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Mitochondrial modulation of store-operated Ca^{2+} entry in model cells of Alzheimer's disease

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ABSTRACT

Mitochondrial malfunction and calcium dyshomeostasis are early pathological events considered as important features of the Alzheimer's disease (AD) brain. Recent studies have suggested mitochondrion as an active regulator of Ca^{2+} signaling based on its calcium buffering capacity. Herein, we investigated the mitochondrial involvement in the modulation of store-operated calcium entry (SOCE) in neural 2a (N2a) transgenic AD model cells. Results showed that SOCE was significantly depressed in N2a cells transfected with wild-type human APP695 (N2a APPwt) compared with empty vector control (N2a WT) cells. Pharmacological manipulation with mitochondrial function blockers, such as FCCP, RuR, or antimycin A/oligomycin, could inhibit mitochondrial calcium handling, and then impair SOCE pathway in N2a WT cells. Furthermore, mitochondria of N2a APPwt cells exhibited more severe swelling in response to Ca^{2+}, which is an indication of mitochondrial membrane permeability transition (MPT), than the wild-type controls. Additionally, treatment with cyclosporin A, a potent inhibitor of cyclophilin D, which can block MPT, could significantly restore the attenuated SOCE in N2a APPwt cells. Therefore, inhibition of cyclophilin D might be a therapeutic strategy for Alzheimer's disease.

1. Introduction

Nowadays, more than 35 million people worldwide are suffering from Alzheimer's disease (AD), the most common form of dementia characterized by pathological features of senile plaques (SP), intracellular neurofibrillary tangles (NFT) and neuronal loss [1]. Senile plaque is mainly composed of β-amyloid (Aβ) peptides, and the imbalance between the production and clearance of Aβ peptides results in their deposition and initiate the disease progression [1]. While the β-amyloid hypothesis has been the basis for plenty of researches on AD pathogenesis, other hypotheses claim that dysregulation of calcium homeostasis or mitochondrial malfunction might also play a key role in this pathological process [2,3].

Calcium, an important second messenger, participates in a wide variety of cellular processes. It is tightly controlled in time, space, and intensity. Sustained disturbance of calcium homeostasis in AD has been thought to be a primary cause inducing the neurodegenerative disease [4]. Stored-operated calcium entry (SOCE) is one of the most prevalent pathways allowing calcium entry into cells, which is activated when the depletion of intracellular calcium store (i.e. endoplasmic reticulum, ER) occurs, facilitating ER refilling process [5]. Recently, SOCE has been related to the pathogenesis of AD. Reports showed that mutant presenilin 1 (PS1), a component of γ-secretase, directly attenuates SOCE [6], and our previous results also suggested that accumulation of Aβ peptide could potentiate SOCE in AD model cells, contributing to Aβ neurotoxicity [7]. Meanwhile, several other cellular signaling pathways and organelles are associated with the regulation of SOCE. In fact, mitochondria have been reported to act as an important physiological regulator for SOCE [8].

Mitochondria are elementarily involved in various cellular processes and functions. Over the last few years, the mitochondrial cascade hypothesis of sporadic AD has been proposed [2], and evidence indicated that interactions between mitochondria and some other factors might contribute to the disease pathogenesis, one of which might be the intimate relationship between mitochondrial physiology and Ca^{2+} signaling cascades. It has been well documented that mitochondria could physiologically regulate and shape Ca^{2+} signals by its Ca^{2+} buffering capacity in limited cellular areas, depending on their energy state and their ability to uptake and accumulate Ca^{2+} within the matrix [9]. Recently, it has been found that mitochondria are actively involved in the maintenance of SOCE in various cell types and disease models [8,10]. However, mitochondrial regulatory role of SOCE in the pathological process of AD has not been ascertained.

In view of the important actions of both mitochondria and Ca^{2+} signaling in AD pathogenesis, here, we investigated mitochondrial modulation of SOCE in neural 2a (N2a) transgenic AD model cells

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stably expressing wild-type human APP695 (APPwt) and their counterparts, the N2a cells transfected with the empty plasmid vectors (N2a wild-type, N2a WT) as controls. Our results showed that mitochondrial Ca^{2+} handling was impaired in N2a APPwt cells, which resulted in a significant reduction of SOCE. The mitochondria-related reduction of SOCE in N2a APPwt cells could be antagonized by treatment with cyclosporin A (CsA), a potent inhibitor of cyclophilin D which is an integral component of mitochondrial membrane permeability transition pore (mPTP) [11,12], suggesting that mitochondrial membrane permeability transition is involved in the impairments of mitochondrial modulation of SOCE in AD pathology.

2. Materials and methods

2.1. Reagents

Fluo3/AM, thapsigargin, carbonyl cyanide 4-(trifluoromethoxyl)s-phenyl-hydrazone (FCCP), cyclosporin A (CsA), ruthenium red (RuR), oligomycin, and rhodamine 123 were purchased from Sigma, USA. Antimycin A was from ENZO Lifesciences, Germany. Penicillin and streptomycin were obtained from Amresco, USA. Fetal bovine serum was from Hyclone, USA.

2.2. Cell cultures

Mouse neural 2a (N2a) cells stably expressing wild-type human APP695 (APPwt) and N2a wild-type controls transfected with empty plasmid vectors (N2a WT) were kindly supplied by Dr. Huaxi Xu (Burnham Institute for Medical Research, La Jolla, USA). All the cells were maintained in medium composed of 50% DMEM and 50% OPTI-MEM, supplemented with 5% fetal bovine serum (FBS), 150 μg/mL G418 and 0.1% antibiotics (penicillin and streptomycin).

2.3. Cytosolic Ca^{2+} measurement

Cells were loaded with Fluo3/AM to monitor cytosolic calcium concentration [7]. The fluorescent dye Fluo3/AM was solubilized and stored in DMSO. When used to load cells, Fluo3/AM was diluted in HBSS (145 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, 10 mM glucose, 1.8 mM $CaCl_2$) containing 1% bovine serum albumin (BSA). The cells were then incubated by Fluo3/AM-containing HBSS (5 μM) at 37 °C for 30 min. After loading, the cytosolic Ca^{2+} concentration was monitored using a confocal laser scanning microscope (FV500, Olympus, Japan) at room temperature. Fluorescence data were collected at 1 s intervals.

2.4. Mitochondrial membrane potential determination

To determine mitochondrial membrane potential (MMP) in N2a cells, Rhodamine 123 was added to cell culture medium at a final concentration of 1 μM and the cells were kept at 37 °C for 30 min. After washing three times with Kreb's-Ringer's buffer (100 mM NaCl, 2.6 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 11 mM glucose), the fluorescence was detected with confocal laser scanning microscopy (FV500, Olympus, Japan). The Image-Pro Plus software (ver 6.0) was used to analyze obtained digital images.

2.5. Mitochondria isolation

Preparation of mitochondrial fraction from cultured N2a cells was achieved using a commercial mitochondrial isolation kit (Beyond time, China). Briefly, N2a cells were harvested and washed with ice-cold PBS. The cells were incubated in lysis buffer at 4 °C for 10 min and then homogenized with a homogenizer. The cell lysate was subjected to centrifuging (600*g*, at 4 °C) for 10 min to remove unbroken cells, and the supernatant was further centrifuged (11,000*g*, at 4 °C) for 10 min to deposit mitochondrial fraction. For mitochondrial protein determination, the mitochondrial pellets were resuspended and lysed in mitochondrial lysis buffer at 4 °C and then the protein concentration was measured by a BCA kit from Pierce, USA with bovine serum albumin used as standard.

2.6. Mitochondrial swelling assay

Ca^{2+}-induced mitochondrial swelling assay was performed to assess mitochondrial membrane permeability transition according to previously published protocols with modifications [12,13]. Isolated mitochondria from N2a WT or APPwt cells were resuspended in 200 μL swelling assay buffer (500 μg/mL protein, 150 mM KCl, 5 mM HEPES, 2 mM K_2HPO_4, 5 mM glutamate, 5 mM malate, pH 7.2). Mitochondrial swelling was induced by the admission of Ca^{2+} (1 μmol/mg protein). Changes in absorbance at 540 nm (OD_{540nm}) were collected by a microplate reader (FLUOstar Omega, BMG Labtech, Germany) for 12 min at 30 s intervals, which indicated mitochondrial swelling induced by the membrane permeability transition.

2.7. Statistical analysis

Data were expressed as means ± SD. Analysis of variance (ANOVA) was carried out. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. SOCE was depressed in N2a APPwt cells

SOCE was activated according to previously published protocols [7]. Briefly, after loading with Fluo-3/AM, cells were incubated in Ca^{2+}-free medium in the presence of 1 μM thapsigargin, a SERCA calcium pump inhibitor, to deplete the ER calcium store. Then 2 mM Ca^{2+} was readmitted into the dish and SOCE was estimated by the amplitude of peak $[Ca^{2+}]_{cyto}$ signal. A dramatically attenuated SOCE was observed in N2a APPwt cells compared with that in N2a WT control cells (Fig. 1A and B). Next, 1 μM thapsigargin was applied to deplete ER store and the area under the thapsigargin-induced Ca^{2+} release curve was used to evaluate ER calcium store content of the cells (Fig. 1C). It was shown that there were no significant differences in the ER calcium store contents between N2a APPwt and N2a WT cells (Fig. 1D), suggesting that the attenuation of SOCE in N2a APPwt cells was not due to the alteration of ER calcium store content.

The mitochondrial modulatory function of Ca^{2+} signaling is based on their ability to sequester and accumulate Ca^{2+} in matrix [8]. In order to study the differences of mitochondrial calcium handling capacity between N2a WT and APPwt cells, FCCP, a protonophore dissipating mitochondrial membrane potential (MMP) and subsequently releasing mitochondrial Ca^{2+} stores, was added during the plateau phase of SOCE. $[Ca^{2+}]_{cyto}$ in both APPwt and WT cells was increased (Fig. 1E). Differences between FCCP-induced $[Ca^{2+}]_{cyto}$ amplitude and cytosolic calcium concentration during SCOE plateau phase were attributed, at least in part, to the release of intracellular mitochondrial calcium stores [14]. The increase of cytosolic calcium in N2a APPwt cells was significantly lower than that in N2a WT cells (Fig. 1F), suggesting that mitochondria in N2a APPwt cells take a smaller load of sequestered calcium than that in N2a WT cells.

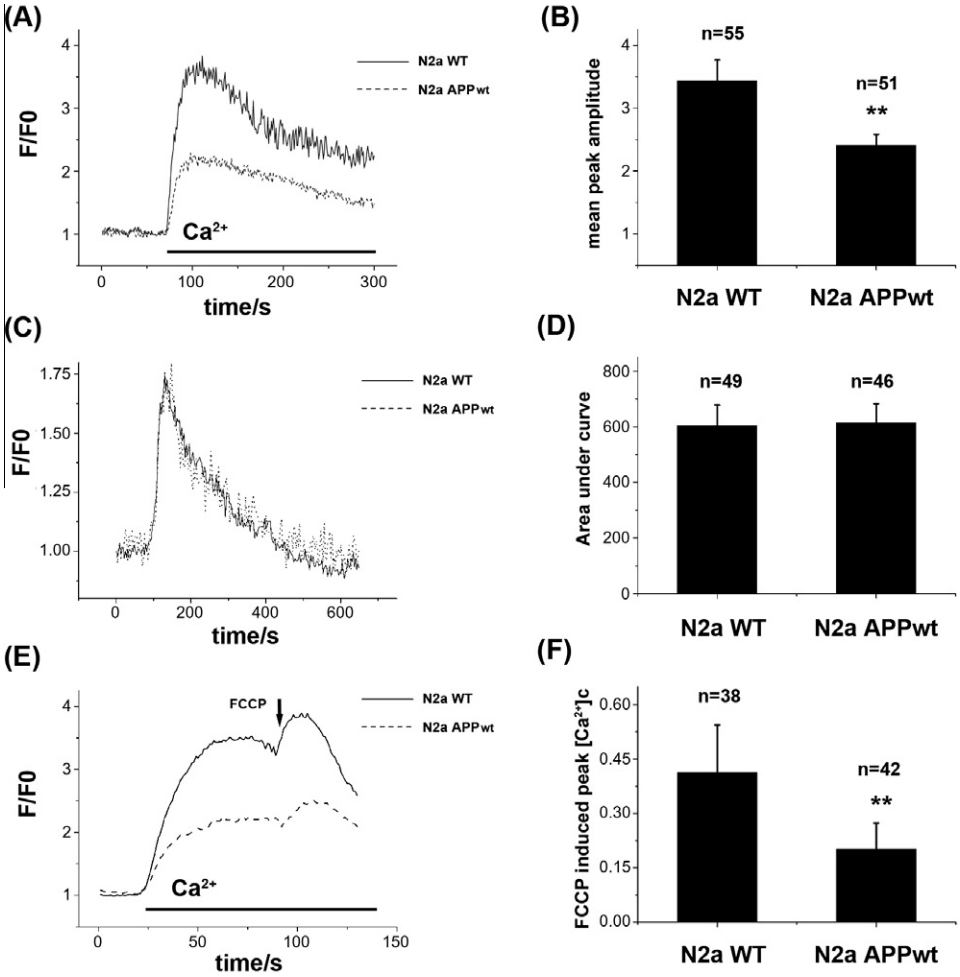

Fig. 1. Store-operated calcium entry and mitochondrial Ca^{2+} sequestration were impaired in N2a APPwt cells. (A) Representative $[Ca^{2+}]_{cyto}$ traces indicating SOCE in N2a WT and APPwt cells, respectively. (B) Averaged peak amplitudes of SOCE in N2a WT and APPwt cells. (C) Representative $[Ca^{2+}]_{cyto}$ traces indicating Ca^{2+} release responses of N2a WT and APPwt cells to 1 μM thapsigargin, respectively. (D) Averaged area under the thapsigargin-induced Ca^{2+} release curve of N2a WT and APPwt cells. (E) Representative $[Ca^{2+}]_{cyto}$ traces showing the release of mitochondrial sequestrated Ca^{2+} following FCCP stimulus during SOCE plateau phase. N2a WT and APPwt cells were exposed to 10 μM FCCP (indicated by arrow) during SOCE platform phase. (F) Averaged FCCP-induced $[Ca^{2+}]_{cyto}$ elevations during SOCE plateau phase in N2a WT and APPwt cells. *n* = number of cells; $^{**}P < 0.01$, compared with N2a WT controls.

3.2. Mitochondrial modulation of SOCE in N2a cells

Mitochondrial calcium uptake mainly depends on the electrical driving force allowing calcium entrance that is built up by mitochondrial membrane potential (MMP) across the inner mitochondrial membrane. Dissipation of the proton across inner mitochondrial membrane by uncouplers, such as carbonyl cyanide 4-(trifluoromethoxyl)s-phenyl-hydrazone (FCCP), would cause immediate depolarization of MMP and result in reduced driving force for Ca^{2+} entry, and as a consequence would strongly diminish mitochondrial calcium buffering capacity [15]. To verify the participation of mitochondria in the process of SOCE, MMP in N2a cells was monitored using mitochondria-selective fluorescent dye, Rhodamine 123, through confocal laser scanning microscopy. Results showed that MMP was obviously lower in N2a APPwt cells than in N2a WT cells [Fig. 2A, (a and b)], which was in consistent with our previous results [16]. Treatment of 10 μM FCCP could efficiently dissipate MMP in both N2a WT and N2a APPwt cells [Fig. 2A, (c–e)]. After FCCP treatment, the readmission of Ca^{2+} induced an attenuated SOCE in N2a WT cells (Fig. 2B and C), suggesting that mitochondrial malfunction in Ca^{2+} handling leads to reduction of SOCE.

Most calcium cations enter mitochondria through mitochondrial calcium uniporters (MCU), which are voltage dependent, highly selective ion channels and could be inhibited by ruthenium red (RuR) [15]. To further confirm whether mitochondria are involved in the regulation of SOCE in N2a cell, the model neuron, we treated N2a WT cells with RuR. After RuR treatment, an obvious decline of SOCE in N2a WT cells was observed (Fig. 2D and E). Moreover, inhibition of mitochondrial electron transport chain (mETC) with 5 μg/mL antimycin A, a selective inhibitor of complex III, together with 5 μg/mL oligomycin, a blocker of mitochondrial ATP-synthase/ATPase, could also lead to a significantly decreased SOCE in N2a WT cells (Fig. 2D and E). These results confirm that SOCE in the N2a cells used herein is regulated by mitochondrial Ca^{2+} handling.

3.3. Cyclosporin A potentiated SOCE in N2a APPwt cells

One of the most important pathological mitochondrial functions involves the mitochondrial membrane permeability transition, which is defined as a sudden increase in permeability of the inner mitochondrial membrane and characterized by the formation of mitochondrial permeability transition pore (mPTP). Formation of mPTP is often considered as the initial step leading to apoptosis, necrosis and cell death as a result of the collapse of mitochondrial membrane potential and the release of small solutes and molecules out of mitochondria [11,12]. Accumulating

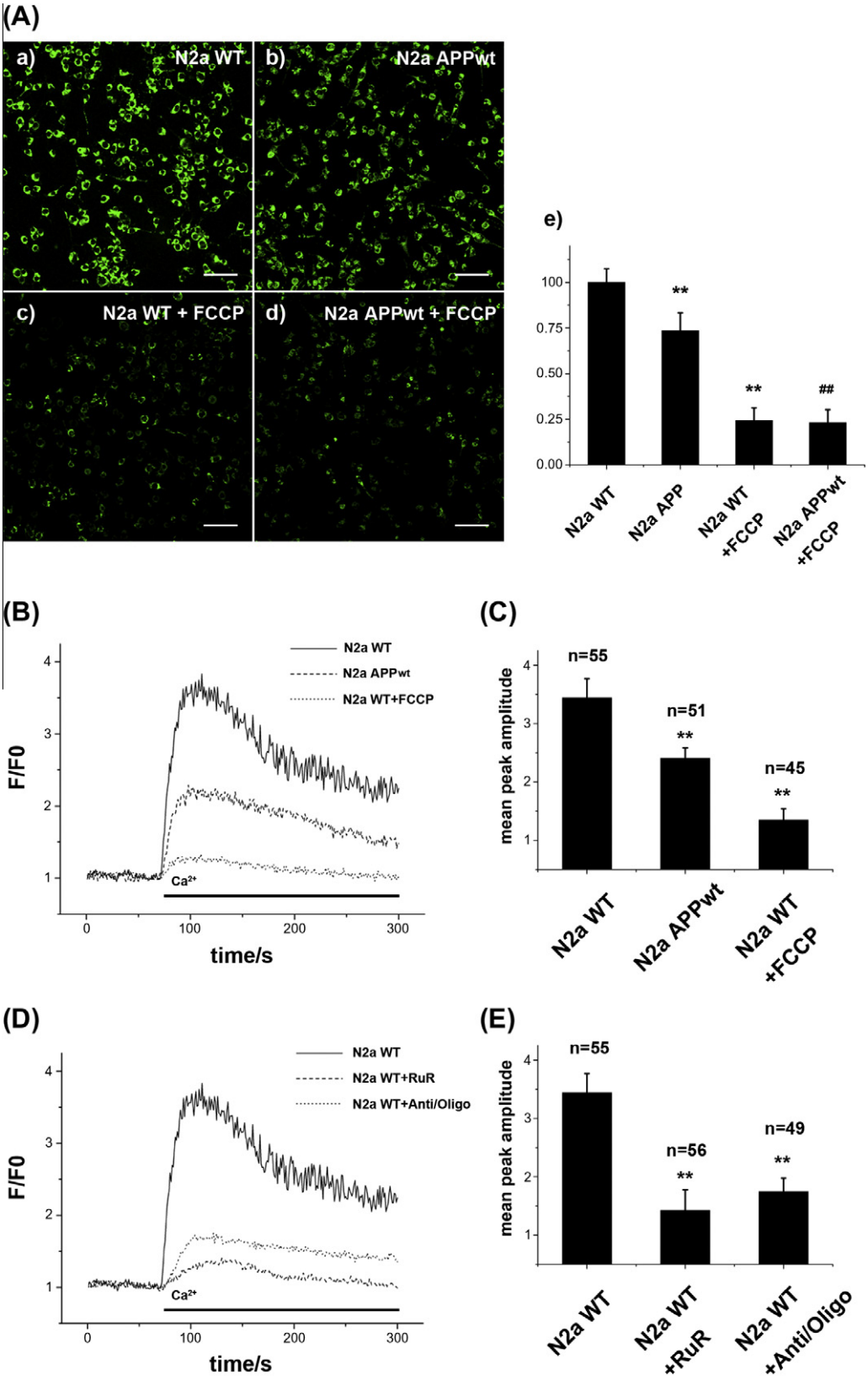

Fig. 2. Mitochondria participated in SOCE modulation in N2a cells. (A) Mitochondrial membrane potential was declined in N2a APPwt cells [(a)] compared with N2a WT cells [(b)], which could be dissipated by FCCP [(c) and (d)]. Statistical analysis between groups is shown in (e). $n = 5$ for each group. (B) Representative $[Ca^{2+}]_{cyto}$ traces indicating SOCE in N2a WT cells, APPwt cells, and WT cells pre-treated with FCCP, respectively. FCCP (10 μM) were applied 30 min prior to thapsigargin-induced SOCE in N2a WT cells. (C) Averaged peak amplitudes of SOCE in N2a WT cells, APPwt cells, and WT cells pre-treated with FCCP. (D) Representative $[Ca^{2+}]_{cyto}$ traces indicating SOCE in N2a WT cells, and WT cells pre-treated with ruthenium red (RuR) or antimycin A/oligomycin (Anti/Oligo), respectively. RuR (10 μM) or Anti/Oligo (5 μg/mL each) was applied 30 min prior to thapsigargin-induced SOCE in N2a WT cells. (E) Averaged peak amplitudes of SOCE in N2a WT cells and WT cells pre-treated with RuR or Anti/Oligo. n = number of cells; $^{**}P < 0.01$, compared with N2a WT controls; $^{\#\#}P < 0.01$, compared with N2a APP wt cells.

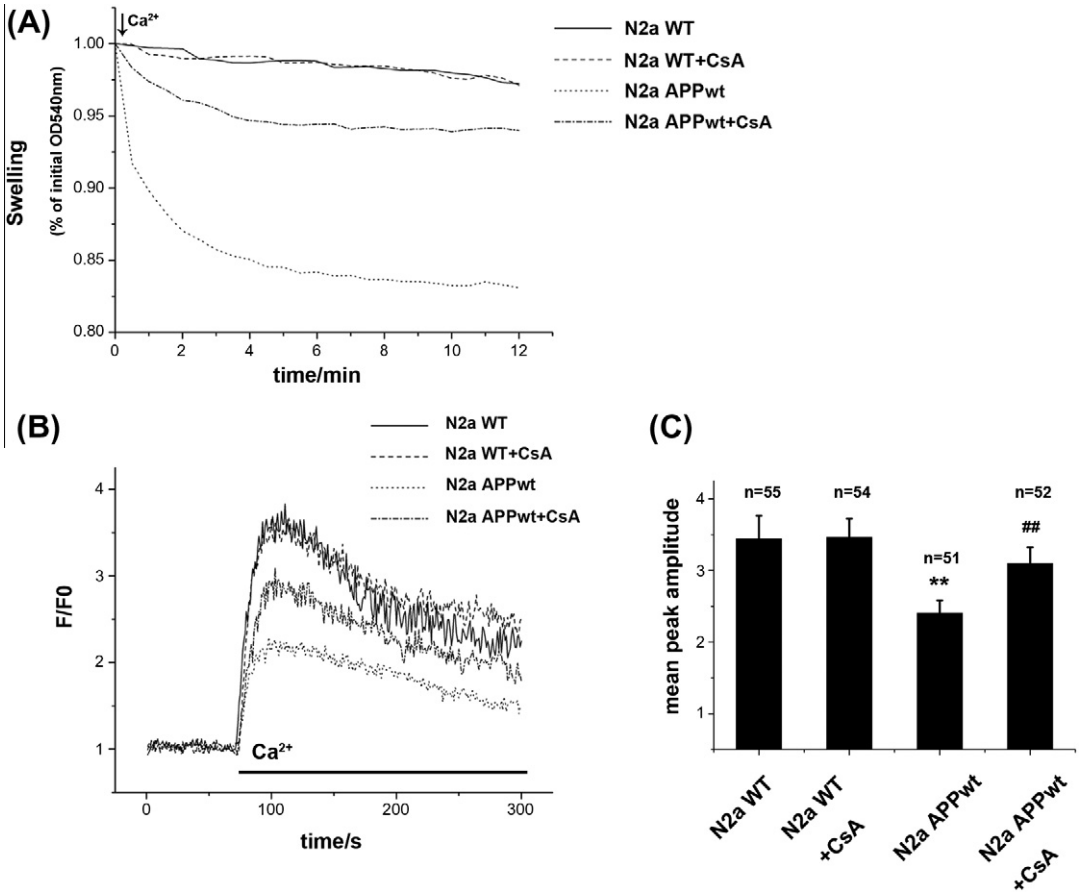

Fig. 3. Cyclosporin A mitigated mitochondrial swelling and SOCE impairment in N2a APPwt cells. (A) Swelling of isolated mitochondria from N2a WT and APPwt cells in response to Ca^{2+}, in the presence or absence of cyclosporin A (CsA) (representative of three independent experiments). (B) Representative $[Ca^{2+}]_{cyto}$ traces indicating SOCE in N2a WT cells, APPwt cells, in the presence or absence of CsA. CsA (1 μM) were applied 30 min prior to thapsigargin-induced SOCE in N2a WT cells. (C) Averaged peak amplitudes of SOCE in N2a WT cells, APPwt cells, in the presence or absence of CsA. *n* = number of cells; $^{**}P < 0.01$, compared with N2a WT controls; $^{\#\#}P < 0.01$, compared with N2a APPwt cells.

evidences have shown that transient opening of mPTP might be a sufficient pathway of Ca^{2+} efflux from mitochondria [15]. It has been also well documented that MPT-induced increase in the permeability of mitochondrial membrane could further result in a dramatic decline in mitochondrial membrane potential (MMP), mitochondrial swelling and rupture of the mitochondrial outer membrane. In order to verify whether mitochondrial permeability transition is involved in the SOCE decline in N2a APPwt cells, we measured mitochondrial swelling of the cells in response to Ca^{2+}. As an early event in MPT, mitochondrial swelling can be monitored as the change in the light absorbance of isolated mitochondria at 540 nm [12,13]. Mitochondria from N2a WT cells showed slight swelling in response to Ca^{2+}, and N2a APPwt mitochondria exhibited much more severe swelling (Fig. 3A). Notably, when treated with cyclosporin A (CsA), an efficient mPTP blocker, mitochondria from N2a APPwt cells were more resistant to the swelling and permeability transition induced by Ca^{2+}, while the same treatment did not affect N2a WT mitochondria obviously (Fig. 3A). Furthermore, when pre-treated with 1 μM CsA for 30 min, SOCE in N2a APPwt cells was significantly potentiated (Fig. 3B and C), suggesting that mPTP formation is involved in the SOCE decline in N2a APPwt cells. These results suggest that aberrant mitochondrial modulation contributes to the SOCE impairment in N2a APPwt cells.

4. Discussion

Calcium dyshomeostasis is believed to be one of the key factors accelerating other pathological alterations in Alzheimer's disease (AD) [17]. As one of the important calcium signaling pathways, store-operated calcium entry (SOCE) has been found to be altered in AD and be associated with the disease pathogenesis [6,7,18]. Recent studies suggested that SOCE was directly attenuated by PS1 mutants in transgenic AD model cells [6] and PS1-evoked inhibition of SOCE altered APP processing, producing more Aβ1-42 peptides [18]. Our previous studies also showed that Aβ overproduction had a potentiating effect on SOCE in AD model cells, which contributed to the molecular mechanism of Aβ toxicity [7]. Studies of SOCE modulation might lead to useful hints for understanding the role of calcium signaling in AD pathogenesis.

Mitochondrial malfunction is another factor actively involved in the pathological process of AD. It has also been well documented that mitochondrial functions were injured in many aspects, such as ATP production and reactive oxygen species (ROS) generation, in AD cases [16]. Recently, postulations have emerged that mitochondria are important regulators of Ca^{2+} signaling based on the evidences that mitochondria within intact neural cells could accumulate Ca^{2+} when the intracellular Ca^{2+} is above some set-point and release these ions into cytosol when calcium elevation disappears [19]. Mitochondria could perform as temporary reversible calcium stores modulating cellular events related to calcium ions. Moreover, a growing body of evidence has indicated that mitochondria act as calcium buffers to protect SOCE against feedback inhibition and account for the long lasting maintenance of SOCE signaling [8]. To investigate whether mitochondrial modulation of SOCE is injured in AD pathogenesis and if this modulation is associated with other pathogenic factors, we compared SOCE in

N2a APPwt cells with that in N2a WT cells. Our results showed that SOCE in N2a APPwt cells was significantly depressed compared with their wild-type counterparts. FCCP, a protonophore abolishing mitochondrial membrane potential, has been long shown to depress calcium entry through store-operated calcium channels [20]. When treated with FCCP, SOCE signaling in N2a WT cells was dramatically depressed. Additionally, mETC blockers antimycin A/oligomycin could also lead to declined SOCE in N2a WT cells. In addition to mitochondrial depolarization, the blocker of mitochondrial Ca^{2+} uniporters, ruthenium red, could also inhibit mitochondrial Ca^{2+} uptake and further prevent the maintenance of SOCE in N2a WT cells, which was in consistent with previous reports in other cell types [15]. Based on the above evidence, we confirm that mitochondria act as an active regulator of SOCE in N2a cells, and mitochondrial malfunction might be one of the mechanisms contributing to the declined SOCE signaling in N2a APPwt cells compared with the WT control cells.

It has been convincingly reported that mitochondria play a central role in the initiation of apoptosis, which has also been attributed to neurodegenerative diseases including AD [15,16]. The release of cytochrome *c* from mitochondria is considered as a key event initiating the cascade in most apoptosis pathways that lead to cellular self-destruction with caspases. Mitochondrial permeability transition would allow cytochrome *c* to release into cytoplasm through the mitochondrial permeability transition pore (mPTP), which consist of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocase (ANT) in the inner membrane and cyclophilin D in mitochondrial matrix [11]. Besides cytochrome *c*, mPTP has also been considered as an ideal route for other mitochondrial contents, including calcium cations [11,15]. It has been shown that by altering the function and/or the integrity of mPTP complex, fenamates like flufenamic acid (FFA) could promote the release of Ca^{2+} from mitochondria [21]. Herein, as indicated by mitochondrial swelling assay, mitochondrial permeability transition might be involved in the impaired SOCE signaling in N2a APPwt cells. Compared with N2a WT cells, mitochondria from N2a APPwt cells showed obviously much more serious swelling in response to Ca^{2+}, indicating the opening of mPTP in these cells, which might facilitate mitochondrial Ca^{2+} extrusion and result in an impaired mitochondrial Ca^{2+} buffering capacity. Moreover, when treated with cyclosporin A (CsA), N2a APPwt cells showed a significant recovery of SOCE and mitochondria of N2a APPwt cells treated with CsA swelled to a much less extent in response to Ca^{2+} stimulus. Accordingly, we propose that mitochondrial permeability transition occurring in N2a APPwt cells leads to severely impaired mitochondrial calcium buffering capacity, which fail to efficiently uptake Ca^{2+} to keep SOCE from feedback inhibition and ultimately result in a declined SOCE in these cells.

Mitochondrial calcium handling impairment has been found to actively interact with other mitochondrial damages in neurodegenerative process of AD. An important aspect of these mitochondrial damages concerns ROS generation. Our previous studies showed that AD model cells exhibited increased basal level of ROS than wild-type controls [16]. It has been reported that sustained increasing formation of mitochondrial ROS might lead to disturbed mitochondrial free Ca^{2+} concentration [22], which would in turn accelerate ROS generation to build up a positive feedback loop inducing an even worse situation in AD cases. ATP production is the primary mitochondrial function, which has also been shown to be injured in AD model cells [16]. Under normal conditions, mitochondria act as a Ca^{2+} buffering system sufficient to avoid $[Ca^{2+}]_{cyto}$ deviation from the set-point, increasing the electron flux through the mETC and thus activating ATP production. Mitochondiral calcium dyshomeostasis would lead to ATP supply failure, which would further cause damage to cellular metabolism and physiological functions [8,15].

Recent studies have highlighted the role of Aβ peptides in mitochondrial malfunction during AD pathogenesis. It has been shown that Aβ oligomers, the most toxic species, were prone to accumulate in mitochondria in the brains of not only Alzheimer's disease patients but also its transgenic mouse models, which occurs before amyloid deposition [11]. The accumulated Aβ in mitochondria decreases activities of enzymes in matrix and mediates oxidative stress in neurons [12]. Thus, the Aβ accumulation has been linked to mitochondrial malfunction observed in AD. Interestingly, as the key component of mitochondrial membrane permeability transition pore, cyclophilin D has been associated with amyloid pathology because of its colocalization and interaction with Aβ peptides in mitochondria. Moreover, the genetic ablation of cyclophilin D was demonstrated to improve mitochondrial, neuronal and synaptic function [12]. In fact, genetic deletion of cyclophilin D has also been found to limit mPTP formation and greatly increase mitochondrial calcium buffering capacity under stressing circumstances [12,23]. Taken together, these observations have provided hints to understand the mechanisms underlying impairments of mitochondrial modulation of SOCE in N2a APPwt cells. However, further investigations are required to elucidate the detailed mechanisms underlying Aβ-induced mitochondrial malfunction in AD progression.

Our study provides new insights into the early mitochondrial malfunction and calcium dyshomeostasis in AD pathogenesis. Mitochondrial permeability transition might be involved in the impairments of mitochondrial modulation of SOCE pathway. Since CsA could effectively inhibit cyclophilin D and alleviate the impairment of SOCE in N2a APPwt cells, blockade of cyclophilin D may be of benefit for Alzheimer's disease therapy.

Acknowledgments

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