



## Review

# Modulation of mitochondrial calcium as a pharmacological target for Alzheimer's disease

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## ABSTRACT

Perturbed neuronal calcium homeostasis is a prominent feature in Alzheimer's disease (AD). Mitochondria accumulate calcium ions ( $\text{Ca}^{2+}$ ) for cellular bioenergetic metabolism and suppression of mitochondrial motility within the cell. Excessive  $\text{Ca}^{2+}$  uptake into mitochondria often leads to mitochondrial membrane permeabilization and induction of apoptosis.  $\text{Ca}^{2+}$  is an interesting second messenger which can initiate both cellular life and death pathways in mitochondria. This review critically discusses the potential of manipulating mitochondrial  $\text{Ca}^{2+}$  concentrations as a novel therapeutic opportunity for treating AD. This review also highlights the neuroprotective role of a number of currently available agents that modulate different mitochondrial  $\text{Ca}^{2+}$  transport pathways. It is reasoned that these mitochondrial  $\text{Ca}^{2+}$  modulators are most effective in combination with agents that increase the  $\text{Ca}^{2+}$  buffering capacity of mitochondria. Modulation of mitochondrial  $\text{Ca}^{2+}$  handling is a potential pharmacological target for future development of AD treatments.

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## 1. Introduction

As the average life span of human population gradually increases, the prevalence of age-related diseases has significantly increased. Alzheimer's disease (AD) is a fatal neurodegenerative disorder, affecting approximately 35.6 million people worldwide (Prince and Jackson, 2009). AD is the most common form of dementia. The disease is characterized by progressive synaptic dysfunction and neuronal loss in various brain regions, especially in the cortex and hippocampus. Severe neurodegeneration in these brain regions results in cognitive, emotion, social and motor impairments. With more than a 100 years of research, the underlying mechanism of this incurable disease still remains elusive. Perturbed neuronal calcium ( $\text{Ca}^{2+}$ ) homeostasis is a common feature in many neurodegenerative diseases including AD, amyotrophic lateral sclerosis (ALS), ischemic stroke and Parkinson's disease (PD) (Mattson and Chan, 2003). Increasing lines of evidence support the idea that  $\text{Ca}^{2+}$  dysregulation plays a key role in AD pathogenesis

(Bezprozvanny, 2009; Bojarski et al., 2008; LaFerla, 2002; Mattson and Chan, 2003; Yu et al., 2009).

## 2. Neuronal $\text{Ca}^{2+}$ dysregulation and Alzheimer's disease

$\text{Ca}^{2+}$  signaling is essential for life and death processes including neuronal excitability, synaptic plasticity, gene transcription and apoptosis (Berridge, 1998; Berridge et al., 1998). The  $\text{Ca}^{2+}$  dysregulation hypothesis postulates that sustained increase in cytosolic  $\text{Ca}^{2+}$  concentrations can lead to neurodegeneration in AD (Khachaturian, 1994; Toescu and Verkhratsky, 2007). Disturbances in  $\text{Ca}^{2+}$  signaling have been found in both sporadic and familial cases of AD (LaFerla, 2002). Several age-related perturbations in pathways regulating  $\text{Ca}^{2+}$  homeostasis have been reported, suggesting a possible linkage between aging and the development of sporadic AD (Bezprozvanny, 2009). A small proportion of AD patients (~5%) suffer from an early-onset familial form that occurs under age of 65 (Hardy, 2006). The genes involved in familial AD include presenilins (presenilin 1 and 2) and amyloid precursor protein (APP) (Hardy and Gwinn-Hardy, 1998). Both have been shown to play important roles in  $\text{Ca}^{2+}$  signaling (LaFerla, 2002). The mechanisms of how  $\text{Ca}^{2+}$  homeostasis is disrupted in AD have been extensively reviewed (Bezprozvanny, 2009; Bojarski et al., 2008; LaFerla, 2002; Mattson and Chan, 2003; Yu et al., 2009). In the fol-

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lowing sections, we will briefly discuss this issue for readers to understand how  $\text{Ca}^{2+}$  dyshomeostasis is linked with AD.

### 2.1. APP mutation induces $\text{Ca}^{2+}$ influx and elevates cytosolic $\text{Ca}^{2+}$ concentrations

Accumulation of senile plaques and neurofibrillary tangles are two important pathological hallmarks in AD brains. Senile plaques are made of beta-amyloid ( $\text{A}\beta$ ) peptides which are derived from APP. Mutations associated with familial AD result in increased production of the amyloidogenic  $\text{A}\beta$  fragments (Mattson, 1997). APP derivatives such as secreted forms of APP (sAPP),  $\text{A}\beta$ -containing fragments, and APP intracellular domain (AICD) have been shown to modulate cellular  $\text{Ca}^{2+}$  signaling (Leissring et al., 2002; Mattson et al., 1993, 1992).  $\text{A}\beta$  aggregates have been found to form cation-selective ion channels in the plasma membrane, resulting in increased cytosolic  $\text{Ca}^{2+}$  concentrations (Arispe et al., 1993a,b; Kagan et al., 2002). Nevertheless, how  $\text{A}\beta$ -induced membrane pores are related to human AD is still unclear. Oxidative damage is another mechanism by which  $\text{A}\beta$  causes disruption in  $\text{Ca}^{2+}$  homeostasis and neurotoxicity (Hensley et al., 1994; LaFerla, 2002). Accumulation of  $\text{A}\beta$  leads to formation of reactive oxygen species (ROS), which promotes DNA damage, lipid peroxidation, protein carbonylation and nitrosylation. Lipid peroxidation modifies functions of membrane transporters and ion channels (Mark et al., 1995), which in turn further elevates basal cytosolic  $\text{Ca}^{2+}$  concentrations, forming a vicious cycle (LaFerla, 2002; Mattson and Chan, 2003).

### 2.2. Presenilins modulate ER $\text{Ca}^{2+}$ signaling and enhance ER $\text{Ca}^{2+}$ release

Presenilins (PS1 and PS2) are components of the  $\gamma$ -secretase complex which are involved in the proteolytic cleavage of APP. PS1 and PS2 are located in various intracellular compartments such as the endoplasmic reticulum (ER) (Annaert et al., 1999), Golgi apparatus (Annaert et al., 1999), and mitochondria (Ankarcrona and Hultenby, 2002). Notably, presenilins are highly enriched in a specific region where the ER membranes are in close contact with mitochondria namely the ER-mitochondrial-associated membranes (MAM) (Area-Gomez et al., 2009).

FAD-linked presenilin mutations are believed to alter the activity of  $\gamma$ -secretase such that more  $\text{A}\beta$  are produced, especially the fibrillogenic  $\text{A}\beta_{1-42}$  peptides (Xia et al., 1997). FAD-related mutant presenilins can also affect ER  $\text{Ca}^{2+}$  handling independent of  $\text{A}\beta$  by exaggerating  $\text{Ca}^{2+}$  release from the ER in response to agonist stimulation. FAD mutant PS1 and PS2 have been shown to interact with the inositol 1,4,5-triphosphate receptor ( $\text{InsP}_3\text{R}$ )  $\text{Ca}^{2+}$ -releasing channels and enhance their gating activity by a gain-of-function effect (Cheung et al., 2010, 2008).  $\text{InsP}_3\text{Rs}$  are more likely to be in a high-probability burst mode, resulting in enhanced ER  $\text{Ca}^{2+}$  release (Cheung et al., 2010). However the molecular mechanism of this modulation remains elusive.

Depletion of ER  $\text{Ca}^{2+}$  store triggers  $\text{Ca}^{2+}$  influx from extracellular space via store-operated  $\text{Ca}^{2+}$  channels (Putney, 1986). This is known as capacitive  $\text{Ca}^{2+}$  entry (CCE or store-operated  $\text{Ca}^{2+}$  entry). Stromal interacting molecule 1 (STIM1) protein acts as  $\text{Ca}^{2+}$ -sensors on the ER which interacts with Orai1/TRPC channels in the plasma membrane and activates store-operated channels for  $\text{Ca}^{2+}$  entry (Ong et al., 2007; Zhang et al., 2005). CCE has been shown to be attenuated by presenilin mutants, possibly due to increased  $\text{Ca}^{2+}$  in the ER store (Herms et al., 2003; Leissring et al., 2000; Yoo et al., 2000). Moreover, increased levels of STIM1 have been found in mouse embryonic fibroblasts lacking presenilins, implicating that expression of STIM1 may be presenilin-dependent (Bojarski et al., 2009).

### 2.3. $\text{Ca}^{2+}$ -dependent tau phosphorylation and dephosphorylation

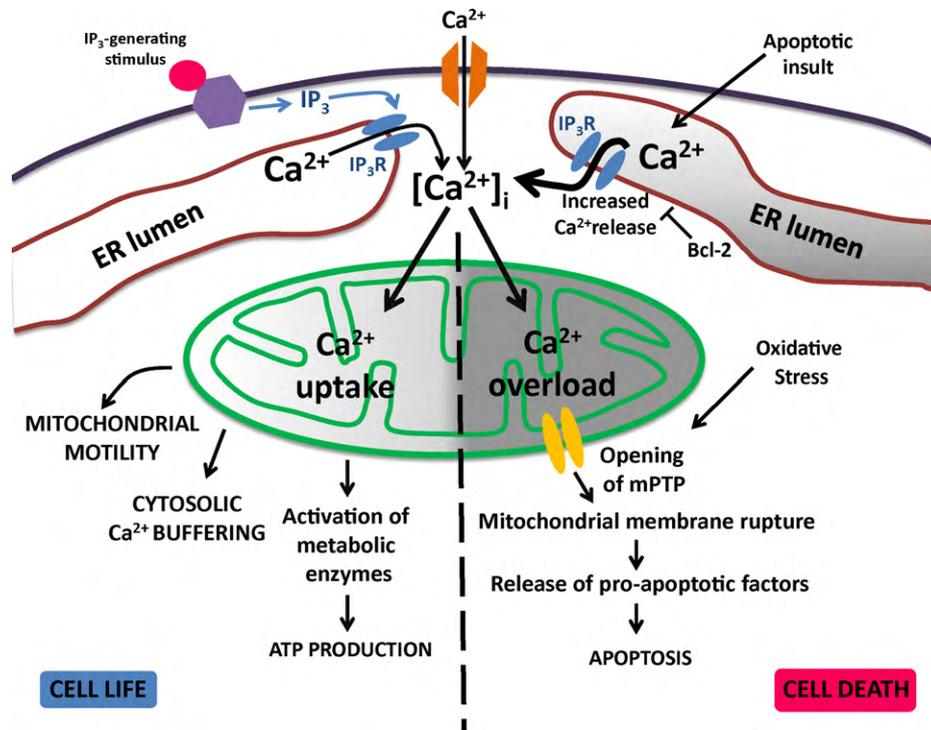
Neurofibrillary tangles formed by hyperphosphorylation of the microtubule-associated protein tau are another hallmark in AD. The phosphorylation state of tau is highly  $\text{Ca}^{2+}$ -dependent. Tau phosphorylation is regulated by  $\text{Ca}^{2+}$ -dependent calmodulin-dependent protein kinase II (CaMKII) and calpain (Litersky et al., 1996; Maccioni et al., 2001). Activation of cyclin-dependent protein kinase 5 (Cdk5) by calpain via p25 has been suggested to play a role in tau hyperphosphorylation (Maccioni et al., 2001). On the other hand, calcineurin, a  $\text{Ca}^{2+}$ /calmodulin-dependent protein phosphatase is involved in tau dephosphorylation (Fleming and Johnson, 1995). Tau dephosphorylation was completely attenuated in rat cerebral-cortical slice pre-treated with the calcineurin inhibitor Cyclosporin A (Fleming and Johnson, 1995). Injection of FK506 (a calcineurin inhibitor) has been reported to enhance tau phosphorylation at various phosphorylation sites in mouse brain (Luo et al., 2008). On the other hand, calcineurin inhibitors have also been shown to increase phosphorylation of glycogen synthase kinase-3 beta (GSK-3 $\beta$ ) at serine-9 (Kim et al., 2009). Phosphorylation of GSK-3 $\beta$  at serine-9 inhibits tau phosphorylation by GSK-3 $\beta$  (Hughes et al., 1993). Hence, both increase and decrease cytosolic  $\text{Ca}^{2+}$  concentrations contribute to tau phosphorylation, therefore perturbed  $\text{Ca}^{2+}$  homeostasis may associate with the tau pathology in AD.

### 2.4. Sporadic AD: ApoE4 and CALHM1

Apolipoprotein E is involved in transporting cholesterol from the blood to the cells. Individuals with the allele for the E4 isoform of apolipoprotein E (ApoE4) have an increased risk of sporadic AD (Mahley et al., 2006). ApoE 4 was found to disrupt  $\text{Ca}^{2+}$  homeostasis by triggering extracellular  $\text{Ca}^{2+}$  influx and amplifying neuronal  $\text{Ca}^{2+}$  responses (Hartmann et al., 1994; Tolar et al., 1999). Recent research has identified polymorphism of a gene called calcium homeostasis modulator 1 (CALHM1) that may link with sporadic AD. CALHM1 encodes for a protein which forms a  $\text{Ca}^{2+}$  channel on the plasma membrane and controls  $\text{A}\beta$  levels (Dreses-Werringloer et al., 2008). Since then several studies have shown that the P86L polymorphism of CALHM1 is associated with AD (Boada et al., 2010; Cui et al., 2010), whilst other studies failed to find a link between CALHM1 and risk of AD (Bertram et al., 2008; Minster et al., 2009; Nacmias et al., 2010; Slegers et al., 2009). The relevance of CALHM1 in AD remains unclear.

### 2.5. Current " $\text{Ca}^{2+}$ -targeted" drugs

As illustrated above, it is clear that  $\text{Ca}^{2+}$  signaling pathways are highly involved in AD pathogenesis. Several FAD-approved drugs and drugs tested in clinical trials therefore aim to target different  $\text{Ca}^{2+}$  signaling pathways in order to re-establish the cytosolic  $\text{Ca}^{2+}$  homeostasis. Memantine (Namenda) is the most common drug for moderate to severe AD. Memantine is a non-competitive N-methyl D-aspartate (NMDA) antagonist. It inhibits  $\text{Ca}^{2+}$  entry into neurons through the NMDA receptors and therefore reduces excitotoxicity (Bezprozvanny, 2009). However, currently it only provides limited benefits for AD patients. Hu et al. (2009) found that specific antagonists targeting at NMDA receptors containing the GluN2B subunit e.g. ifenprodil and Ro 25-6981, might be effective in protecting neurons from  $\text{A}\beta$ -induced inhibition of synaptic plasticity *in vivo*. EVT-101 (Evotec AG, Hamburg, Germany; <http://www.evotec.com/>) is a newly developed NMDA receptor subunit 2B specific antagonist. Phase I trial of EVT-101 is completed and cognitive performance of patients was improved (NCT00526968). This specific NMDA receptor antagonist is believed to greatly reduce the chance of



**Fig. 1.** Life and death pathways of mitochondrial  $\text{Ca}^{2+}$  accumulation. Left: Under normal conditions,  $\text{Ca}^{2+}$  influx from extracellular matrix or  $\text{Ca}^{2+}$  release from the ER causes increase in cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ). Mitochondria rapidly take up cytosolic  $\text{Ca}^{2+}$ , which is crucial for life processes such as mitochondrial movement,  $\text{Ca}^{2+}$  homeostasis and bioenergetic metabolism. Right: When mitochondria are overloaded with  $\text{Ca}^{2+}$ , mitochondrial permeability transition pores will be triggered to open. Several pro-apoptotic factors will be released to the cytosol, thereby inducing apoptosis.

side effects caused by the unspecific NMDAR antagonist memantine.

Nimodipine is an isopropyl  $\text{Ca}^{2+}$  channel blocker which has been shown to improve cognitive performance of dementia patients including AD (Lopez-Arrieta and Birks, 2002). MEM-1003 (Memory Pharmaceuticals, Montvale, New Jersey, USA; <http://www.Memrypharma.com/>) is a nimodipine-related neuronal L-type  $\text{Ca}^{2+}$  channel antagonist. Phase IIa clinical trial has recently been completed (NCT00257673), but failed to show significant improvements in patients (Hareyan, 2007). Evidence from NMDA receptor antagonists and  $\text{Ca}^{2+}$  channel blockers indicates that decreased  $\text{Ca}^{2+}$  flux into neurons may benefit AD patients.

Indeed, classic therapies which aim to compensate the level of acetylcholine in AD patients also cause alteration in  $\text{Ca}^{2+}$  homeostasis. FAD-approved acetylcholinesterase (AChE) inhibitors e.g. Donepezil, Galatamine, and Rivastigmine inhibit degradation of acetylcholine and therefore increase acetylcholine concentrations in the brain which is believed to associate with improvement in cognitive functions. In fact, the AChE inhibitors will cause an increase opening of acetylcholine receptors, which are receptor-activated  $\text{Ca}^{2+}$  channels themselves. The two major classes of FAD-approved AD drugs (NMDA receptor antagonists and AChE inhibitors) apparently will have opposite effects on cytosolic  $\text{Ca}^{2+}$  concentration, implying that there is evidence for both increased and decreased cytosolic  $\text{Ca}^{2+}$  in AD.

Dimebon (Latrepirdine) (Medivation Inc., San Francisco, CA) is an antihistamine drug used in Russia (Bachurin et al., 2001). Recent studies have discovered the novel role of Dimebon as a neuroprotective agent as well as a cognition-enhancing agent (Bachurin et al., 2001). As an antagonist of NMDAR and  $\text{Ca}^{2+}$  channels, Dimebon protects neurons by preventing NMDA and  $\text{Ca}^{2+}$ -induced neurotoxicity (Bachurin et al., 2001). On the other hand, it also increases the level of acetylcholine by inhibiting the AChE (Bachurin et al., 2001). Phase II clinical trial reported that Dimebon is well tolerated and exhibit significant improvements in patients with mild to moder-

ate AD (Doody et al., 2008). However, a recent Phase III clinical trial failed to show the same promising results (Neale, 2010). Additional Phase III clinical trials of Dimebon are still on-going at the moment; therefore the effectiveness of Dimebon in AD remains debatable.

Most of the current AD treatments such as AChE inhibitors can provide a one-time elevation of cognitive performance. However, the decline of cognitive ability from this elevated level will occur with the same speed as in non-treated patients. This urges researchers to seek for disease-modifying drugs.

### 3. Mitochondrial $\text{Ca}^{2+}$ governs neuronal life and death pathways

Mitochondria are important in maintaining neuronal  $\text{Ca}^{2+}$  homeostasis. Normal mitochondrial functions are extremely important for neurons, as neuronal activities such as synaptic transmission and axonal transport require high level of energy. In particular, mitochondrial  $\text{Ca}^{2+}$  levels are crucial for maintaining cellular functions including bioenergetic metabolism. On the other hand, excessive  $\text{Ca}^{2+}$  uptake into mitochondria results in rupture of the outer mitochondria membrane, which may then lead to initiation of apoptosis. However, this phenomenon is likely to occur only *in vitro*. The regulatory systems maintaining the mitochondrial  $\text{Ca}^{2+}$  homeostasis thus provide an attractive therapeutic target in treating AD. In the following sections we will explain how mitochondrial  $\text{Ca}^{2+}$  is involved in life and death pathways in the cell (Fig. 1), and how mitochondrial  $\text{Ca}^{2+}$  is linked to AD.

#### 3.1. The cell life pathway: physiological roles of mitochondrial $\text{Ca}^{2+}$ uptake

$\text{Ca}^{2+}$  uptake into mitochondria plays a key role in cellular ATP production and mitochondrial motility. Bioenergetic metabolism in mitochondria highly relies upon  $\text{Ca}^{2+}$ . In the mitochondrial matrix, activity of the metabolic enzymes involved in the Krebs

cycle (pyruvate,  $\alpha$ -ketoglutarate, and isocitrate dehydrogenases) is all  $\text{Ca}^{2+}$ -dependent (Rizzuto et al., 2000).  $\text{Ca}^{2+}$  directly regulates  $\alpha$ -ketoglutarate and isocitrate dehydrogenases, whilst pyruvate dehydrogenases are activated by  $\text{Ca}^{2+}$ -dependent phosphatases (Rizzuto et al., 2000).  $\text{Ca}^{2+}$  concentration in mitochondria therefore determines the rate of ATP synthesis for the cell.

Mitochondria are mobile organelles which travel along the axons to regions of increased energy need in the cell, such as synapses (Chang et al., 2006; Hollenbeck and Saxton, 2005). Microtubules-dependent mitochondrial motility is regulated by the kinesin1/Miro/Milton complex (Glater et al., 2006; Guo et al., 2005; Stowers et al., 2002). Miro (mitochondrial Rho GTPase) is a mitochondrial outer membrane protein. The activity of Miro is  $\text{Ca}^{2+}$ -dependent due to the presence of a pair of  $\text{Ca}^{2+}$ -binding EF hand motifs (Frederick et al., 2004). Milton is a cytoplasmic protein which binds with Miro to form a protein complex that links kinesin-1 to mitochondria for anterograde transport (Glater et al., 2006; Guo et al., 2005; Stowers et al., 2002). The  $\text{Ca}^{2+}$ -binding EF-hand domain of Miro is essential for  $\text{Ca}^{2+}$ -dependent mitochondrial movement. Elevated  $\text{Ca}^{2+}$  causes kinesin heavy chain to dissociate with microtubules, suppressing mitochondrial motility (Wang and Schwarz, 2009).  $\text{Ca}^{2+}$ -dependent mitochondrial motility is crucial for distribution of mitochondria in neurons. It recruits mitochondria to cellular regions with the need of ATP supply and  $\text{Ca}^{2+}$  buffering e.g. activated synapses (Macaskill et al., 2009).

In addition, Miro is essential for regulation of mitochondrial morphology. At resting low cytosolic  $\text{Ca}^{2+}$  levels, Miro facilitates the formation of elongated mitochondria by inhibiting dynamin-related protein 1 (Drp-1 or dynamin-like protein 1, DLP-1)-mediated fission (Saotome et al., 2008). On the other hand, high cytosolic  $\text{Ca}^{2+}$  triggers fragmentation and shortening of mitochondria (Saotome et al., 2008). Miro-mediated redistribution of mitochondria has also been shown to increase their ability to accumulate  $\text{Ca}^{2+}$  (Saotome et al., 2008). Evidence from the above studies demonstrates that Miro acts as a cytosolic  $\text{Ca}^{2+}$ -dependent regulator of mitochondrial dynamics. Meanwhile, calcineurin, a  $\text{Ca}^{2+}$ -dependent phosphatase, has been shown to regulate the translocation of cytosolic Drp-1 via dephosphorylation during fission (Cereghetti et al., 2008).

Clearly,  $\text{Ca}^{2+}$  regulates motility, distribution, morphology and functions of mitochondria in physiological conditions. It is therefore crucial to maintain mitochondrial  $\text{Ca}^{2+}$  homeostasis for normal cellular functioning. If this homeostasis is disrupted, a death signal can be resulted.

### 3.2. The cell death pathway: mitochondrial $\text{Ca}^{2+}$ overload triggers intrinsic apoptosis

The physiological  $\text{Ca}^{2+}$  signal can switch to a death signal when the  $\text{Ca}^{2+}$  level is beyond the threshold. Hence, excessive  $\text{Ca}^{2+}$  uptake into mitochondria can be lethal to neurons. The intrinsic (mitochondrial) pathway of apoptosis is triggered by intracellular stress, such as  $\text{Ca}^{2+}$  overload and oxidative stress (Galluzzi et al., 2009). Mitochondria integrate pro- and anti-apoptotic signals and determine the fate of the cell. If death signals predominate, mitochondrial-membrane-permeabilization (MMP) occurs, and large conductance permeability-transition-pores (PTP) opens (Galluzzi et al., 2009). PTP opening allows uncontrolled entry of solutes and water into the mitochondrial matrix by osmotic forces (Galluzzi et al., 2009). This causes mitochondria to swell and leads to rupture of the outer mitochondria membrane, releasing proteins from the intramembrane space e.g. cytochrome c into the cytosol (Galluzzi et al., 2009). MMP results in mitochondrial depolarization, uncoupling of oxidative phosphorylation, overproduction of ROS and release of pro-apoptotic proteins to the cytosol, eventually leading to cell death. When MMP is permanent and numerous mito-

chondria are continuously affected, neurons can no longer cope with the stress and apoptosis is initiated (Galluzzi et al., 2009). Physiological mitochondrial  $\text{Ca}^{2+}$  concentrations do not induce PTP opening, but will work in synergy with pro-apoptotic stimuli (Rizzuto et al., 2009). The “double hit” hypothesis proposes that apoptotic stimuli have dual targets (Pinton et al., 2008). On one hand, it causes  $\text{Ca}^{2+}$  release from the ER and subsequent  $\text{Ca}^{2+}$  uptake by mitochondria. On the other hand, it makes mitochondria more sensitive to potential  $\text{Ca}^{2+}$  damaging effects (Pinton et al., 2008).

The above pathways are summarized in Fig. 1. Given the dual roles of mitochondria  $\text{Ca}^{2+}$  in neurons, we will critically discuss the possibility of modulating  $\text{Ca}^{2+}$  in mitochondria as a potential pharmacological target for AD in this review.

## 4. Mitochondrial $\text{Ca}^{2+}$ handling and AD

Mitochondrial dysfunction is a prominent feature in AD.  $\text{A}\beta$  has been found in mitochondria of AD brain and transgenic mouse model of AD overexpressing  $\text{A}\beta$ .  $\text{A}\beta$  peptides accumulate in mitochondria and are associated with oxidative stress, disrupted  $\text{Ca}^{2+}$  homeostasis, impaired energy metabolism and induction of apoptosis (Mattson et al., 2008). Mitochondria from aged cerebellar granular neurons are depolarized and less efficient in handling  $\text{Ca}^{2+}$  load (Toescu and Verkhratsky, 2007). Cortical mitochondria from 12-month-old mice also show a reduced capacity for  $\text{Ca}^{2+}$  uptake when challenged with  $\text{CaCl}_2$  pulses, compared to that of 6-month-old mice (Du et al., 2008). Mitochondria isolated from fibroblasts of AD patients exhibit reduced  $\text{Ca}^{2+}$  uptake compared to age-matched control, suggesting that  $\text{Ca}^{2+}$  buffering ability may be impaired in the mitochondria of AD fibroblasts (Kumar et al., 1994). Following oxidative stress, the increase in  $\text{Ca}^{2+}$  uptake in mitochondria of AD fibroblasts is much greater than that in control, implicating that mitochondria from AD fibroblasts have a higher sensitivity towards oxidative stress (Kumar et al., 1994). Mitochondria with over-expression of human APP also show a lower  $\text{Ca}^{2+}$  capacity compared to non-transgenic mitochondria (Du et al., 2008).  $\text{A}\beta_{1-42}$  oligomer induces  $\text{Ca}^{2+}$  overload in mitochondria in both cortical and cerebellar granular neurons (Sanz-Blasco et al., 2008). The increase is limited to a pool of mitochondria close to the sites of  $\text{Ca}^{2+}$  entry and release (Sanz-Blasco et al., 2008).  $\text{Ca}^{2+}$  overload in mitochondria causes increased ROS production and impairment of bioenergetic metabolism which eventually leads to cell death. Mutations in presenilins may promote mitochondrial dysfunction by perturbing ER  $\text{Ca}^{2+}$  handling, which promotes synaptic mitochondrial  $\text{Ca}^{2+}$  overload and in turn triggers apoptosis. A recent study has also shown that mutated CALHM1 may cause slower kinetics of mitochondrial  $\text{Ca}^{2+}$  uptake and release, increasing the risk of mitochondrial  $\text{Ca}^{2+}$  overload (Moreno-Ortega et al., 2010).

The importance of mitochondrial  $\text{Ca}^{2+}$  in apoptosis has been emphasized in neuronal death in AD. However, mitochondrial  $\text{Ca}^{2+}$  is also important in earlier stages of the disease. The rupture of mitochondrial membrane caused by  $\text{Ca}^{2+}$  overload reduces the number of “healthy” mitochondria, and this will affect crucial neuronal functions including synaptic transmission and axonal transport. This could perhaps account for some of the early symptoms of the disease e.g. memory impairment. In this notion, the maintenance of mitochondrial  $\text{Ca}^{2+}$  homeostasis is important for both early and later stages of the disease. In the following paragraphs, we will illustrate different influx and efflux pathways regulating the mitochondrial  $\text{Ca}^{2+}$  homeostasis, and how different agents targeting these pathways can provide neuroprotection in AD.

## 5. Mitochondria in neuronal $\text{Ca}^{2+}$ signaling

$\text{Ca}^{2+}$  signaling causes transient changes in cytosolic  $\text{Ca}^{2+}$  concentration. Mitochondria rapidly take up  $\text{Ca}^{2+}$  when a physiological



dria. Hence, MAM also involves in intracellular communication and delivery of  $\text{Ca}^{2+}$  between the organelles. Outside the mitochondria, glucose-regulated protein 75 (grp75) mediates the interactions of VDAC and  $\text{IP}_3\text{R}$  on the ER membrane to regulate  $\text{Ca}^{2+}$  uptake into mitochondria (Szabadkai et al., 2006). The interaction of sigma-1 localizes on the MAM and grp 78 (BiP) is crucial in regulating the integrity between the ER and mitochondria (Hayashi and Su, 2007). A family of fission and fusion proteins regulating mitochondrial morphology is also important for maintaining ER-mitochondrial  $\text{Ca}^{2+}$  coupling. Genetic ablation of mitofusin 2 causes an increase in distance between the ER and mitochondria, resulting in less efficient mitochondrial  $\text{Ca}^{2+}$  uptake (de Brito and Scorrano, 2008). This provides genetic evidence supporting the  $\text{Ca}^{2+}$  microdomains theory, which proposes that mitochondria preferentially accumulate at “microdomains” of high  $\text{Ca}^{2+}$  concentrations (Rizzuto and Pozzan, 2006).  $\text{Ca}^{2+}$  microdomains refer to localized areas with increased cytosolic  $\text{Ca}^{2+}$  that does not generalize to the whole cell cytoplasm (Rizzuto and Pozzan, 2006). Microdomains are enriched in  $\text{IP}_3\text{Rs}$  and can be found between mitochondria and the cytosolic mouth of  $\text{Ca}^{2+}$  channels, localized either in the neighboring ER or in the plasma membrane (Rizzuto and Pozzan, 2006). These microdomains allow efficient  $\text{Ca}^{2+}$  uptake into mitochondria. Increased levels of  $\text{Ca}^{2+}$  in those contact points will then be rapidly diffused into other mitochondria.

## 6. Potential targets for mitochondrial $\text{Ca}^{2+}$ modulation

### 6.1. Modulating mitochondrial calcium uptake via VDAC to attenuate calcium overload

VDAC is highly permeable at low potentials (10 mV) (Shoshan-Barmatz and Gincel, 2003), and is relatively “closed” at higher potentials. VDAC can also be modulated by various proteins and cytosolic compounds, including Bcl-2 family of proteins (Shimizu et al., 2000, 1999; Vander Heiden et al., 2001), metabolic enzymes such as hexokinase (Pastorino and Hoek, 2008), and the cytoskeletal protein tubulin (Rostovtseva et al., 2008).

Minocycline is an antibiotic derived from tetracycline and is a potential therapeutic agent in various neurological diseases (Garcia-Martinez et al., 2010). It has been shown that minocycline can act as a modulator of VDAC (Garcia-Martinez et al., 2010). Minocycline reduces the conductance and voltage dependence state of VDAC (Garcia-Martinez et al., 2010). However, it is unclear if these modulations can reduce  $\text{Ca}^{2+}$  influx via VDAC.

### 6.2. Reduce mitochondrial $\text{Ca}^{2+}$ uptake by mitochondrial membrane depolarization to inhibit calcium overload

As mentioned earlier,  $\text{Ca}^{2+}$  entry to the mitochondria is highly dependent on  $\Delta\Psi_m$ . FCCP [carbonyl cyanide-p-(trifluoromethoxy) phenylhydrazone] is a protonophore and potent uncoupler of oxidative phosphorylation. It depolarizes the mitochondrial membrane and inhibits mitochondrial  $\text{Ca}^{2+}$  uptake. FCCP has been shown to inhibit mitochondrial  $\text{Ca}^{2+}$  elevation triggered by  $\text{A}\beta_{1-42}$  oligomers (Sanz-Blasco et al., 2008). FCCP-induced inhibition of mitochondrial  $\text{Ca}^{2+}$  uptake also attenuates both cytochrome c release and cell death without affecting cellular levels of ATP (Sanz-Blasco et al., 2008). These results suggest a possible neuroprotective mechanism against  $\text{A}\beta$ -induced neurotoxicity by depolarizing the mitochondrial membrane, thereby attenuating mitochondrial  $\text{Ca}^{2+}$  overload. Indeed, uncouplers such as FCCP and 2–4 dinitrophenols are dangerous drugs due to their high risk of intoxication. Allosteric modulators of uncoupling proteins would be a much safer alternative approach to induce pharmacological reduction of mitochondrial membrane potential.

An early report showing that patients suffering from rheumatoid arthritis has a low risk of developing AD leads to a hypothesis that there is chronic neuroinflammation in AD brains and anti-inflammatory agents maybe neuroprotective (McGeer et al., 1990). Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin have been shown to reduce the degree of cognitive decline in AD patients (Rogers et al., 1993). The effectiveness of NSAIDs in AD has been challenged by negative results from clinical trials (McGeer et al., 2006). Nevertheless, a recent study has shown a novel neuroprotective mechanism by NSAIDs. Salicylate, R-flurbiprofen and indomethacin induce depolarization of the mitochondrial membrane, which then reduce  $\text{Ca}^{2+}$  entry into mitochondria (Sanz-Blasco et al., 2008). However, a direct action of NSAIDs on mitochondrial membrane potential has not been well established. In addition, a recent Phase III clinical trial with R-flurbiprofen showed negative results to treat AD patients. The failure from using NSAIDs as an AD treatment suggest that a more specific but mild potent compound which modulates uncoupling proteins may be the future therapeutic target.

KB-R7943 is a selective inhibitor of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger. It causes depolarization of isolated brain mitochondria and reduces mitochondrial  $\text{Ca}^{2+}$  uptake (Storozhevsky et al., 2009). Furthermore, KB-R7943 has been shown to inhibit mitochondrial  $\text{Ca}^{2+}$  uptake in permeabilized HeLa cells (Santo-Domingo et al., 2007). However, the mechanism of how KB-R7943 induces depolarization is not clear (Storozhevsky et al., 2009). Similarly, minocycline has also been shown to induce depolarization of the mitochondrial membrane which reduces NMDA-induced  $\text{Ca}^{2+}$  overload in the mitochondria (Garcia-Martinez et al., 2010).

Taken together, depolarization of the mitochondrial membrane can be a possible way to inhibit  $\text{Ca}^{2+}$  uptake into mitochondria. By reducing  $\text{Ca}^{2+}$  entry, the risk of mitochondrial  $\text{Ca}^{2+}$  overload can be lowered. However, the underlying mechanism of the depolarizing effect by the above drugs is still awaited to be elucidated. A possible target would be the components of the ETC which regulate  $\Delta\Psi_m$ . Further studies on how the membrane is depolarized by the drugs are required.

### 6.3. Modulation of uniporter calcium uptake efficiency attenuates excessive calcium entry

$\Delta\Psi_m$  establishes a driving force for  $\text{Ca}^{2+}$  entering mitochondria via the uniporter on the IMM. The activity of uniporter is regulated by extra-mitochondrial  $\text{Ca}^{2+}$  (Kroner, 1986), and increase in cytosolic  $\text{Ca}^{2+}$  can both activate and inactivate mitochondrial  $\text{Ca}^{2+}$  uptake (Rizzuto et al., 2009). The uniporter is readily inhibited by Ruthenium Red and is also regulated by adenine nucleotides (Bernardi, 1999; Litsky and Pfeiffer, 1997) and plant flavonoids (Montero et al., 2004). Protein kinases are also important regulators of the uniporter. Treatment with SB202190, a specific inhibitor of  $\alpha$  and  $\beta$  isoforms of p38 mitogen-activated protein (MAP) kinase has been shown to increase the rate of  $\text{Ca}^{2+}$  uptake by mitochondria (Montero et al., 2002). The results suggest that p38 MAP kinase may inhibit the opening of uniporter. Protein kinase C has dual effects on  $\text{Ca}^{2+}$  uptake by the uniporter: while the  $\zeta$  isoform activates the uniporter, the  $\beta$  and  $\delta$  isoforms inactivate it. Taken the above reports together, uniporter on mitochondria can be modulated by numerous pharmacological interventions. Careful consideration has to be taken regarding the specificity of these interventions.

### 6.4. Inhibition of permeability transition pore opening to inhibit induction of apoptosis

Dimebon has been shown to inhibit the opening of PTP induced by  $\text{A}\beta_{25-35}$  (Bachurin et al., 2003). However, the mechanism of Dimebon is not specific to mitochondria (Bachurin et al., 2001). In

addition, it is not known whether inhibition of PTP opening would have any effect on mitochondrial  $\text{Ca}^{2+}$  homeostasis.

The abundance of CypD is associated with the vulnerability of the mitochondrial PTP to  $\text{Ca}^{2+}$  (Du et al., 2008). The immunosuppressant Cyclosporine A (CsA) binds to CypD and inhibits its translocation to the IMM and subsequent induction of PTP opening (Rizzuto et al., 2009). Pre-treatment of CsA has been shown to increase mitochondrial  $\text{Ca}^{2+}$  buffering capacity in wild type and mutant amyloid precursor protein (mAPP) transgenic mice (Du et al., 2008). Moreover, mitochondria isolated from CypD deficient mAPP mice have a higher  $\text{Ca}^{2+}$  uptake capacity than that of mAPP mice (Du et al., 2008). CypD deficient mitochondria are resistant to both  $\text{A}\beta$ - and  $\text{Ca}^{2+}$ -induced mitochondrial swelling and PTP opening (Du et al., 2008). This result shows that the absence of CypD protects neurons from  $\text{A}\beta$ -induced cell death. Blockade of CypD also improves learning and memory in AD mice (Du et al., 2008), implying that inhibition of CypD can be a potential therapeutic target for treatment of AD.

#### 6.5. Modifying calcium release from the ER to reduce calcium uptake into mitochondria

Bcl-2 family proteins can form ion channels in membranes (Minn et al., 1997; Schendel et al., 1998), and affect  $\text{Ca}^{2+}$  homeostasis in the ER and mitochondria. Over-expression of Bcl-2 causes an increase in  $\text{Ca}^{2+}$  leak and thereby reducing the amount of  $\text{Ca}^{2+}$  stored in the ER (Pinton et al., 2000). This in turn reduces the risk of  $\text{Ca}^{2+}$  overload in mitochondria, as there is less  $\text{Ca}^{2+}$  available for uptake. Agents reducing ER  $\text{Ca}^{2+}$  release may thus reduce the risk of mitochondrial  $\text{Ca}^{2+}$  overload.

#### 6.6. Enhancement of mitochondria activity as a drug target for AD

Mitochondrial defects are implicated in many neurodegenerative diseases including PD and AD. New therapeutic approaches have now begun to target mitochondria as a potential drug target (Chaturvedi and Beal, 2008). So far, we have mentioned different ways to reduce  $\text{Ca}^{2+}$  uptake in order to prevent excessive  $\text{Ca}^{2+}$  from entering mitochondria. As mitochondria act as  $\text{Ca}^{2+}$  buffers in the cell, a second approach to prevent  $\text{Ca}^{2+}$  overload is to increase the buffering capacity of mitochondria.

Agents such as Creatine protect neurons from glutamate- and  $\text{A}\beta$ -induced toxicity by providing energy reserves (Brewer and Wallimann, 2000). In PD animal models, antioxidants such as mitoQ (mitoquinone) and Coenzyme Q10 (CoQ10) selectively prevent mitochondrial oxidative damage (Chaturvedi and Beal, 2008). CoQ10 has also been shown to exhibit anti-amyloidogenic effects (Chaturvedi and Beal, 2008). These antioxidant agents may enhance the efficiency of ETC, hence results in better maintenance of mitochondrial membrane potential and therefore ATP production. Mitochondrial  $\text{Ca}^{2+}$  overload is not just dependent on mitochondrial  $\text{Ca}^{2+}$  concentration but may also depend on mitochondrial energy and redox state. These antioxidants may therefore indirectly increase the mitochondrial buffering capacity by indirectly preventing the induction of PTP opening through increased mitochondrial calcium. Taurine is another example that can increase the capacity of mitochondria to sequester  $\text{Ca}^{2+}$  when the cells are challenged by agents that cause an increase in cytosolic  $\text{Ca}^{2+}$  (El Idrissi, 2008). As all the proposed drug candidates above have not gone through Phase III clinical trials of PD or AD, their relevance to the diseases remains obscure.

#### 6.7. Tournefortic acid B

Tournefortic acid B (TAB) is a polyphenolic anti-oxidative compound extracted from *Tournefortia sarmentosa* Lam, which is widely

used as deoxidants and anti-inflammatory agents in Taiwan (Chi et al., 2008). TAB significantly decreases the  $\text{A}\beta_{25-35}$ -induced elevation of mitochondrial  $\text{Ca}^{2+}$  in cortical neurons (Chi et al., 2008). TAB also blocks the  $\text{A}\beta_{25-35}$ -induced cytochrome c release from mitochondria and the generation of mitochondrial protein tBid (Chi et al., 2008). The exact mechanism of how TAB attenuates mitochondrial  $\text{Ca}^{2+}$  uptake remains unclear.

## 7. Discussions and future directions

Mitochondria play a crucial role in determining the fate of cells. When mitochondrial  $\text{Ca}^{2+}$  concentration is within the physiological limit,  $\text{Ca}^{2+}$  activates ATP production and regulates other mitochondrial functions. However, when the cell is challenged by apoptotic stimuli and mitochondria become overwhelmed with  $\text{Ca}^{2+}$ , the intrinsic pathway of apoptosis is initiated. Mitochondrial  $\text{Ca}^{2+}$  concentration therefore plays a key role in switching between life and death signal. There is evidence that mitochondrial  $\text{Ca}^{2+}$  homeostasis is altered in AD and may even contribute to the cognitive deficits in AD. This leads to the hypothesis that modulating the level of mitochondrial  $\text{Ca}^{2+}$  by various pathways can be beneficial for patients suffering from AD. Mitochondrial  $\text{Ca}^{2+}$  handling provides an exciting and interesting drug target. Of all the drugs we have discussed so far, up-to-date, FCCP and Cyclosporine A are the drugs which have a specific and clearly identified action on mitochondria.

Nonetheless, at the moment it is not clear whether altering mitochondrial  $\text{Ca}^{2+}$  homeostasis represents a viable therapeutic strategy for AD. The biggest challenge now is to understand more about mitochondrial  $\text{Ca}^{2+}$  homeostasis at a molecular level, especially the molecular identity of the  $\text{Ca}^{2+}$  uniporter,  $\text{Na}^+/\text{Ca}^{2+}$  and  $\text{H}^+/\text{Ca}^{2+}$  exchangers. Moreover, the molecular composition of PTP is unclear. Additional  $\text{Ca}^{2+}$  uptake mechanisms such as the rapid mode of  $\text{Ca}^{2+}$  uptake and mitochondrial ryanodine receptors have been demonstrated in mitochondria from other tissues in the body e.g. the heart. Nevertheless, the role of these  $\text{Ca}^{2+}$  uptake modes in neuronal mitochondria is yet to be explored. Regarding the role of  $\text{Ca}^{2+}$  in mitochondria, there is so much to be explored: e.g. how  $\text{Ca}^{2+}$  can be switched from physiological to pathological and how mitochondrial  $\text{Ca}^{2+}$  signaling is affected when the tethering between ER and mitochondria is disrupted? With more research in these areas, it is more likely for us to design viable drugs targeting the mitochondrial  $\text{Ca}^{2+}$  pathways. Designing drugs that can specifically target mitochondrial  $\text{Ca}^{2+}$  homeostasis in neurons is challenging. It is important that the drug can be specifically delivered to neurons; otherwise it is likely to alter mitochondrial  $\text{Ca}^{2+}$  homeostasis in other tissues as well, including heart, muscle and liver. This will result in severe side effects. In this case, special central nervous system (CNS) drug delivery systems such as intranasal administration provides a potential drug delivery method (Illum, 2004).

Moreover, majority of the studies investigating the neuroprotective effect of modulating mitochondrial  $\text{Ca}^{2+}$  handling are based on AD models induced by  $\text{A}\beta$ . Future studies on the molecular basis of mitochondrial  $\text{Ca}^{2+}$  handling in other areas in AD e.g. tau and AD animal models will definitely give us a clear picture.

In addition to the points above, there are some important questions we have to critically consider when designing drugs that alter mitochondrial  $\text{Ca}^{2+}$ :

#### 7.1. Decrease or increase mitochondrial $\text{Ca}^{2+}$ uptake?

A number of studies mentioned have shown that by reducing  $\text{Ca}^{2+}$  influx into mitochondria, the risk of  $\text{Ca}^{2+}$  overload is lowered and induction of apoptosis can be attenuated (Garcia-Martinez et al., 2010; Sanz-Blasco et al., 2008). However, other studies have shown that by increasing the  $\text{Ca}^{2+}$  buffering capacity of mitochon-

dria, more  $\text{Ca}^{2+}$  can be sequestered from the cytoplasm, and thus neurons can be protected (Du et al., 2008; El Idrissi, 2008). It is still unclear whether increasing or reducing mitochondrial  $\text{Ca}^{2+}$  uptake is a better approach for neuroprotection. Both approaches have their own reasons, but there are a few points we have to carefully consider. If the modulations allow less  $\text{Ca}^{2+}$  entering the mitochondria, it is important to make sure that the reduced  $\text{Ca}^{2+}$  uptake will not affect  $\text{Ca}^{2+}$ -dependent physiological functions such as ATP production. At the same time, an important question is how the excessive cytosolic  $\text{Ca}^{2+}$  will be extruded if there is less  $\text{Ca}^{2+}$  uptake by mitochondria. In this case, additional  $\text{Ca}^{2+}$  buffering system in the cytoplasm would be needed. For the latter approach, it is crucial to ensure that the increased mitochondrial  $\text{Ca}^{2+}$  uptake will not exceed the threshold which triggers cell death pathways. In this case, neuroprotective agents that can increase or retain the activity of mitochondria will be useful to ensure normal mitochondrial function. The excessive  $\text{Ca}^{2+}$  taken by mitochondria can then be used for metabolic activities of mitochondria.

In either case, we have to make sure that the normal  $\text{Ca}^{2+}$ -dependent mitochondrial functions such as ATP production and mitochondrial dynamics will not be affected while we are manipulating mitochondrial  $\text{Ca}^{2+}$  concentrations.

## 7.2. Heterogeneity of mitochondrial response

The microdomain hypothesis suggests that those mitochondria close to  $\text{Ca}^{2+}$  channels and ER stores are vulnerable to take up  $\text{Ca}^{2+}$  (Csordas et al., 2006; Rizzuto and Pozzan, 2006). It is interesting to study if the distance between the ER and mitochondria determines the vulnerability of mitochondria to  $\text{Ca}^{2+}$  overload? Moreover, how does the  $\text{Ca}^{2+}$  overload in one mitochondrion spread to other mitochondria? When considerable amount of mitochondria undergo membrane permeabilization, irreversible cell death mechanism is initiated. In this notion, would it be possible to attenuate  $\text{Ca}^{2+}$  overload among mitochondria to avoid cell death? Mitochondria have a quality control mechanism called mitophagy in which damaged mitochondria are selectively eliminated by autophagy (Lemasters, 2005). Recent work has demonstrated that NIX, ULK1 and Parkin are involved in regulation of mitophagy in mammalian cells (Tolkovsky, 2009). However the exact molecular mechanism and how mitophagy is initiated remains unclear. It is important to understand whether mitophagy can serve as a protective mechanism prior initiation of apoptosis.

## 8. Conclusions

At this point, there is still no single drug that can provide a cure for AD. Although there is evidence supporting the role of modulating mitochondria  $\text{Ca}^{2+}$  in neuroprotection, whether this approach can be an effective treatment for AD remains obscure. A combination with other drugs which aim to increase the ability of neurons for synaptic transmission and modulate the cytosolic calcium homeostasis may be beneficial in treating AD. For future development of drugs targeting mitochondrial  $\text{Ca}^{2+}$ , agents that can enhance the activity of mitochondria should also be applied to increase the ability of mitochondria to buffer the excessive  $\text{Ca}^{2+}$ .

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