathology underlying AD.

We provide here an update on recent progress in understanding of the mechanisms underlying mitochondrial Ca^{2+} dysregulation in AD, and discuss how these findings may inform new therapeutic approaches linked to mitochondrial dysfunction in AD and other pathologies.

AD: Calcium Homeostasis Implications

AD is the neurodegenerative disorder most commonly associated with age. It entails progressive cognitive impairment and dementia. The pathological hallmarks of AD are (i) the deposition of extracellular amyloid β (Aβ) plaques formed by **Aβ peptide**, a byproduct of the amyloid β precursor protein (APP) resulting from its sequential cleavage by β- and γ-secretases, (ii) the formation of intraneuronal fibrillary tangles composed of abnormally hyper-phosphorylated and misfolded **tau**, and (iii) massive atrophy of the cerebral cortex as a result of synapse loss and neuronal death. Rare cases of genetically determined, autosomal dominant, **early-onset familial AD** (fAD) are caused by genetic mutations in genes encoding **presenilin** (*PSEN*) *1*, *PSEN2*, or *APP* that are involved in the Aβ generation pathway. This genetic evidence and other lines of evidence led to the 'amyloid cascade hypothesis', which posits that Aβ peptide accumulation – owing to its overproduction and/or the failure of its clearance mechanisms – is a trigger upstream of tau dysregulation and neurodegeneration, and causes the most common, late-onset form of sAD [3,4]. However, the negative outcomes from clinical trials targeting Aβ plaques [5], and questions regarding the correlation between amyloid accumulation in the brain and cognitive decline [6], have led the research community to consider other (possibly non-mutually exclusive) hypotheses for the etiology of AD, including the 'cholinergic hypothesis' [7], the 'tau propagation hypothesis' [8], the 'mitochondrial cascade hypothesis' [9], the 'inflammatory hypothesis' [10], and the 'glymphatic system hypothesis' [11].

Studies spanning several decades support the idea that Ca^{2+} homeostasis is altered in both sAD [12,13] and fAD [14], and also in neurons and astrocytes in AD animal models [15–22], as well as

Glossary

Aβ peptide: a peptide of 38–43 amino acids that is the main component of amyloid plaques. $A\beta_{1-42}$ is more hydrophobic and prone to aggregation, and is the most predominant peptide in amyloid plaques. It has been proposed that soluble Aβ oligomers (Aβo), rather than amyloid plaques, are the most neurotoxic species of Aβ.

Ca^{2+} microdomains: subcellular regions of localized high Ca^{2+} concentration that are key elements of Ca^{2+} signaling, particularly in neurons and cardiac cells. They are formed at sites where Ca^{2+} enters the cytoplasm through Ca^{2+} channels either in the plasma membrane or in internal stores such that, when the channel opens, Ca^{2+} concentration increases up to several hundred micromolar. They can be visualized using fluorescence/bioluminescence reporters such as aequorins.

Early-onset familial AD (fAD): AD caused by mutations in the genes encoding amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), or presenilin 2 (*PSEN2*). These mutations are extremely rare (<1% of people with AD carry one of these mutations), and result in early-onset symptoms of disease (in people as young as 45 years of age).

Late-onset sporadic AD (sAD): the most common form of AD. It is detectable in people aged 65 years and older. Little is known about the cause of the onset of sAD, but is likely a combination of genetic risk factors, diet, and environment.

Mitochondrial Ca^{2+} uniporter (MCU): the main mitochondrial pathway for Ca^{2+} uptake. It constitutes a macromolecular complex of proteins (named the mitochondrial calcium uniporter complex), including the pore and several regulatory subunits. The MCU defines the pore domain of the complex.

Mitochondrial Ca^{2+} uptake (MICU) family: the gatekeepers of MCU. MICU1 and MICU2 are Ca^{2+} sensors (given their two Ca^{2+}-binding EF-hand motifs). The combination of the two regulates the MCU complex to prevent Ca^{2+} overload at low extramitochondrial Ca^{2+} concentrations. MICU3, mainly expressed in the CNS, enhances mitochondrial Ca^{2+} uptake in neurons.

Mitochondrial membrane potential ($\Delta\Psi m$): the driving force for cytosolic Ca^{2+} to accumulate in the mitochondrial matrix via the MCU. It is generated by

in induced pluripotent stem cell (iPSC)-derived neurons from AD patients [23,24]. Ca^{2+} dysregulation is thought to be one of the earliest events in AD, and this observation together with the aforementioned lines of evidence led to the longstanding 'Ca^{2+} dyshomeostasis in AD hypothesis' [25]. This postulates that activation of the amyloidogenic pathway causes remodeling of the neuronal Ca^{2+} signaling pathway, thereby altering Ca^{2+} homeostasis and consequently impairing mechanisms involved in learning and memory. In addition, and as an extension of the Ca^{2+} hypothesis of AD, hyperphosphorylated tau also influences Ca^{2+} homeostasis because it impairs neuronal circuits [22], activates voltage-gated Ca^{2+} channels (VGCCs) [26], and depletes nuclear Ca^{2+} [27].

A wide variety of neuronal functions depend on intracellular Ca^{2+} signaling. Taking into consideration that a high and sustained Ca^{2+} increase, especially in mitochondria, induces the production of free radicals and ROS, it seems logical to assume that the disturbance of Ca^{2+} homeostasis contributes to enhanced oxidative stress in neurons in AD, resulting in amplification of free radical formation and Ca^{2+}-mediated degenerative processes. One could even hypothesize that altered intracellular Ca^{2+} could be at the origin of the damage and neuronal dysfunction that takes place in AD. However, whether mitochondrial dysfunction leads to AD or the pathologies underlying the disease, or whether it is a consequence of Aβ and pathological tau accumulation, remains a much-debated topic.

Impaired Mitochondrial Ca^{2+} Homeostasis in AD

Alterations in Ca^{2+} homeostasis, particularly in mitochondrial Ca^{2+} homeostasis (Box 2), are deleterious to proper cell function and survival. AD brains are characterized by reduced ATP levels, increased ROS production, and impaired mitochondrial function [28]. Peripheral tissues derived from AD patients also display mitochondrial damage. Indeed, fibroblasts from AD patients show defects in mitochondrial dynamics and bioenergetics as well as Ca^{2+} dyshomeostasis [29,30]. Before mitochondrial Ca^{2+} overload results in cell death, mitochondrial Ca^{2+} dyshomeostasis triggers other mitochondrial Ca^{2+}-related disturbances, including mitochondrial dynamics (trafficking, fission, fusion), mitochondrial Ca^{2+} buffering, and mitophagy (a selective process for mitochondrial autophagy), processes that are proposed to be altered in AD [28,31–36]. Figure 1 (Key Figure) summarizes the main findings regarding mitochondrial Ca^{2+} dyshomeostasis in AD, and these are discussed further below.

Aβ Accumulation Can Cause Mitochondrial Ca^{2+} Dyshomeostasis

Both APP and Aβ accumulate in the mitochondrial matrix, via translocase of the outer mitochondrial membrane (TOM) 40 and translocase of the inner mitochondrial membrane (TIM) 23 import channels [37,38], where they interact with specific intramitochondrial proteins and decrease the activity of respiratory chain complexes, leading to mitochondrial dysfunction. Aβ has been found in mitochondria of human postmortem AD brains, in AD Tg mouse models, and following exogenous application to cells [39–41], and it has been proposed that intraneuronal Aβ peptide accumulation in mitochondria precedes its deposition as extracellular plaques [42].

The presence of Aβ itself impairs mitochondrial Ca^{2+}. Direct exposure of neurons to soluble Aβ oligomers (Aβo) *in vitro* leads to mitochondrial Ca^{2+} uptake and subsequent mitochondrial Ca^{2+} overload. This increase in mitochondrial Ca^{2+} activates the **mitochondrial permeability transition pore** (mPTP), cytochrome *c* release, and apoptosis [43,44]. Interestingly, mild mitochondrial depolarization with uncouplers [such as carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone, FCCP] or non-steroidal anti-inflammatory drugs (NSAIDs) can prevent these events, suggesting a key role for mitochondrial Ca^{2+} in cell death independently of cytosolic Ca^{2+}, and supporting a novel mechanism of neuroprotection by NSAIDs against Aβo via mitochondrial depolarization independently of their anti-inflammatory effect [44]. Furthermore, this

proton pumps in the electronic transport chain and is negative inside mitochondria.

Mitochondrial permeability transition pore (mPTP): a megachannel that allows the efflux of metabolites with a molecular weight of up to 1.5 kDa from the mitochondrial matrix. It is activated by multiple effectors, especially Ca^{2+} in the presence of phosphate and reactive oxygen species (ROS). The mPTP is mainly formed by ATP synthase and is regulated by cyclophilin D (CypD). Its inhibition by cyclosporine A (CsA) leads to closure of the pore.

$Na^+/Ca^{2+}/Li^+$ exchanger (NCLX): the major pathway for Ca^{2+} efflux from the mitochondrial matrix. Ca^{2+} extrusion is coupled to Na^+ influx from the cytosol into the mitochondrial matrix; however, Na^+ can be effectively replaced by Li^+ (and hence its name).

Presenilin (PSEN): an integral membrane protein located in the endoplasmic reticulum (ER). In the brain, neurons mainly contain PSEN1 and PSEN2. They participate in Aβ generation as catalytic enzymes within the γ-secretase complex.

Tau: a protein contained within the axons of nerve cells that promotes the assembly and stabilization of microtubules, the main components of the cytoskeleton and key players in the transport of vesicles, organelles, and proteins. Hyperphosphorylation of tau results in misfolding and the formation of neurofibrillary tangles.

Voltage-dependent anion channel (VDAC): the most abundant channel in the outer mitochondrial membrane (OMM) that provides an aqueous pathway from the cytosol and across the OMM. It allows the entry of substrates for mitochondria to produce ATP, such as pyruvate, succinate, and NADH, as well as Ca^{2+}, Na^+, and K^+.

effect is enhanced *in vitro* with aging, the most important risk factor for sAD [45]. Experiments in isolated mitochondria have demonstrated that Aβ can induce mitochondrial depolarization via mPTP activation in the presence of mitochondrial Ca^{2+} overload and ROS [46], supporting the argument that Aβ induces mitochondrial Ca^{2+} overload. Mechanistically, it has been proposed that Aβ interacts with CypD, an mPTP regulator, to open the mPTP, leading to depolarization (reduced **mitochondrial membrane potential**, ΔΨm), reduced mitochondrial Ca^{2+} buffering capacity, and increased ROS production, thereby causing neuronal injury and decline of cognitive function, as shown using an AD mouse model overexpressing a mutant form of human APP and Aβ [47]. Genetic deletion of CypD in the mouse rescues mitochondrial and neuronal perturbation and improves learning and memory [47]. Aβ can also promote excessive Ca^{2+} release from the ER to mitochondria and induce mitochondrial Ca^{2+} overload [48], triggering mPTP opening and neuronal cell death.

A study from our group recently confirmed, using multiphoton microscopy, the existence of mitochondrial Ca^{2+} overload *in vivo* in an animal model of AD. Specifically, neurons of a Tg AD mouse model (APP^{swe}/$PSEN1^{\Delta E9}$, APP/PSEN1), engineered to develop Aβ plaques, exhibit higher levels of mitochondrial Ca^{2+} than wild-type (WT) controls, both in somas and neurites. Elevated Ca^{2+} levels were observed in neuronal mitochondria only at an age after significant plaque deposition [43]. In this study, no correlation between high levels of mitochondrial Ca^{2+} and the distance to Aβ plaques was found. This contrasts with a previous *in vivo* study which showed that the

Box 2. Mitochondrial Calcium Homeostasis

Mitochondria play a central role in intracellular Ca^{2+} signaling as Ca^{2+} sensors or buffers. By handling Ca^{2+} signaling, they control important physiological processes such as the synthesis of hormones and neurotransmitter metabolism. In turn, increases in mitochondrial Ca^{2+} upregulate the activity of several Ca^{2+}-sensitive enzymes to stimulate the rate of ATP production. The different pathways involved in mitochondrial Ca^{2+} homeostasis (summarized in Figure I) are discussed below.

(i) Mitochondrial Ca^{2+} Influx

The main Ca^{2+} uptake pathway in mitochondria is the mitochondrial Ca^{2+} uniporter (MCU) [128,129] complex, although a rapid uptake mode (RaM) for short Ca^{2+} pulses activated by lower external Ca^{2+} concentration is another route [130]. Two other proteins participate in the Ca^{2+}-permeable pore: MCUb [131], whose presence in the pore complex inhibits mitochondrial Ca^{2+} uptake, and EMRE [132], which keeps the regulatory subunits MICU1 and MICU2 (see below) attached to the MCU complex. The response of MCU to extramitochondrial Ca^{2+} is regulated by the **mitochondrial Ca^{2+} uptake (MICU) family** of proteins, MICU1 and MICU2 [133], which are in the intermembrane space. These regulatory subunits confer to the MCU a characteristic sigmoidal response to extra mitochondrial Ca^{2+} (i.e., cytosolic Ca^{2+}), with a very low response rate to low extracellular Ca^{2+} (resting conditions) and a large capacity at high Ca^{2+} levels, explaining the Ca^{2+}-buffering function of mitochondria. Other regulatory subunits of the MCU pore are the mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1) [134] and the small calcium-binding mitochondrial carrier protein (SCaMC) [135].

(ii) Mitochondrial Ca^{2+} Efflux

The efflux of Ca^{2+} from the mitochondrial matrix depends on two main mechanisms: (i) electrogenic exchange of Na^+/Ca^{2+} (likely 3–4 Na^+ ions per each Ca^{2+}) known as the Na^+/Ca^{2+} Li^+ exchanger (NCLX) [136], and (ii) ubiquitous H^+/Ca^{2+} exchange (likely an electroneutral process of 2 H^+ for each Ca^{2+}), known as leucine zipper and EF-hand containing transmembrane protein 1 (Letm1) [137], that functions as a second Ca^{2+} extrusion mechanism when Ca^{2+} levels are elevated in the mitochondrial matrix.

The Mitochondrial Permeability Transition Pore (mPTP)

When pathological Ca^{2+} concentrations are reached inside mitochondria, this large-conductance channel opens in the IMM, leading to loss of the mitochondrial membrane potential (ΔΨm) [138]. If pore opening is sustained, it leads to organelle swelling, mitochondrial membrane depolarization, and arrest of ATP synthesis. The cristae unfold as the matrix swells, causing the OMM to rupture. After mitochondrial permeabilization, various proapoptotic factors are released into the cytoplasm through the OMM, and each has different downstream targets, many of them triggering caspase activity. In addition, the collapse of the ΔΨm together with the leakage of pyridine nucleotides (i.e., NADH), leads to the generation of ROS. Eventually, neuronal death via apoptosis takes place. Mitochondrial Ca^{2+} overload is one of the main inducers of mPTP opening, suggesting that the mPTP is a key player in the maintenance of mitochondrial Ca^{2+} homeostasis in cells. In addition, mPTP may contribute to mitochondrial Ca^{2+} homeostasis by venting Ca^{2+}.

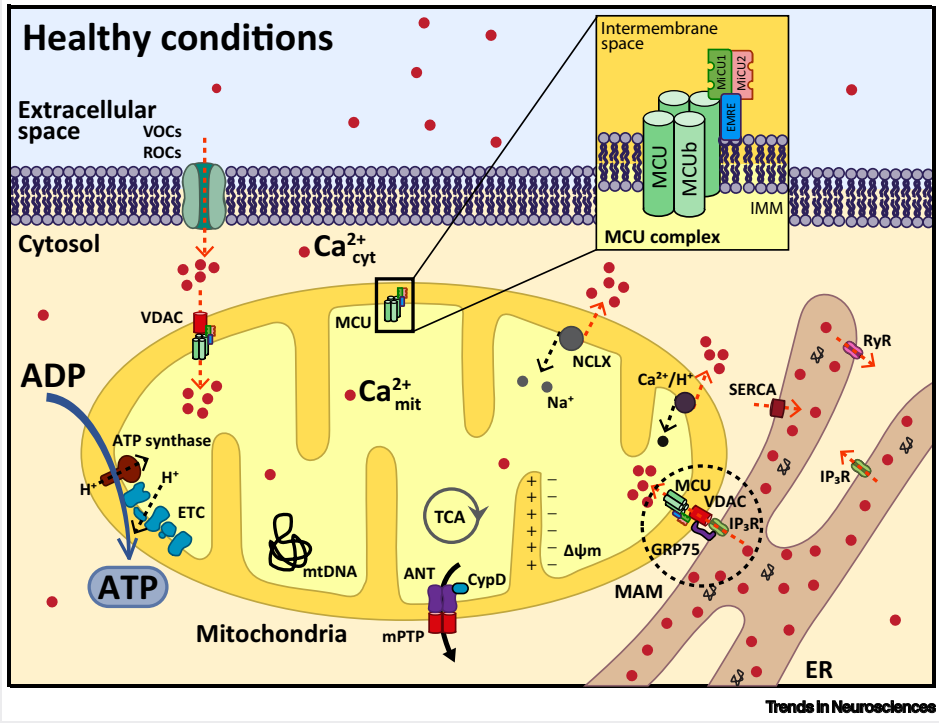

Figure I. Main Components of Mitochondrial Ca^{2+} Homeostasis. The physiological pathway for Ca^{2+} influx into the mitochondria is the MCU complex. The two main Ca^{2+} efflux pathways from the mitochondria are the NCLX and H^+/Ca^{2+} exchangers. The mPTP might contribute to venting Ca^{2+}. The ETC extrudes H^+ from the matrix and generates the high electrical gradient of the IMM, namely the ΔΨm. In return, the ATP synthase uses the generated electrical gradient to synthesize ATP from ADP and phosphate. The ER transfers Ca^{2+} to mitochondria via MAMs. Abbreviations: ΔΨm, mitochondrial membrane potential; ANT, adenine nucleotide translocator; CypD, cyclophilin D; cyt, cytoplasmic; 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, mitochondria-associated membranes; MCU, mitochondrial Ca^{2+} uniporter; MICU, mitochondrial Ca^{2+} uptake proteins; mit, mitochondrial; 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 cycle; VDAC, voltage-dependent anion channel; VOCs, voltage-operated Ca^{2+} channels.

likelihood of finding cytosolic Ca^{2+} overload is increased in the vicinity of Aβ plaques, although such overload was detectable throughout the cortex [16]. In our view, mitochondria could be sensing and buffering the higher Ca^{2+} concentration everywhere (close to and far from Aβ plaques, due to Aβo), whereas to observe increased Ca^{2+} concentration in the cytosol, the neurons might need to be closer to plaques, where the concentration of Aβo is believed to be higher [43]. In addition, mitochondria are mobile and undergo mitophagy, and affected neurites could be rapidly pruned, diluting the local effect of plaques, all of which may contribute to the observed effect. Simultaneous assessment of Ca^{2+} levels in both compartments (cytosol and mitochondria) *in vivo* in the same cell could help in assessing the relative contributions of these effects. The study discussed earlier [43] also showed that direct application of naturally secreted soluble Aβo to the naïve WT brain *in vivo* recapitulated the increase in mitochondrial Ca^{2+} concentration. Blocking the **mitochondrial Ca^{2+} uniporter** (MCU, the main pathway for Ca^{2+} uptake in mitochondria; Box 2) – using the specific channel blocker Ru360 – abolished this increase, implying, from our standpoint, that an intact MCU is required for Aβ-driven mitochondrial Ca^{2+} uptake [43]. Importantly, neurons with elevated mitochondrial Ca^{2+} in their cell somas – a rare event observed *in vivo* in this

Key Figure

Mitochondrial Ca^{2+} Homeostasis Is Impaired in Alzheimer's Disease

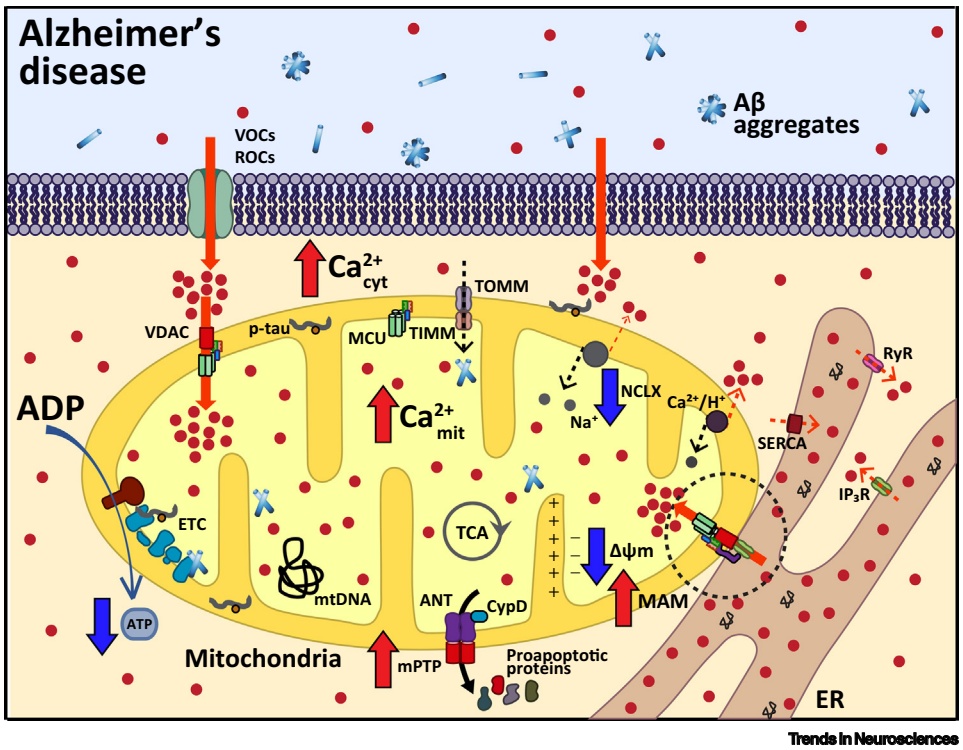

Figure 1. Schematic of the neurotoxic effects of Aβ aggregates and phosphorylated tau (p-tau) on mitochondrial Ca^{2+} homeostasis. Aβ can overactivate channels and/or form pores in the cytosolic plasma membrane, allowing massive influx of Ca^{2+} from the extracellular space into the cytosol. To regulate buffering of the excessive levels of cytosolic Ca^{2+}, mitochondria take up Ca^{2+} from the cytosol via the mitochondrial Ca^{2+} uniporter (MCU) complex that is required for Aβ-induced Ca^{2+} uptake. This leads to mitochondrial Ca^{2+} overload. In addition, Aβ can trigger Ca^{2+} release from the endoplasmic reticulum (ER) via IP_3Rs or RyRs. Ca^{2+} transfer from ER to mitochondria via mitochondria-associated ER membranes (MAMs) is enhanced, likely because of a decrease in the optimal ER–mitochondria distance or increased contacts, contributing to mitochondrial Ca^{2+} increase. Ca^{2+} extrusion from mitochondria via NCLX is reduced. Extracellular tau also contributes to mitochondrial Ca^{2+} overload in neurons. Excessive levels of Ca^{2+} in mitochondria can increase reactive oxygen species (ROS) generation, decrease the mitochondrial membrane potential (ΔΨm), and activate the mitochondrial permeability transition pore (mPTP), leading to the release of proapoptotic factors into the cytosol and activation of caspases. Eventually, neuronal cell death via apoptosis occurs. In addition, both Aβ and tau can be transported to mitochondria via the TOMM40 (translocase of the outer mitochondrial membrane import machinery) complex and the TIMM23 (translocase of the inner mitochondrial membrane) complex, and are found in the outer mitochondrial membrane (OMM) and in the inner mitochondrial membrane (IMM), as well as in the matrix. Once inside mitochondria can they interact with mitochondrial proteins (such as the complexes of the electron transport chain, ETC) and affect proper mitochondrial function and the production of ATP. Abbreviations: ANT, adenine nucleotide translocator; Ca^{2+}/H^+, Ca^{2+}/H^+ exchanger; CypD, cyclophilin D; cyt, cytoplasmic; IP_3R, inositol trisphosphate receptor; mit, mitochondrial; mtDNA, mitochondrial DNA; NCLX, $Na^+/Ca^{2+}/Li^+$ exchanger; ROCs, receptor-operated Ca^{2+} channels; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; TCA, tricarboxylic acid cycle; VDAC, voltage-dependent anion channel; VOCs, voltage-operated Ca^{2+} channels.

mouse model – undergo apoptosis in the APP/PSEN1 Tg mouse model, suggesting a connection between the two processes *in vivo*, although it cannot be completely determined whether one is a cause or a consequence of the other. Rapid neuronal cell death preceded by Aβ-triggered oxidative

stress was previously shown in the same mouse model [49]. Hence, these studies demonstrate a link between mitochondrial Ca^{2+} overload, oxidative stress, and neuronal apoptosis *in vivo*. Other studies have shown that dendritic and axonal processes are more susceptible to AD-related Ca^{2+} accumulation in the cytosol [16] and in the ER *in vivo* [50]. Mitochondrial Ca^{2+} overload was observed in both somas and neurites in the Tg mouse [43]. However, cells die only when mitochondria with Ca^{2+} overload accumulate in the neuronal soma, a very rare event, but not when they accumulate in neurites. From our point of view, this suggests that mitochondrial Ca^{2+} overload within the neuronal soma constitutes an 'all or none' phenomenon rather than an isolated event in individual mitochondrion. Interestingly, in neurons that subsequently die, caspase activation co-occurs with oxidative stress in neuronal soma, but not in the dystrophic neurites surrounding Aβ plaques [49]. It would be interesting to assess whether cells with elevated Ca^{2+} in other compartments also undergo cell death or whether this is a particularly mitochondria-related event *in vivo*, as suggested by *in vitro* experiments [44,45].

Another proposed mechanism for Aβ-mediated mitochondrial Ca^{2+} overload is impaired Ca^{2+} extrusion through reduced levels of the **$Na^+/Ca^{2+}/Li^+$ exchanger** (NCLX) – the main pathway for Ca^{2+} efflux from mitochondria (Box 2). Recent work has reported decreased expression of NCLX in mitochondria from human AD brains and in the 3×Tg-AD mouse AD model (which develops both amyloid plaques and neurofibrillary tangles), linked to impaired mitochondrial Ca^{2+} efflux and mitochondrial Ca^{2+} overload, that could contribute to the progression of the disease [51].

Presenilins have also been proposed to regulate mitochondrial Ca^{2+} homeostasis and synaptic plasticity [52]. Fibroblasts from fAD patients display increased Ca^{2+} release from intracellular stores after stimulation compared with controls [53]. Presenilins have been proposed to act as low-conductance ER leak channels in a γ-secretase-independent fashion, thus contributing to maintaining physiological Ca^{2+} levels within this organelle, a function they fulfill either by themselves or indirectly by increasing the activity of different ER receptors (inositol trisphosphate receptors, IP_3Rs; and ryanodine receptors, RyRs) [54]. fAD-linked mutations in presenilins could result in a loss of function of the ER Ca^{2+} leak channel, and impair Ca^{2+} release from the ER through these channels, resulting in accumulation of Ca^{2+} in this organelle and increased vulnerability to degeneration [55] (the ER–mitochondria relationship is discussed in the section on Mitochondria-Associated ER Membranes). Other studies, however, have failed to support these observations [56,57]; and other groups [58,59] proposed that fAD presenilin-linked mutations reduce, rather than increase, ER and Golgi Ca^{2+} levels. Despite broad investigation, how mutant presenilins affect Ca^{2+} signaling remains a matter of controversy in the field. Presenilin mutations could also lead to neurodegeneration by dysregulating mitochondrial metabolic activity, as shown in a *Caenorhabditis elegans* model [60].

Pathological Tau Can Cause Mitochondrial Ca^{2+} Dyshomeostasis

Tau has been found to localize at the outer mitochondrial membrane (OMM) and within the inner mitochondrial space, as shown *in vitro* [61], and to interact with mitochondrial transporters and complexes, as shown in cortical brain tissue from AD versus control subjects and in mouse AD models [62,63], suggesting that tau-dependent modulation of mitochondrial functions might also be a trigger for the neurodegenerative process in AD.

To meet specific subcellular demands, mitochondria are transported within neurons, traveling long distances within the axon. Mitochondrial transport is regulated by Ca^{2+} and several mitochondrial and cytoplasmic proteins, including tau and kinases [64]. High local Ca^{2+} concentrations arrest mitochondrial transport, retaining them at sites of high energy demand or where Ca^{2+} buffering is

immediately required [65]. Tau is a primarily axonal unfolded monomeric protein that stabilizes microtubules and allows smooth axonal transport of organelles. Abnormally hyperphosphorylated and misfolded tau detaches from microtubules, aggregates, and translocates to the somatodendritic compartment of the neuron where it forms neurofibrillary tangles, resulting in disruption of mitochondrial transport, energy deprivation, and oxidative stress at the synapse, and eventually in neurodegeneration [66]. Excessive cytosolic Ca^{2+} causes tau hyperphosphorylation via activation of kinases [67] or microsomal prostaglandin E synthase 1 (mPGES1) [68].

Several studies support the idea that pathological tau causes mitochondrial Ca^{2+} dyshomeostasis. Both cells that overexpress tau and cells exposed to extracellular tau aggregates show disruptions of mitochondrial Ca^{2+} buffering and cellular Ca^{2+} homeostasis [69,70]. A recent study showed that, in cortical neurons exposed to tau protein and in patient-derived human iPSC neurons bearing a specific mutation in the gene encoding tau (10+16 *MAPT* mutation), basal mitochondrial Ca^{2+} levels are elevated, probably due to inhibition of NCLX by tau, resulting in increased vulnerability to Ca^{2+}-induced cell death [71]. In addition, tau dysregulates the mitochondrial complex I in the 3×Tg AD mouse model [72], and affects mitochondrial respiration and ATP synthesis (complex V) in a Tg mouse model of tauopathy ($MAPT^{P301L}$) [73]. Interestingly, the expression of a truncated tau isoform (Asp421-cleaved tau or TauC3), compared with full-length tau, induces mitochondrial dysfunction in immortalized cortical neurons *in vitro*, including fragmented mitochondria, decreased $\Delta\Psi m$, and impaired mitochondrial membrane integrity, that were associated with high ROS and reduced mitochondrial Ca^{2+}-buffering capacity, events that can be prevented by inhibitors of calcineurin (a Ca^{2+}-sensitive tau phosphatase) inhibitors such as cyclosporine A [69].

Aβ and tau likely work cooperatively to impair mitochondrial function, although the underlying mechanisms of the interplay between Aβ and tau remain unresolved. Tau is required for Aβ to trigger negative effects on neurons and mitochondria. Lack of tau protein in tau knockout (KO) mice may protect from Aβ-induced neurotoxicity and cognitive impairment compared to WT mice [74,75], supporting a key role for tau in the mechanisms leading to neurodegeneration induced by Aβ. Relatedly, tau may facilitate Aβ toxicity to mitochondria because overexpression of a pathologic form of tau in primary cortical neurons enhanced $\Delta\Psi m$ loss triggered by Aβ-mediated impairment of Ca^{2+} buffering capacity of mitochondria, whereas tau deletion rescued this phenotype [76].

Of note, the expression of many of the genes involved in mitochondrial Ca^{2+} transport is altered in AD, according to bulk RNA-seq and microarray analyses from AD and control brains [77–79]. Specifically, most genes encoding proteins involved in mitochondrial Ca^{2+} influx (MCU complex) are downregulated, whereas the gene encoding NCLX (mitochondrial Ca^{2+} efflux) is upregulated [43]. Although this could result in an overall decrease in mitochondrial Ca^{2+} levels, depending on the protein activity, we believe that it may also suggest compensatory changes to avoid Ca^{2+} overload in mitochondria in human AD brain. In any case, these data demonstrate significant alterations in genes related to mitochondrial Ca^{2+} homeostasis in human AD samples (Figure 2).

AD Risk Factors Influence Mitochondrial Ca^{2+} Homeostasis

Several AD risk factors may also impair mitochondrial Ca^{2+} homeostasis. Apolipoprotein E4 (ApoE4), the most prevalent genetic risk factor for sAD, is a lipid transport protein that participates in the regulation of plasma cholesterol. APOE4-expressing neurons show decreased NAD^+/NADH ratio, increased ROS, and elevated mitochondrial Ca^{2+} levels [80]. Supporting these results, transcriptomic analysis of the entorhinal cortex of *APOE4* versus *APOE3* mice revealed upregulation of genes related to OXPHOS [81], whereas genetic removal of APOE4 improved

	Gene	Analysis	Brain region	LogFC	*P*adj
Efflux	*SLC8B1*	RNA-seq	Parahippocampal gyrus	0.438	9.60E–05
	SLC8B1	RNA-seq	Inferior frontal gyrus	0.248	0.0244
	SLC8B1	Microarray	Middle temporal gyrus	0.172	0.1660
	SLC8B1	Microarray	Frontal pole	0.157	0.2542
	SLC8B1	Microarray	Occipital visual cortex	0.138	0.1660
Influx	*MICU1*	RNA-seq	Parahippocampal gyrus	–0.033	0.0035
	MCU	RNA-seq	Parahippocampal gyrus	–0.080	0.0501
	MICU2	RNA-seq	Parahippocampal gyrus	–0.085	0.2296
	SMDT1	RNA-seq	Frontal pole	–0.091	0.2484
	MICU1	RNA-seq	Superior temporal gyrus	–0.093	0.1936
	MICU1	RNA-Seq	Inferior frontal gyrus	–0.112	0.0035
	SMDT1	RNA-seq	Inferior frontal gyrus	–0.128	0.0533
	SMDT1	RNA-seq	Dorsolateral prefrontal cortex	–0.136	0.0877
	MCU	RNA-seq	Inferior frontal gyrus	–0.136	0.0393
	MCU	Microarray	Posterior cingulate cortex	–0.137	0.1985
	MCU	RNA-seq	Frontal pole	–0.143	0.2445
	MICU2	RNA-seq	Superior temporal gyrus	–0.163	0.1950
	MICU2	RNA-seq	Frontal pole	–0.178	0.0126
	MICU3	RNA-seq	Inferior frontal gyrus	–0.219	0.0899
	MICU1	RNA-seq	Frontal pole	–0.221	0.0039
	MICU3	RNA-seq	Superior temporal gyrus	–0.250	0.1332
	SMDT1	Microarray	Posterior cingulate cortex	–0.325	0.2140
	MICU3	RNA-seq	Frontal pole	–0.339	0.0103
	SMDT1	Microarray	Superior temporal gyrus	–0.388	0.1806
	SMDT1	Microarray	Putamen	–0.472	0.1806
	SMDT1	Microarray	Occipital visual cortex	–0.491	0.0964
	MICU3	RNA-seq	Parahippocampal gyrus	–0.558	0.0000
	MICU3	Microarray	Inferior frontal gyrus	–0.652	0.1553
	MICU3	Microarray	Superior temporal gyrus	–0.659	0.1247
	MICU3	Microarray	Hippocampus	–0.693	0.1048
	MICU3	Microarray	Posterior cingulate cortex	–0.718	0.0899
	MICU3	Microarray	Frontal pole	–0.925	0.0103
	MICU3	Microarray	Middle temporal gyrus	–0.954	0.0103

Figure 2. The Expression of Genes Encoding Proteins Involved in Mitochondrial Ca^{2+} Transport Is Altered in Alzheimer's Disease (AD) Post-Mortem Human Brains. Heatmap analysis of gene expression changes in the human AD brain based on post-mortem tissue analyses. Comparisons between AD subjects (B3, Braak stages V–VI) and control individuals (B1, Braak stages 0–I–II) were made for *MCU*, *MCUB*, *MCUR1*, *MICU1*, *MICU2*, *MICU3*, *SMDT1* (all encoding mitochondrial Ca^{2+} influx MCU complex subunits: MCU, MCUb, MICU1, MICU2, MICU3, and EMRE) and *SLC8B1* (mitochondrial Ca^{2+} efflux, NCLX). Note that influx genes are downregulated (blue colors in the heatmap), whereas the only efflux gene is upregulated (red colors in the heatmap). The original data were published as a bubble chart in Calvo-Rodriguez *et al.* [43]. Differential gene expression data that are publicly available at the Accelerating Medicines Partnership – Alzheimer's Disease (AMP-AD) knowledge portal was used [78]. Twenty-five publicly available microarray and RNA-seq datasets from the Mount Sinai Brain Bank (MSBB) and the Religious Orders Study and Memory and Aging Project (ROSMAP) cohorts were included [77,79]. Differentially expressed genes were identified at a false discovery rate (FDR) of 25%. Data were adjusted for neuronal loss with the pan-neuronal marker MAP2. Log fold-change (LogFC) values and *P* values adjusted (adj) for multiple comparisons (Benjamini–Hochberg) are given. Grey color indicates statistically non-significant differences. Modified, with permission, from [43].

neuronal cytosolic Ca^{2+} responses in an AD mouse model, as shown by two-photon imaging [82], which suggest alternative mechanisms that underlie the pathological effects of *APOE4*.

The calcium homeostasis modulator 1 (CALHM1) is a Ca^{2+} channel involved in the regulation of cytosolic Ca^{2+} and Aβ levels [83]. The *CALMH1* polymorphism P86L has been proposed as a risk factor for late-onset sAD [13,83]. The P86L polymorphism alters the channel Ca^{2+} permeability

[83], suggesting an additional role of Ca^{2+} signaling in AD. However, other studies have not supported this hypothesis [84,85]. Studies in HeLa cells showed that mitochondria show slower kinetics when sensing Ca^{2+} entering through P86L-CALHM1 relative to CALHM1 [86], implying that mutated CALHM1 may cause mitochondrial Ca^{2+} overload and render cells more susceptible to apoptotic stimuli.

TREM2 (triggering receptor expressed on myeloid cells 2) is an immunomodulatory receptor that is crucial for myeloid cell activation and survival. A variant of *TREM2* has been found to increase the risk of AD and other neurodegenerative diseases [87], and recent work in human patient iPSC-derived microglia has shown that the expression of TREM2 loss-of-function variants in these cells impairs mitochondrial respiratory capacity [88]. These findings may offer a possible explanation to prior work in mice, which reported reduced mitochondrial number in *Trem2*$^{-/-}$ mutant mice microglia [89]. However, whether mitochondrial Ca^{2+} plays a role in these phenomena is unknown, given that currently no studies (to our knowledge) have directly examined the relationship between TREM2 and mitochondrial Ca^{2+}.

Mitochondria-Associated ER Membranes (MAMs) and the MAM Hypothesis of AD

The MCU transports Ca^{2+} from the cytosol into mitochondrial matrix, thus dissipating the $\Delta\Psi m$ generated in the inner mitochondrial membrane (IMM). However, its affinity for Ca^{2+} is very low, which means that, under physiological conditions, the cytosolic Ca^{2+} concentration should be 5–10 μM to open the MCU. These values are never reached in the cytosol of a healthy cell, and thus it was long believed that the mitochondrial Ca^{2+} transporters would only take up Ca^{2+} under conditions of cellular Ca^{2+} overload [90]. The use of aequorin and other GFP-based fluorescent probes demonstrated that mitochondria do not sense bulk Ca^{2+} levels in the cytosol, and instead sense regions of high Ca^{2+} in their vicinity. Mitochondria can be juxtaposed to Ca^{2+} channels on the ER [91], where transient regions of high Ca^{2+} concentration (>10 μM), known as **Ca^{2+} microdomains** or hotspots, are formed [92]. Microdomains are generated at the mouth of the Ca^{2+} channel and its immediate neighborhood, on the inner side of the plasma membrane or on the Ca^{2+} channels of the ER. The generation of these hotspots controls several cellular functions, such as the secretion of neurotransmitters/synaptic vesicles at the presynaptic membrane [93]. Mitochondria are primary targets of Ca^{2+} microdomains, whereas the ER is one of the main generators.

The OMM and ER establish contact sites, known as MAMs. These sites of juxtaposed ER and OMM are tethered by proteins without undergoing fusion, and are biochemically distinct from pure ER and pure mitochondria. MAMs are intracellular and dynamic lipid rafts that are enriched in cholesterol and sphingomyelin, as well as in proteins associated with Ca^{2+} dynamics, including RyRs and IP_3Rs. MAMs regulate key cellular events, including lipid synthesis and transport, Ca^{2+} transport from the ER to mitochondria, and several metabolic pathways such as glucose metabolism [94], all of which are impaired in AD. Many proteins have been proposed among the tethers linking ER and mitochondria, including mitofusin 2 and GRP75 (glucose-regulated protein 75) [95,96]. **Voltage-dependent anion channels** (VDACs) cluster at the ER–mitochondria contact sites and participate in the rapid Ca^{2+} transfer [97], and σ1 receptor promotes Ca^{2+} transport from ER to mitochondria by interacting with the IP_3Rs [98]. ER–mitochondria interplay at the MAMs is an efficient strategy for Ca^{2+} transfer from ER to mitochondria at the opening of IP_3R Ca^{2+} channels [92]. Under normal conditions, mitochondria take up Ca^{2+} that is directly released from the ER, and Ca^{2+} remains local or the Ca^{2+} wave dissipates from close to distant mitochondria, avoiding mitochondrial Ca^{2+} overload [93,99]. In turn, the ER controls mitochondrial energy metabolism. Neuronal Ca^{2+} homeostasis depends crucially on this ER–mitochondria Ca^{2+} transfer.

However, excessive Ca^{2+} release from the ER might overfill mitochondria with Ca^{2+} under abnormal conditions.

Several studies have investigated the role of MAM-dependent mitochondrial dysfunction in AD. During cellular stress, MAMs alter their regulatory proteins and functions, which can lead to mitochondrial dysfunction. Expression of MAM-associated proteins is upregulated in post-mortem AD brains, in APP Tg AD mouse models, and in primary hippocampal neurons exposed to Aβ [100]. Moreover, it has been proposed that there is an optimal distance between the ER and mitochondria, which if disturbed can lead to higher levels of ER–mitochondria Ca^{2+} transfer, thus triggering apoptosis. Increased MAM activity and ER–mitochondria connectivity have been reported in human fibroblasts from fAD and sAD patients [101], and in primary neurons exposed to Aβo, leading to enhanced ER–mitochondria Ca^{2+} transfer [102,103].

Of note, PSEN1 and PSEN2, although localized at the ER, are enriched in MAMs [104]. C99 – the C-terminal fragment of APP that generates Aβ upon its cleavage by γ-secretase – was found not only in endosomes but also in MAMs. Therefore, the amyloidogenic processing of C99 to $Aβ_{42}$ was proposed to take place in MAMs [105,106]. *In vitro* models of AD, and cells from fAD and sAD patients, exhibit significant increases in C99 in MAMs that correlate with alterations in MAM structure and function [105]. Based on this, it has been proposed that mitochondrial dysfunction lies downstream of C99 accumulation in MAMs, suggesting an early role of mitochondrial dysfunction in AD. This framework has been coined the 'MAM hypothesis of AD' [107].

Tau also interferes with ER–mitochondria communication and contributes to neurodegeneration. Increased ER–mitochondria association has been reported in a Tg mouse model of tauopathy compared to WT control mice [108], an association that correlated with increased colocalization of tau at the surface of the ER, but not at the surface of mitochondria, both in the mouse model and in post-mortem AD brains. Similarly, *in vitro* experiments showed that ER–mitochondria interactions were affected by the expression of $2N4RΔC_{20}$ tau (another truncated form of tau), suggesting that, in addition to the effects of the Aβ pathway on ER–mitochondria communication, this might be an important pathological event in tau-related dysfunction [61].

In addition, APOE4 may upregulate MAM activity (ER–mitochondria communication and function), as shown by measuring the synthesis of phospholipids and cholesterol esters in cells treated with conditioned media from APOE4 astrocytes. According to the authors, these data support the role of upregulated MAM function in AD, and imply that APOE4 may also modulate ER–mitochondria communication, while contributing to the mechanism of APOE4 as a risk factor for AD [109].

Therapeutic Potential of Mitochondrial Ca^{2+} in Alzheimer's Disease

Given that mitochondrial Ca^{2+} dysregulation can trigger neurodegeneration via apoptosis/necrosis, identifying targets to maintain Ca^{2+} balance and mitochondrial function is paramount for developing disease-modifying drugs. However, the development of compounds and small molecules aimed at protecting mitochondrial function and mitochondrial Ca^{2+} homeostasis as a strategy for neuroprotection remains underdeveloped. The different Ca^{2+} influx and efflux pathways in mitochondria (see Figure I in Box 2) provide attractive targets for manipulating the Ca^{2+} concentration within the organelle, and several lines of research with particular drugs have been already tested (Table 1), although most are designed to protect from the neurotoxic effects of Aβ.

Ca^{2+} dysregulation in mitochondria could be consequence of increased influx (MCU complex), decreased efflux (NCLX), altered capacity of Ca^{2+} buffering, or increased ER–mitochondria Ca^{2+}

Table 1. Mitochondrial Ca^{2+} Homeostasis Components Proposed as Druggable Targets in AD

Mitochondrial target	Treatment	Experimental model	Drug effect	Refs
MCU	Ru360	Fibroblasts isolated from fAD patients	Decreased ROS levels	[60]
		Topical application of naturally secreted Aβo to the mouse brain surface *in vivo*	Decreased mitochondrial Ca^{2+} uptake	[43]
		Primary mouse microglia and BV-2 cells exposed to $Aβ_{25–35}$	Attenuated apoptosis, mitochondrial ROS production, and ER stress	[114]
	TG-2112x	Cocultures of cortical or hippocampal neurons and astrocytes exposed to glutamate	Decreased mitochondrial Ca^{2+} overload and cell death	[115]
ΔΨm	FCCP	Cultured rat hippocampal neurons exposed to oligomeric $Aβ_{1–42}$	Decreased mitochondrial Ca^{2+} uptake and neuronal death via mild mitochondrial depolarization	[44,45]
	NSAIDs	Cultured rat hippocampal neurons exposed to oligomeric $Aβ_{1–42}$	Decreased mitochondrial Ca^{2+} uptake and neuronal death via mild mitochondrial depolarization	[44,45]
	Berberine	Mouse hippocampal neurons exposed to oligomeric $Aβ_{1–42}$	Maintenance of ΔΨm, prevention of ATP decrease, and improvement of mitochondrial motility and trafficking	[116]
	Folic acid and memantine	Neuroblastoma SH-SY5Y cells exposed to $Aβ_{25–35}$	Attenuation of Aβ-elicited mitochondrial membrane depolarization, reduced cytochrome *c* release into the cytosol and ROS generation	[117]
ΔΨm/VDAC	Minocycline	Cultured rat cerebellar granule cells exposed to NMDA	Decreased mitochondrial Ca^{2+} uptake and ROS production	[118]
mPTP	Dimebon	Mitochondria isolated from rat brain cerebral cortex exposed to high Ca^{2+}	Reduction of Ca^{2+}-induced mitochondrial swelling	[119]
mPTP (CypD)	CsA	Isolated mitochondria or primary cultured neurons exposed to Aβo	Decreased neuronal death and inhibition of mPTP opening	[43,44,120]
		Fibroblasts isolated from sAD patients	Decreased mitochondrial superoxide levels, improved mitochondrial and cytosolic Ca^{2+} dysregulation	[29]
	Pyridyl/pyrazinyl thiourea derivatives	HT-22 hippocampal cell line exposed to $Aβ_{1–42}$	Protection against Aβ-induced neurocytotoxicity	[121]
	C-9 (4-aminobenzenesulfonamide derivative)	Isolated mouse cortical mitochondria and SK-N-SH cells exposed to oligomeric $Aβ_{1–42}$	Disruption of Ca^{2+}-mediated-induced mitochondrial swelling. Increase of cytochrome c oxidase activity.	[122]
	2-(3-arylureido)pyridines and 2-(3-arylureido)pyrazines	HT-22 hippocampal cell line exposed to $Aβ_{1–42}$	Protection against Aβ-induced neurocytotoxicity	[123]
Mitochondrial Ca^{2+} levels/unknown	Tournefolic acid B	Primary cultures of neonatal rat cortical neurons exposed to $Aβ_{25–35}$	Decreased mitochondrial Ca^{2+} uptake and reduced cell death	[124]
Mitochondrial Ca^{2+} levels/ΔΨm	Linalool	HT-22 cells and mouse organotypic hippocampal slices exposed to glutamate and NMDA	Reduction of mitochondrial Ca^{2+} levels and mitochondrial ROS	[125]
NCLX	CGP37157	Primary rat cortical neurons exposed to acetyl choline, KCl, and NMDA	Blocking of voltage-gated calcium channels (VGCCs), decreased cytosolic and mitochondrial Ca^{2+} overload	[126]
MAMs	Methyl B12 (methylcobalamin)	PC12 cells exposed to $Aβ_{25–35}$	ROS scavenging, reduced ER–mitochondria Ca^{2+} flux through IP_3R, and protection against apoptosis and cell death	[127]

transfer (MAMs). To date, the MCU subunit of the mitochondrial uniporter complex is one of the best studied. MCU KO mice were generated to ascertain the role of the MCU in biologic processes. As expected, skeletal muscle and heart mitochondria from MCU KO mice did not take up Ca^{2+} [110]. However, other groups found that deletion of MCU incompletely inhibits mitochondrial Ca^{2+} uptake and mPTP induction in brain mitochondria [111], suggesting the presence of a Ca^{2+}

uptake pathway that is independent of MCU, and that mediates residual Ca^{2+} influx and mPTP induction in a fraction of the mitochondrial population. MCU overexpression *in vitro* exacerbates *N*-methyl-D-aspartate (NMDA)-induced loss of ΔΨm and cell death, whereas MCU knockdown shows protection against neuronal cell death following NMDA receptor activation, revealing a key role of MCU and mitochondrial Ca^{2+} in excitotoxicity [112]. Reducing mitochondrial Ca^{2+} uptake via MCU deletion improved mitochondrial function and rescued neurodegeneration in a *C. elegans* line expressing a mutated version of the gene encoding a PSEN homolog [60], and knockdown of endogenous MCU rendered primary cultured neurons resistant to oxidative stress following histamine stimulation [113]. In addition, inhibition with the MCU inhibitor Ru360 may prevent mitochondrial Ca^{2+} uptake in mice *in vivo* after stimulation with naturally secreted Aβo [43]. This rapidly accumulating evidence points to MCU as a potential therapeutic target in AD. However, further research will be necessary to ascertain the therapeutic candidacy of the other subunits of the uniporter complex, and of NCLX and the MAMs, including overexpression and KO mouse models and/or virus-mediated gene transfer approaches.

Concluding Remarks and Future Directions

In AD, mitochondrial dysfunction is physically and temporally connected to Aβ and pathological tau accumulation, which act independently and synergistically on this organelle. Recent advances in imaging techniques and models of AD have allowed new approaches for a better understanding of the mitochondrial Ca^{2+} dyshomeostasis that is associated with these two proteins. Bulk RNA-seq data from human AD post-mortem brain tissue has identified alterations in the genes encoding proteins involved in Ca^{2+} transport in mitochondria compared with control subjects, suggesting an alteration in mitochondrial Ca^{2+} in the human AD brain. In addition, multiphoton and confocal microscopy combined with Ca^{2+} reporters targeted to mitochondria have allowed direct evaluation of Ca^{2+} levels in mitochondria, as well as Ca^{2+} buffering capacity and ER–mitochondria Ca^{2+} transfer via MAMs. Nevertheless, several questions need to be addressed to clarify the role of mitochondrial Ca^{2+} in AD (see Outstanding Questions). Furthermore, as discussed earlier, if mitochondrial Ca^{2+} is key in the process of neuronal cell death, finding drugs that target mitochondrial Ca^{2+} seems to be essential for the treatment of AD. The latest studies point to MCU as a candidate therapeutic target for AD and other neurodegenerative diseases in which mitochondrial Ca^{2+} is impaired. Future work will focus on the clinical relevance of these findings, and, in our view, a combination of the modulation/removal of both Aβ and tau deposition, together with strategies aiming at restoring mitochondrial function/energy metabolism, appear to be promising in the development of a therapy for AD.

Outstanding Questions

Mitochondrial impairment has been recognized as an early event in AD in terms of metabolic energy production. Recent reports show that amyloid plaque deposition is necessary for mitochondrial Ca^{2+} overload in AD. However, does mitochondrial Ca^{2+} elevation occur concomitantly with amyloid plaque formation? Does it occur before or after neurofibrillary tangle accumulation?

Mitochondrial trafficking and mitochondrial fission/fusion are known to be impaired in AD, compromising normal neuronal physiology and eventually leading to apoptosis. Are mitochondria with high Ca^{2+} also impaired in terms of their dynamics/mobility? The use of multiphoton microscopy experiments *in vivo* in mouse models of AD as well as analyses in patient-derived iPSC neurons will be instrumental in addressing this question.

ER and mitochondria are physically connected via MAMs, and both Ca^{2+} transfer and ER–mitochondria connectivity are impaired in AD. What is the physiological role of ER–mitochondria Ca^{2+} transfer, and how can its disruption in AD be prevented?

Mitochondrial Ca^{2+} and mitochondrial function in AD have been mostly explored in the context of neuronal function. Are they also impaired in other cell types in the brain (e.g., astrocytes, microglia) in AD? Can therapeutics target the different cell types to individually correct mitochondrial Ca^{2+} dyshomeostasis?

Acknowledgments

This work was supported by National Institutes of Health grants R01AG0442603, S10 RR025645, and R56AG060974 (to B.J.B.) and by the Tosteson & Fund for Medical Discovery and BrightFocus Foundation fellowship award A2019488F (to M.C.R.). We thank Dr Alberto Serrano-Pozo for critical review of the manuscript.

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