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Review

Neuronal Calcium Signaling, Mitochondrial Dysfunction, and Alzheimer's Disease

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Abstract. Alzheimer's disease (AD) is the most common neurodegenerative disorder among the aged worldwide. AD is characterized by extensive synaptic and neuronal loss that leads to impaired memory and cognitive decline. The cause of AD is not completely understood and no effective therapy has been developed. The accumulation of toxic amyloid-β_{42} (Aβ_{42}) peptide oligomers and aggregates in AD brain has been proposed to be primarily responsible for the pathology of the disease, an idea dubbed the 'amyloid hypothesis' of AD etiology. In addition to the increase in Aβ_{42} levels, disturbances in neuronal calcium (Ca^{2+}) signaling and alterations in expression levels of Ca^{2+} signaling proteins have been observed in animal models of familial AD and in studies of postmortem brain samples from sporadic AD patients. Based on these data, the 'Ca^{2+} hypothesis of AD' has been proposed. In particular, familial AD has been linked with enhanced Ca^{2+} release from the endoplasmic reticulum and elevated cytosolic Ca^{2+} levels. The augmented cytosolic Ca^{2+} levels can trigger signaling cascades that affect synaptic stability and function and can be detrimental to neuronal health, such as activation of calcineurin and calpains. Here we review the latest results supporting the 'Ca^{2+} hypothesis' of AD pathogenesis. We further argue that over time, supranormal cytosolic Ca^{2+} signaling can impair mitochondrial function in AD neurons. We conclude that inhibitors and stablizers of neuronal Ca^{2+} signaling and mitochondrial function may have therapeutic potential for AD treatment. We also discuss latest and planned AD therapeutic trials of agents targeting Ca^{2+} channels and mitochondria.

Keywords: Alzheimer's disease, calcium, Dimebon, endoplasmic reticulum, excitotoxicity, mitochondria

THE ROLE OF INTRACELLULAR Ca^{2+} DYSREGULATION IN ALZHEIMER'S DISEASE

The most prevalent idea of Alzheimer's disease (AD) pathogenesis is based on the "amyloid cascade hypothesis", first penned in 1992 by Hardy and Higgins [1], which states that accumulation of amyloid-β (Aβ) peptide, altered processing, or lack of clearance is the initiating molecular event that ultimately leads to amyloid plaques, neurofibrillary tangles, inflammation, synaptic loss, and neurodegeneration in both sporadic (late-onset AD, LOAD) and familial (genetically-linked) AD (FAD) [2]. In patients, evidence to support the "amyloid cascade hypothesis" is demonstrated by the accumulation of amyloid plaques in the AD brain; the FAD cases resulting from missense mutations in the amyloid-β protein precursor (AβPP) or presenilin (PSEN1/2), transmembrane proteins which comprise the catalytic subunit of the AβPP-cleaving enzyme γ-secretase [3]. All mutations responsible for FAD affect the proteolysis of the type 1 transmembrane glycoprotein AβPP and result in the overproduction of the hydrophobic, aggregation-prone 1-42 Aβ fragment, increased A$\beta_{42/40}$ ratio, and plaque deposition. Thus, "amyloid-targeting" therapies have been the main fo-

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cus of AD drug development for the past 30 years. They include immunotherapies that target Aβ, γ-/β-secretase inhibitors to block Aβ production, selective Aβ_{42}-lowering agents, statins to enhance α-secretase activity, anti-Aβ aggregation agents, and others. However, the considerable failure rate of Aβ-targeting drugs in clinical trials [4] suggest that reduction of Aβ alone might be insufficient to significantly modify AD outcomes and that alternative therapeutic targets must be considered.

Accumulating data suggests that neuronal Ca^{2+} dysregulation plays an important role in AD pathogenesis. Given the ubiquitous nature of intraneuronal Ca^{2+} signaling, it is necessary to maintain strict regulation of cellular [Ca^{2+}] but at the same time, to do so is energetically expensive. In aged neurons, ATP-generating mechanisms are less efficient and Ca^{2+} handling mechanisms become compromised, leading to excessive free Ca^{2+}, increased intracellular [Ca^{2+}] over time (or Ca^{2+} overload), activation of Ca^{2+}-dependent proteases, reactive oxygen/nitrogen species formation (ROS/RNS), mitochondrial dysfunction, oxidative damage, and apoptosis/necrosis. This is the basis for the 'Ca^{2+} hypothesis of brain aging and AD' first proposed by Khatchaturian in 1989 [5], which states that age-dependent, subtle changes to Ca^{2+} homeostasis would account for the age-related changes in neuronal function [6–8] and that the accumulation of these changes to Ca^{2+} handling could account for the neuronal damage and cognitive decline in AD. The common features of aging neurons is increased Ca^{2+} release from intracellular stores via inositol 1,4,5 triphosphate receptors (IP_3R) and ryanodine receptors (RyanR), increased Ca^{2+} influx via L-type VGCC, increased slow afterhyperpolarization due to activation of Ca^{2+}-dependent K^+ channels, reduced contribution of NMDAR-mediated Ca^{2+} influx, reduced cytosolic Ca^{2+} buffering capacity and activation of calcineurin and calpains. Resulting changes in neuronal Ca^{2+} dynamics lead to augmented susceptibility to induction of long-term depression and an increase in the threshold frequency for induction of long-term potentiation in aging neurons, which may contribute to age-related memory decline [9].

Recent evidence supporting the 'Ca^{2+} hypothesis of AD' was compiled in several recent reviews [10–15]. Changes to intracellular Ca^{2+} signaling in patients were first described in fibroblasts isolated from those at risk for AD. Cells from familial AD patients harboring PSEN1 mutations displayed enhanced IP_3-induced Ca^{2+} responses when compared to cells from healthy subjects [16]. Expression of Ca^{2+}-handling genes was significantly altered in brain tissues from AD patients [17]. The only genetic factor that consistently influences risk or onset age in sporadic AD is the apolipoprotein E ε4 allele (ApoE4), with the risk increasing as the number of copies an individual carries increases [18]. Studies in cell lines and primary cortical neurons expressing recombinant ApoE4 showed elevated cytosolic Ca^{2+} levels by efflux through plasma membrane Ca^{2+} channels [19,20], however, it is not known if such changes are present in ApoE4 carriers. Finally, a single nucleotide polymorphism in CALHM1, a newly identified plasma membrane Ca^{2+} channel, interferes with Ca^{2+} permeability and slightly increases susceptibility to sporadic, late-onset AD [21, 22]. The role of CALHM1 in AD is controversial, with recent studies showing no association between the two [23–25], therefore further study of CALHM1 function is required. Recently, other genes that increase the risk of developing sporadic AD have been identified [26–28] but their effect(s) on intracellular Ca^{2+} are unknown. However, Ca^{2+} dysregulation in neurons appears to be a genuine consequence of AD pathology and further investigations regarding drugs or drug targets that can modulate intraneuronal Ca^{2+} are warranted. The goal of this review is to discuss if the endoplasmic reticulum (ER) and mitochondria, two organelles intimately involved in both intracellular Ca^{2+} signaling (see Fig. 1) and AD pathogenesis, could offer new opportunities for the modulation of intracellular Ca^{2+} and design of disease-modifying therapies.

NEURONAL Ca^{2+} DYSFUNCTION IN ALZHEIMER'S DISEASE

The ER is the largest intracellular organelle which functions to regulate post-translational protein processing. In addition, the ER participates in intraneuron Ca^{2+} signalling and serves as a dynamic store and source of Ca^{2+} ions [29] (Fig. 1). Upon generation of IP_3, Ca^{2+} is released from the ER via IP_3R and amplification of the Ca^{2+} signal is be mediated by the RyanRs, termed Ca^{2+} -induced Ca^{2+} release (CICR). To refill depleted stores, sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA) sequesters cytosolic Ca^{2+} into the ER where it is bound by Ca^{2+} binding proteins such as calreticulin and calnexin [30,31]. Disruption of ER Ca^{2+} homeostasis can affect protein folding by intra-ER chaperones, cellular function and can initiate cell survival and/or death programs [32]. Because ER Ca^{2+}

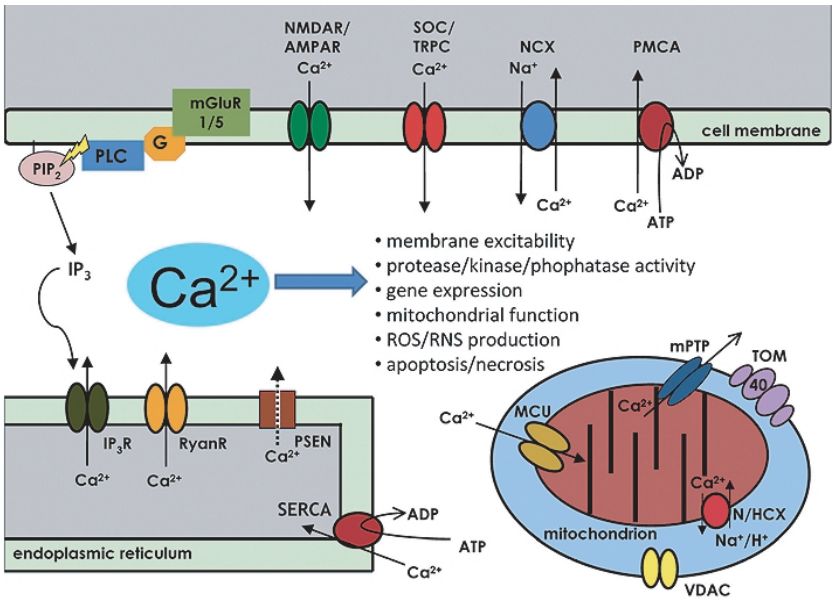

Fig. 1. The compartmentalization of intracellular Ca^{2+} signalling in neurons and AD pathogenesis. Calcium (Ca^{2+}) is a key regulator of many neuronal processes and serves as the critical link between environmental stimuli and the intracellular effectors that result in a physiological response [121]. Gene expression, protein processing, ATP production, neurotransmitter release, action potential generation, modulation of membrane excitability, short-term and long-term synaptic plasticity, neurite outgrowth and control of cell death mechanisms are Ca^{2+}-regulated processes that are imperative for neuronal function. The proteins that bind free Ca^{2+}, such as calmodulin (CaM), and activate Ca^{2+}-dependent cellular processes are expressed in membrane enclosed compartments such as the cytoplasm, the endoplasmic reticulum (ER) or the mitochondria (mt). The concentration of free Ca^{2+} ($[Ca^{2+}]$) and the spatio-temporal pattern of Ca^{2+} microdomains determines the activation of particular cellular processes [122]. Thus, the $[Ca^{2+}]$ in each compartment is tightly regulated. Plasma membrane Ca^{2+} ATPases (PMCA), sodium/calcium exchangers (NCX) and sarco-/endoplasmic reticulum Ca^{2+} ATPases (SERCA) set up an electrochemical gradient which, upon neuronal activation, Ca^{2+} ions can passively move between cellular compartments through voltage- and/or ligand-gated channels. Calcium influx from the extracellular matrix can happen through voltage-gated Ca^{2+} channels (VGCC), *N*-methyl-*D*-aspartate receptors (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), store-operated channels (SOC, e.g., transient receptor potential channels (TRPC)). Calcium efflux from intracellular ER stores is mediated by inositol 1,4,5-trisphosphate receptors (IP_3R), ryanodine receptors (RyanR) and presenilins (PSEN) which facilitate "ER Ca^{2+} leak" [44]. Mitochondria participate in Ca^{2+} signaling by taking up Ca^{2+} from cytosolic or ER microdomains across the outer mitochondrial membrane (OMM) through unknown mechanisms, likely through the voltage-dependent anion channel (VDAC) into the inner mitochondrial membrane (IMM) lumen through the mitochondrial Ca^{2+} uniporter (MCU). Recently, a Ca^{2+}/H^{+} anti-porter (leucine zipper EF-hand–containing transmembrane protein 1, Letm1) that transports Ca^{2+} from the cytosol into the IMM lumen in was identified in HeLa cells [123] but its function in neurons is unknown. Polymorphisms in *TOMM40* gene encoding outer mitochondrial membrane component of the TOM complex have been linked with the probability of developing late-onset AD [28,90–92]. Calcium equilibrium is maintained along the IMM by NCX or hydrogen/calcium exchangers (HCX). Opening of the mitochondrial permeability transition pore (mPTP) allows large efflux of Ca^{2+} from the IMM lumen and is often a trigger for the cell death signalling cascade [66]. Under normal circumstances following neuronal stimulation, active Ca^{2+} transport returns $[Ca^{2+}]$ in each compartment to homeostatic levels. Both active and passive Ca^{2+} handling mechanisms are subject to regulation, in fact, Ca^{2+} itself is an important regulator of Ca^{2+} channel activity.

signalling is essential to intracellular Ca^{2+} homeostasis, the involvement of aberrant ER Ca^{2+} signalling in AD has received much attention and has been recently reviewed [12].

Interest in the role of aberrant ER Ca^{2+} signalling in AD began when it was discovered that mutations responsible for FAD also affected ER Ca^{2+} signalling, which predominantly resulted in exaggerated release of Ca^{2+} from overloaded ER stores. Skin fibroblasts from human patients that harbour a mutation in PSEN1-A246E showed exaggerated Ca^{2+} release from IP_3-gated stores compared to controls after treatment with bombesin and bradykinin [16]. Alterations in Ca^{2+} signalling were detected before the development of overt clinical symptoms and such changes were not present in cells from subjects that failed to develop AD [33]. These initial results were recapitulated experimentally in various model systems expressing FAD-related mutations in PSEN and the data suggested that in addition to contributing to altered γ-secretase function, PSEN mutations had a significant impact on Ca^{2+} signaling in AD models. The PSEN1-M146V mutation augmented Ca^{2+} release from IP_3- and caffeine- gated stores in hippocampal and cortical neurons in 3XTg-

AD mice [34,35]. In human post-mortem tissue, ryanodine binding (indicative of increased RyanR protein) was elevated in hippocampal regions (subiculum, CA2 and CA1) of AD brain in the early stages of the disease prior to extensive neurodegeneration and overt Aβ plaque deposition [36]. RyanR protein levels and channel function are increased in mouse models of containing PSEN mutations PSEN1-M146V and PSEN2-N141I [37–39]. Clinical mutations of PSEN2 also enhanced Ca^{2+} release from IP_3R-gated ER stores [40]. PSEN1 mutations or genetic deletion attenuate capacitative Ca^{2+} entry (CCE), a refilling mechanism for depleted ER Ca^{2+} stores [41–43]. To explain these data, it has been hypothesized that PSENs able to function as ER Ca^{2+} leak channels and that FAD mutations in PSEN1 and PSEN2 disrupt this function [44]. This idea was supported by demonstration of overloaded ER Ca^{2+} stores and exaggerated ER Ca^{2+} release in double PSEN knock-out mouse fibroblasts and in fibroblasts transfected with PSEN1 and PSEN2 FAD mutant constructs [44,45]. Additional mechanisms which may contribute to abnormal Ca^{2+} signaling in PSEN FAD cells may include increased RyanR expression and recruitment [35,38,46], directly affect gating of RyanR [47] or IP_3R [48,49], or affect function of the SERCA pump [50]. Taken together, these results indicate that presenilins play a direct role in Ca^{2+} signaling and affect activity and/or expression of many proteins involved in ER Ca^{2+} signalling. Thus, it is not surprising that many PSEN FAD mutations have major effects on intracellular Ca^{2+} homeostasis [15].

In contrast, few studies have identified changes to ER Ca^{2+} signaling in cells expressing APP mutations. It has been documented that fibroblasts from AD patients harboring the Swedish double mutation, APP-K670N/M671L, showed reduced bombesin-induced intracellular Ca^{2+} elevations compared to controls while all other pools of Ca^{2+} were unaffected [51]. Primary cortical neurons from TgCRND8 mice that express both APP-KM670/671NL and APP-V717F (Indiana) demonstrated elevated release of Ca^{2+} from upregulated RyanR type 3 [52] while global Ca^{2+} handling was unaffected [53]. The effects of APP mutations on ER Ca^{2+} signaling appear to be more subtle compared to the effects of PSEN mutations. The cumulative result of these small changes over time could have a significant effect on neuronal function that may contribute to cognitive decline. For example, *in vivo* Ca^{2+} imaging experiments revealed that neurons of aged AβPP-expressing Tg2567 and AβPP/PS1ΔE9 mice that were in close proximity to Aβ plaques were overloaded with Ca^{2+} when compared to neurons in the young mice prior to plaque formation and to neurons from the PSEN1-M146V and wild-type mice [54]. The augmented cytosolic Ca^{2+} leads to a loss of Ca^{2+} compartmentalization in dendritic spines and distorted neurite morphologies mediated by activation of Ca^{2+}-dependent phosphatase calcineurin [54–56]. Resulting functional and structural modifications of synaptic connections could negatively impact neuronal networks and memory function. Consistent with this idea, inhibitors of calciunerin exerted positive effects in memory tests with Tg2567 mice [57,58]. Activation of calciunerin and resulting changes in synaptic connectivity can be induced by relatively modest Ca^{2+} elevations. More pronounced cytosolic Ca^{2+} increase may lead to activation of calpains, Ca^{2+}-dependent proteases which can degrade cellular signaling proteins that are involved in learning and memory [59,60].

The dysregulation of cytosolic Ca^{2+} by aberrant ER Ca^{2+} signaling is an important aspect of AD pathogenesis, yet ER Ca^{2+} handling machinery are underutilized targets for AD therapeutics. The only exceptions are PSENs, which are targeted for their γ-secretase activity rather than their ER Ca^{2+} signaling function [61]. The inherent problems associated with targeting ER Ca^{2+} channels and pumps are drug specificity, subtype selectivity and maintenance of biological function. For example, blockade of PSEN via γ-secretase inhibitors could hinder their ability to process Notch, which is important for neurodevelopment, and they may also affect their ER Ca^{2+} leak function. In addition, some of the observed Ca^{2+} signaling changes may in fact be compensatory and exert beneficial effect in AD (reviewed in [12]). For example, blockade of RyanR in dantrolene-fed AβPP/PS1 mice actually attenuated Aβ plaque deposition and promoted synaptic loss in these mice [62]. Moreover, the up-regulation of RyanR type 3 is protective in cultured primary cortical neurons from TgCRND8 mice [53] and upregulation of RyanR2 maintains synaptic function in 3XTg-AD mice [46]. Therefore, efficacious targeting of ER Ca^{2+} signaling may require further understanding of these changes and sophisticated drug design to achieve the required specificity.

MITOCHONDRIAL DYSFUNCTION IN ALZHEIMER'S DISEASE

Mitochondria (mt) are dynamic ATP-generating organelles which contribute to many cellular functions

including intracellular Ca^{2+} regulation, alteration of reduction-oxidation potential of cells, free radical scavenging and activation of caspase-mediated programmed cell death (Fig. 1). ATP generation is accomplished through oxidative phosphorylation and because the activity of rate-limiting mitochondrial dehydrogenases (pyruvate, isocitrate, oxoglutarate) located in the inner mitochondrial matrix (IMM) are regulated by Ca^{2+}, increases in mitochondrial Ca^{2+} correlate to enhanced ATP generation [63]. The Ca^{2+} dependency of mitochondrial bioenergetics enables mt to decode Ca^{2+} signals and thus, to tune ATP synthesis to the energetic requirements of cells, including neurons [64]. The driving force for Ca^{2+} entry into the mt is the mitochondrial membrane potential (ψ) and Ca^{2+} can be taken up by the low affinity mitochondrial Ca^{2+} uniporter MCU) on the IMM [65]. Ca^{2+} equilibrium is maintained along the IMM by sodium or hydrogen/calcium exchangers (N/HCX). If intramitochondrial Ca^{2+} levels become too high, the ψ can collapse and cause the opening of the mitochondrial permeability transition pore (mtPTP), which allows efflux of Ca^{2+} with high conductance from the IMM lumen and is often a trigger for the cell death signaling cascade [66], though brief openings could serve as a rapid Ca^{2+} release mechanism [67]. The alterations to neuronal Ca^{2+} homeostasis in AD can therefore negatively affect mitochondrial Ca^{2+} signaling (or vice versa), trigger mitochondrial dysfunction and ultimately compromise neuronal function and health.

Mt can participate in intracellular Ca^{2+} signaling in several capacities. They can buffer changes to local [Ca^{2+}] near the plasma membrane or the ER, enhance or decrease Ca^{2+} flux and modulate the frequency of Ca^{2+} oscillations in many cell types [66]. In neurons, mt Ca^{2+} uptake occurs in presynaptic terminals and during periods of high Ca^{2+} activity such as epileptiform discharges or excitotoxic episodes [68–70]. Neurons employ mt Ca^{2+} efflux mechanisms to shape cytoplasmic Ca^{2+} kinetics in response to intense electrical stimulation by slowly releasing the accumulated Ca^{2+} [71] or by allowing the efficient refilling of the ER and therefore modulating Ca^{2+} oscillations [72]. It was demonstrated recently using mitochondrial-targeted ratiometric pericam (2 mtRP) to monitor mitochondrial Ca^{2+} transients that hippocamal mt located at the synapse were more sensitive to synaptic activation compared to mt located in the soma and that the mitochondrial Ca^{2+} transients were independent of ER cross talk [73]. Given the involvement of mt in intraneuronal Ca^{2+} signaling it is conceivable then that alterations in mitochondrial Ca^{2+} handling could contribute to Ca^{2+} dysregulation in AD. The extent to which this occurs and contributes to AD pathogenesis is currently not known. One notable observation is that non-steroidal anti-inflammatory drugs (NSAIDs) have the ability to reduce mitochondrial Ca^{2+} uptake, which may account for their apparent benefits in warding off AD [74].

Recently it has been demonstrated that certain aspects of AD pathology can significantly alter mitochondrial Ca^{2+} signaling and trigger mitochondrial dysfunction in experimental models. The Aβ peptide has been shown to inhibit mitochondrial respiration [75] and in the presence of Ca^{2+} cause the opening of the mtPTP in isolated mt [76]. Cyclophilin D (CypD) is a mt protein located in the IMM lumen that associates with the mtPTP and regulates its open probability [67]. Recently, it has been shown *in vitro* that Aβ oligomers target and form a complex with CypD, resulting in increased vulnerability to mtPTP opening [77]. Moreover, mt from CypD knock-out mice were insensitive to cyclosporine A, a strong inhibitor of Ca^{2+}-induced mtPTP opening, and displayed a higher Ca^{2+} threshold than wild-type mt [77]. Interestingly, Aβ-induced alterations to long-term potentiation were attenuated in CypD knock-out mice compared to wild-type, which provided further evidence that potentially links mitochondrial Ca^{2+} signaling to neuronal dysfunction in AD. Aβ42 oligomers can indirectly alter mitochondrial Ca^{2+} signaling by inducing massive Ca^{2+} entry into neurons and promote mitochondrial Ca^{2+} overload [74], which lead to opening of the mtPTP, ψ collapse, cytochrome c release, apoptosis and cell death. Depolarization of mt by a series of NSAIDS (including salicylate, sulindac sulphide, indomethacin, ibuprofen and R-flurbiprofen) inhibited mitochondrial Ca^{2+} overload, release of cytochrome c and cell death induced by Aβ [74]. Taken together, these findings suggest that mitochondrial Ca^{2+} signalling could be compromised and play a significant role in AD pathogenesis.

There is extensive data to support an obligatory path to mitochondrial damage and dysfunction in AD, possibly triggered by dysregulated Ca^{2+} signalling, and that these changes can occur early in disease progression. Increased cytosolic cytochrome c oxidase, increased oxidative stress markers and reduced energy metabolism have been described in the brain of AD patients prior to Aβ plaque formation [78,79]. Electron microscopy studies of mt in various regions of AD brain showed significant morphological changes, such as reduced IMM cristae size [80]. Mitochondrial dynamics, such as fusion, fission and motility, may al-

so be affected in AD. Neuronal cells treated with conditioned medium from cells expressing mutant APP lead to increased mitochondrial fission, loss of dendritic spines and cell death [81]. The increased mitochondrial fission observed was mediated by elevated levels of S-nitrosylated dynamin-like protein 1 (SNO-Drp1). Drp1 is a cytosolic protein recruited to mitochondria during fission [82] and SNO-Drp1 is suggested to have increased fission activity due to enhanced dimerization. Increased SNO-Drp1 protein levels were found in brain from AD patients and AD mouse models [81]. In contrast, fibroblasts from sporadic AD patients expressed lower levels of Drp1 and displayed elongated mt [83]. However, the same group found that neuroblastoma cells (M17) over-expressing APP had predominately fragmented mt, decreased levels of Drp1 a defect in neuronal differentiation [84]. Furthermore, M17 cells exposed to oligomeric Aβ diffusible ligands displayed mitochondrial fragmentation and loss of dendritic spine and PSD95 density, which was reversed by Drp1 overexpression [85]. Decreased levels of other mitochondrial enzymes, namely pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase complex, have also been reported in AD brain [86]. Changes in the mRNA expression of mt-encoded genes involved in oxidative phosphorylation were also confirmed in AD brain. The down-regulation of complex I and up-regulation of complex III and IV genes suggested a demand on energy production in brain tissue from early and definite cases of AD [87], which is interpreted as compensatory to the decrease in mt numbers. Oxidative stress was reported in mt of brain, platelets and fibroblasts from AD patients [87,88]. It is clear that mitochondrial dysfunction and oxidative stress are pathological changes observed in AD patients and that mitochondria constitute an attractive therapeutic target for developing AD treatment [89].

Additional support for potential role of mt in AD pathogenesis has been provided by recent genetic analysis of LOAD patients. Several groups demonstrated that polymorphisms in the intron region of the *TOMM40* gene (translocase of outer mitochondrial membrane 40 homolog) protein correlate with probability of developing AD [28,90–92]. The *TOMM40* gene is located on chromosome 19 in the immediate proximity of *APOE* gene, and *TOMM40* and *APOE* genes are in the linkage disequilibrium with each other. Some investigators argued that apparent linkage with TOMM40 can be explained by proximity to APOE allele [93] and that the main effect of polymorphism in *TOMM40* gene is to affect APOE expression levels [94,95]. It is also feasible that that polymorphism in *TOMM40* gene affect expression levels or function of TOMM40 itself. The function of TOMM40 is to mediate protein transport into mt [96] and mitochondrial import complex has been implicated in trafficking APP and Aβ into mt [97,98]. It remains to be determined if TOMM40 polymorphisms affect mtPTP or the ability of mt to handle Ca^{2+} load.

THE CONVERGENCE OF DYSREGULATED Ca^{2+} AND MITOCHONDRIAL DYSFUNCTION IN ALZHEIMER'S DISEASE

The control of intraneuronal Ca^{2+} signaling and neuronal homeostasis requires the participation of both the ER and mt as their functions are interdependent and crucial for neuronal function. Evidence in the literature suggests that the ER and mt are both functionally and structurally coupled [99], therefore it would be logical to hypothesize that Ca^{2+} disturbances in one organelle would affect Ca^{2+} signaling and potentially alter the function of the other. The ER and mt are physically linked by ER-mitochondria-associated membranes (MAM) [100,101] and MAM were initially thought to function as a compartment for the synthesis and transfer of phospholipids between the two organelles [102]. In addition to lipid metabolism, MAM are a site for exchange of Ca^{2+} ions [100,101]. MAMs are enriched in chaperones that stabilize the association between the ER and mt membranes and prolongs Ca^{2+} signaling mediated by the IP_3R type 3 receptor [103]. The mitochondrial chaperone glucose-regulated protein 75 (grp75) regulates IP_3R mediated mt Ca^{2+} signaling by stabilizing the physical interaction of VDAC isoform 1 to IP_3R [104]. These data suggest that under normal circumstances ER and mt Ca^{2+} signaling are closely linked. It would be interesting to determine how MAM function is compromised in aged neurons and in AD.

It was recently discovered that MAM carefully isolated from mouse brain were highly enriched with PSEN1/2 protein, along with the other components of the γ-secretase complex; APH1, nicastrin and presenilin enhancer protein 2 [105]. Moreover, γ-secretase activity was enhanced in the MAM fraction. Studies in the past have localized PSENs and the γ-secretase complex to many subcellular organelles, including the mt [106]. The divergent results regarding PSEN localization to organelles other than the ER could be attributed to the technical difficulty of subcellular fractionation and a lack of accurate MAM antigenic mark-

ers [105]. However, the possibility that the γ-secretase complex may reside at the ER/mt interface could explain how Aβ peptides could be transported to and accumulate in mt [98] and offer a physical coupling of Aβ generation and mitochondrial dysfunction in AD. As discussed above, in addition to γ-secretase PSENs also function as ER Ca^{2+} leak channels [44]. One of the functions of MAM is to mediate ER-mt Ca^{2+} transport [100,101] and it is possible that PSEN FAD mutations may have significant effects on local Ca^{2+} leak rates within MAM domain of the ER, leading to impaired Ca^{2+} coupling between ER and mt.

Many questions remain regarding the interplay between ER and mt and their contribution to dysregulated intracellular Ca^{2+} in AD. How would mutations in PSEN affect this functional and physical coupling? Does the compromised Ca^{2+} leak function of mutant PSEN affect mitochondrial Ca^{2+} uptake at MAM? If so, how? Are the proteins essential for the association of ER and mt compromised in AD? Are other Ca^{2+} handling proteins, such as RyanR and SERCA, important for ER to mitochondrial Ca^{2+} transport? Is Ca^{2+} transport unidirectional? Further studies of MAM, in cellular and animal AD models and using tissue from AD patients, are required to determine their functional significance in AD pathogenesis. However, it is clear that the functional coupling of ER and mitochondrial Ca^{2+} handling could link the effects of dysregulated intracellular Ca^{2+} signaling to Aβ generation and mitochondrial dysfunction in AD. Drugs designed to modulate ER and mitochondrial Ca^{2+} signaling could increase the chances of efficacy as they would have the potential to modify many aspects of AD pathology.

Ca^{2+} BLOCKERS AND MITOCHONDRIAL STABILIZERS AS POTENTIAL ALZHEIMER'S DISEASE THERAPEUTICS

The experimental results discussed above lead to the conclusion that Ca^{2+} blockers and mitochondrial stabilizers are potential AD treatments. Similar conclusions have been reached for other neurodegenerative disorders [11,89]. However, there are only a few drugs targeting these pathways that have been evaluated in AD clinical trials so far. Memantine is a non-competitive NMDAR inhibitor which is already approved by FDA for AD treatment and sold under brand name Namenda. Potentially more specific NMDAR inhibitors such as nitromemantines can be developed [107]. Evotec Inc has developed orally-active NR2B subtype selective NMDA antagonists, EVT101 and EVT103. EVT101 has been determined to be safe based on Phase I trial sponsored by Evotec (NCT00526968). In collaboration with Roche, Evotec is developing EVT101 for treatment-resistant depression and Phase II trial for this condition is planned. EVT101 and EVT103 are also very promising candidates for treatment of AD. Nimodipine, a dihydropyridine derivative and L-VGCC antagonist, has beneficial effects in AD patients and slows the progression of the disease [108]. The L-VGCC inhibitor MEM-1003 with better brain permeability has been developed by Memory Pharmaceuticals and tested in Phase II AD clinical trial (NCT00257673), but the patients failed to show significant improvement in cognitive function after 12 weeks of treatment and development of MEM-1003 for AD has been discontinued. Another L-type VGCC antagonist isradipine (Dynacirc CR) is being tested in on-going Phase II clinical trial in PD (NCT00753636) and may have potential utility for treatment of AD as well.

Perhaps the most promising and also most controversial compound from this class is Dimebon (Latrepirdine). Dimebon is a drug that has been developed and used as an antihistamine in Russia since 1983. Recently Dimebon has been proposed to be useful for treating neurodegenerative disorders [109] and licensed by Medivation for this application. Dimebon demonstrated significant positive effects in six-month randomized, double-blinded, placebo-controlled Phase II trial of 183 patients with mild to moderate Alzheimer's disease (AD) sponsored by Medivation and conducted in Russia (NCT00377715). At conclusion of the trial it was reported that after 12 weeks of taking Dimebon, patients significantly improved over baseline for ADAS-cog score (mean drug-placebo difference -4.0; $p < 0.0001$) [110]. More recently Phase II trial for cognitive effects in Huntington's disease was completed in USA (NCT00497159). The results of the study with 91 patients has been recently reported – after 90 days treatment with Dimebon there was no significant difference in ADAS-cog, but there was an increase in MMSE score (0.97 points difference, $p = 0.03$) for the treatment group at conclusion of the trial [111]. The large Phase III 26 weeks long clinical trial of Dimebon in AD patients sponsored by Medivation and Pfizer has been recently completed (NCT00838110, CONNECTION trial) and the results were disappointing as treatment with Dimebon did not significantly improve ADAS-cog ($p = 0.86$) or meet any other primary or secondary efficacy endpoints (http://investors.medivation.com/releasedetail.cfm?

ReleaseID=448818). The Phase III study of Dimebon in Huntington's disease (NCT00920946, HORIZON trial) sponsored by Medivation and Pfizer is currently recruiting. It is not clear at the moment if development of Dimebon for AD and HD by Medivation and Pfizer will be continued.

Perhaps the disappointing outcomes of the clinical trials involving Dimebon in AD and HD are a result of the fact that the mechanisms responsible for the postulated beneficial actions of Dimebon have never been clarified. It has been initially suggested that Dimebon may act as an inhibitor of NMDA receptors [112], blocker of voltage-gated Ca^{2+} channels [113] or as a blocker of the mitochondrial permeability transition pore [114]. However, these initial experiments were performed with very high concentrations of Dimebon. For example, concentrations as high as 50 μM were required to inhibit mtPTP opening of isolated mitochondria [114]. Nevertheless, these potential targets indicated that Dimebon may act by stabilizing neuronal Ca^{2+} signaling and mtPTP opening. More recent evaluation of Dimebon in experiments with primary neuronal cultures from an HD mouse model demonstrated that concentrations of at least 10 μM were required to inhibit VGCC and NMDAR [115]. At least 50 μM of Dimebon was needed to exert neuroprotective effects in glutamate excitotoxicity model [115]. The concentrations of Dimebon needed to affect Ca^{2+} signaling and mt in all published reports [112–115] were at least 10 μM, which is far above physiological range. In search for more physiologically relevant targets of Dimebon, an unbiased screen was performed [115]. It was discovered that Dimebon very potently inhibits α_{1B} adrenergic receptors, histamine H1 receptors and serotonin 5-HT6 receptors, as well as number of additional receptors [115]. These findings were recently confirmed in an independent study [116]. It is most likely that some cognitive effects of Dimebon observed in AD and HD clinical trials [110,111] are due to interaction with these receptors. In particular, an ability of Dimebon to inhibit 5-HT6 serotonin receptors with high affinity (K_i = 34 nM) [116] is of interest. Serotonin 5-HT6 receptors are known targets for cognitive enhancement which has been previously considered for AD treatment [117]. A recently published evaluation of Dimebon in animal model confirmed the ability of Dimebon to interact with 5-HT6 receptors *in vivo* and to exert acute behavioral effects similar to specific 5-HT6 receptor antagonist SB-399885 [118]. These studies support the hypothesis that cognitive effects of Dimebon are most likely due to its ability to inhibit 5-HT6 serotonin receptors. In addition, other potential effects of Dimebon such as its effects on amyloid metabolism [119] and protein aggregation [120] may have contributed to some of the results observed in the clinic.

CONCLUSION

There is much evidence to suggest that dysregulated Ca^{2+} signaling and mitochondrial dysfunction play a significant role in pathogenesis of AD. Evidence suggests that the various Ca^{2+} handling channels and pumps in the ER are prominent contributors to the alterations of intracellular Ca^{2+} signaling in AD. ER Ca^{2+} levels are increased in ageing neurons. Many AD-causing mutations in PSENs results in Ca^{2+} overload due to impaired ER leak function. Extracellular Aβ oligomers form Ca^{2+}-permeable pores and destabilize neuronal Ca^{2+} signaling. Supranormnal cytosolic Ca^{2+} signals lead to activation of Ca^{2+}-dependent phosphatase calcineurin, Ca^{2+}-dependent protease calpain and changes in synaptic structure and function. Elevated Ca^{2+} signals lead to impaired mitochondrial function and eventually to cell death. Ca^{2+} and mitochondrial inhibitors and stabilizers have utility for AD treatment. Some of these compounds are already being evaluated in AD clinical trials. Additional compounds with increased potency and specificity need to be developed in the future.

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