A reciprocal relationship between reactive oxygen species and mitochondrial dynamics in neurodegeneration

Clara Hiu-Ling Hung, Sally Shuk-Yee Cheng, Yuen-Ting Cheung, Suthicha Wuwongse, Natalie Qishan Zhang, Yuen-Shan Ho, Simon Ming-Yuen Lee, Raymond Chuen-Chung Chang

Laboratory of Neurodegenerative Diseases, School of Biomedical Sciences, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong, China
State Key Laboratory of Brain and Cognitive Sciences, The University of Hong Kong, Pokfulam, Hong Kong, China
Institute of Chinese Medical Sciences, University of Macau, Macau, China
School of Nursing, Faculty of Health and Social Sciences, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China

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ABSTRACT

Mitochondrial fragmentation due to fission/fusion imbalance has often been linked to mitochondrial dysfunction and apoptosis in neurodegeneration. Conventionally, it is believed that once mitochondrial morphology shifts away from its physiological tubular form, mitochondria become defective and downstream apoptotic signaling pathways are triggered. However, our study shows that beta-amyloid (Aβ) induces morphological changes in mitochondria where they become granular-shaped and are distinct from fragmented mitochondria in terms of both morphology and functions. Accumulation of mitochondrial reactive oxygen species triggers granular mitochondrial formation, while mitoTEMPO (a mitochondria-targeted superoxide scavenger) restores tubular mitochondrial morphology within Aβ-treated neurons. Interestingly, modulations of mitochondrial fission and fusion by genetic and pharmacological tools attenuated not only the induction of granular mitochondria, but also mitochondrial superoxide levels in Aβ-treated neurons. Our study shows a reciprocal relationship between mitochondrial dynamics and reactive oxygen species and provides a new potential therapeutic target at early stages of neurodegenerative disease pathogenesis.

1. Introduction

Mitochondria are ubiquitous intracellular organelles that regulate important cellular functions including bioenergetic metabolism, Ca2+ homeostasis and apoptosis. Mitochondrial dysfunction is a prominent pathological feature in various age-related neurodegenerative diseases including Alzheimer’s disease (AD) [1-6].

2. Balance between fission and fusion is required for proper mitochondrial functions due to its involvement in the maintenance of mitochondrial DNA, segregation of damaged mitochondria by mitochondrial fusion, and regulation of mitochondrial morphology [7-9]. Mitochondrial fission is regulated by mitofusins (Mfn1 and Mfn2) and optic atrophy 1 (OPA1) [10-14]; while fission is mediated by dynamin-related protein 1 (Drp1; also known as DLP-1), mitochondrial fission 1 protein (Fis-1) and mitochondrial fission factor (Mff) [15-19]. Recent studies have shown that these proteins contribute to the formation of distinct mitochondrial morphologies under different cell conditions [20,21]. Oxidative stress has also been shown to induce transient changes in mitochondrial morphology as well as fragmentation of the mitochondrial network [22-24]. With prolonged and persistent cellular oxidative damage, interconnected tubular mitochondrial networks are reorganized as small punctate spheres due to extensive fission, resulting in “mitochondrial fragmentation” in apoptosis [25-27].

3. Mitochondrial network remodelling has been proposed to play an important role in neurodegeneration [5,28]. β-amyloid peptide (Aβ) has been shown to promote mitochondrial fragmentation [29-32]. An imbalance of fission and fusion proteins has been detected in AD

Abbreviations: Aβ, β-amyloid; AD, Alzheimer’s disease; Drp1, Dynamin-related protein 1; ER, Endoplasmic reticulum; ER-RFP, Endoplasmic reticulum-targeted dsRed; FCCP, Carbonyl cyanide p-trifluoromethoxy-phenyl-hydrazone; H2O2, Hydrogen peroxide; Mito-KillerRed, KillerRed-dMito; Mito-GFP, Mitochondria-targeted green fluorescent protein; Mito-RFP, Mitochondria-targeted dRFP; Mito-PAGFP, Photoactivable mitochondria-targeted green fluorescent protein; Mito-TEMPO, Mitochondria-targeted superoxide scavenger; Mfn, Mitofusin; Mito-RFP, Mitochondria-targeted green fluorescent protein; Mito-KillerRed, KillerRed-dMito; Mito-GFP, Mitochondria-targeted green fluorescent protein; Mito-TEMPO, Mitochondria-targeted superoxide scavenger; Mfn, Mitofusin; OPA1, Optic atrophy 1; ROS, Reactive oxygen species; SPA-FRAP, Simultaneous photoactivation and fluorescence recovery after photobleaching; TMRE, Tetramethylrhodamine ethyl ester

* Correspondence: Laboratory of Neurodegenerative Diseases, School of Biomedical Sciences, LKS Faculty of Medicine, The University of Hong Kong, Rm. L4-49, Laboratory Block, Faculty of Medicine Building, 21 Sassoon Road, Pokfulam, Hong Kong, China.
E-mail address: R.C.C.Chang@hku.hk (R.C.C. Chang).

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patients’ brains as well [30,33]. Furthermore, disruption in the fission and fusion balance and mitochondrial trafficking have been demonstrated in various AD transgenic mouse models [34–38]. However, the precise mechanism underlying mitochondrial fragmentation in AD remains under debate [31,39]. Previous studies often focus on mitochondrial fragmentation as an indicator for cell death and mitochondrial dysfunction. However, the relationship between mitochondrial morphology and function remains obscure.

Here, we report that Aβ induced unique changes in mitochondrial morphology, which we refer to as granular mitochondria. Aβ-induced granular mitochondria maintained functional integrity and were thus morphologically and functionally distinct from spherical fragmented mitochondria during apoptosis. Oxidative stress induced by Aβ was found to impair mitochondrial dynamics, as revealed by simultaneous photoactivation and fluorescence recovery after photobleaching (SPA-FRAP) imaging. Notably, we show that mitoTEMPO, the mitochondria-targeted superoxide scavenger, and both genetic and pharmacological approaches to inhibit the fission protein Drp1 could abolish the formation of granular mitochondria by Aβ. Interestingly, attenuation of granular mitochondria formation by inhibiting fission could also ameliorate Aβ-induced accumulation of reactive oxygen species (ROS). Our study demonstrates that Aβ induces acute disturbance in mitochondrial dynamics through induction of oxidative stress, which is independent of apoptosis. In addition, mitochondria morphology and oxidative stress reciprocally affects one another in an in vitro model of AD.

2. Materials and methods

2.1. Primary hippocampal culture

Primary culture of hippocampal neurons was prepared from embryonic day 18–19 Sprague-Dawley rats (Laboratory Animal Unit, The University of Hong Kong, accredited by Association for Assessment and Accreditation of Laboratory Animal Care International) as previously described. All experimental protocols involving animals were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) at LKS Faculty of Medicine, HKU. Briefly, hippocampi were isolated and mechanically dissociated in ice-cold phosphate buffered saline (Life Technologies) supplemented with glucose (18 mM, Sigma-Aldrich). Neurons were plated onto poly-L-lysine (25 μg/ml, Sigma-Aldrich) coated glass-bottomed confocal dishes (SPL Life Science) and dish were transfected on DIV 5 in Neurobasal (Evrogen). mito-PAGFP was obtained from Dr. Richard Youle (Addgene). Neurons were plated onto coverglass-bottomed confocal dishes for confocal live cell imaging. Images were acquired every 1 min using the 488 nm and 563 nm laser lines for 30 min. The excitation wavelengths were 488 nm for GFP; and 543 nm for DsRed2, MitoSOX™, TMRE was excited at 543 nm, and Rhod-2. TMRE was excited at 800 nm by two-photon excitation (Chameleon, Coherent Inc.).

2.2. Transfection

Primary hippocampal neurons seeded on glass-bottomed confocal dish were transfected on DIV 5–6 for 2–3 h with Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Drug treatments were performed 24–48 h after transfection. GFP-Mito (mito-GFP) was purchased from Invitrogen (Life Technologies). DsRed2-Mito (mito-RFP, Clontech) was a generous gift from Prof. Siu-Kai Kong (The Chinese University of Hong Kong, Hong Kong). eGFP-BAX and eGFP-Drp1K38A were gifts from Dr. Zheng Dong (Medical College of Georgia, USA). pC-MitoRed-dMito (mito-KillerRed) was purchased from Evrogen (Evrogen). mito-PAGFP was obtained from Dr. Richard Youle (Addgene plasmid 23348, Addgene).

2.3. Transfection and transduction

Primary hippocampal neurons seeded on glass-bottomed confocal dish were transduced with baculovirus-based CellLight™ Mitochondria-GFP, BacMam 2.0 (Invitrogen™) on day 5–6 following seeding. Drug treatments were performed 24–48 h after transfection.

2.4. Preparation of oligomeric Aβ

Aβ peptides were purchased from Yale University and ChinaPeptides, and were prepared as previously described [40]. Briefly, lyophilized Aβ peptide was first dissolved in 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (Sigma-Aldrich). After evaporation of the peptide solution, anhydrous DMSO was added to the preparation to achieve the stock solution. The peptide solution was then sonicated at room temperature for 30 min. After sonication, Aβ peptides were snap-frozen in liquid nitrogen, and stored at −80 °C until the day of experiment. Presence of monomers, dimers, and oligomers was confirmed by Tris-Tricine gel electrophoresis as shown previously [40]. Aβ peptides were diluted to the indicated working concentration on the day of the experiment.

2.5. Drug treatments

All drug treatments were performed on DIV6-7. Neurons were treated with 10 μM oligomeric Aβ for the indicated hours. Mitotracker (2.5 μM, Enzo Life Sciences), mitochondrial division inhibitor (mdivi-1, 5 μM; Sigma-Aldrich), and Mitochondrial Fusion Promoter M1 (MPPM1, 5 μM; Sigma-Aldrich) were pre-incubated for 1 h prior to Aβ treatment. Neurons were incubated with 10 μM carbonyl cyanide p-trifluoro-methoxy-phenyl-hydradine (FCCP; Sigma-Aldrich) for 2 h to induce mitochondrial fragmentation. Neurons were treated with H2O2 (50 μM; BDH Chemicals) and rotenone (25 nM; Sigma-Aldrich) for 1 h to induce oxidative stress.

2.6. Confocal live cell imaging

Primary hippocampal neurons were grown on coverglass-bottom confocal dishes for confocal live cell imaging. Images were acquired with a LSM 510 Meta inverted laser-scanning confocal microscope (Carl Zeiss) using a ×63 1.4 NA Apochromat objective (Carl Zeiss), with an incubation chamber maintained at 37 °C with 5% CO2 during the experiments. Confocal Z-stack images were acquired at 0.4 μm intervals for 7 stacks. The excitation wavelengths were 488 nm for GFP; and 543 nm for DsRed2, MitoSOX™ Red, and Rhod-2. TMRE was excited at 800 nm by two-photon excitation (Chameleon, Coherent Inc.).

For light-induced ROS production experiments using mito-KillerRed, regions of interest were manually defined using the LSM 510 software (Carl Zeiss). To induce light-sensitive ROS production in mitochondria, a small region of one z-plane was irradiated using 100% power of the 800 nm laser for 30 min. The diffusion rate of mito-PAGFP fluorescence was determined by standard deviation of whole-cell fluorescence at each time point using the Multi-measure plugin of Image J (National Institutes of Health, NIH) as previously described [20]. The fluorescence recovery of mito-RFP in the irradiated region was measured using Image J (NIH) and normalized with a non-bleached area.

To study mitochondrial transport in neuronal processes, neurons
were transfected with mito-RFP. Images were acquired with an ×63 1.4 NA oil Apochromat objective (Carl Zeiss) using a Carl Zeiss Axio Observer inverted microscope equipped with a Perkin Elmer UltraView™ spinning disk confocal imaging system. Images were taken every 5 s using the 568 nm laser line of an Argon/Krypton laser (Perkin Elmer) for 150 cycles (~12 min) using the Metamorph software (Molecular Devices). Movement of mitochondria were measured using the Imaris software (Imaris 7.5.1, Bitplane) according to the manufacturer's instructions.

Post-acquisition image processing was performed using Adobe Photoshop (Adobe) and Image J (NIH). Pixel intensities of Rhod-2, MitoSOX™ Red and TMRE were measured using Image J (NIH) after background subtraction.

2.7. Quantitative mitochondrial morphometric analysis

For automated morphometric analysis, aspect ratio and circularity of individual mitochondria were measured using a modified version of the mitochondrial morphology plugin of Image J (NIH) developed by Dagda and colleagues [41]. Volume of mitochondria was measured using the Particle Analyser plugin for Image J (NIH). All measurements were calculated from 2-axis confocal stacks. To measure individual mitochondrial length, five 5 cm × 5 cm grids were randomly selected from each image and lengths of five randomly chosen mitochondria were measured in each grid (Photoshop, Adobe). Individual mitochondria were then sub-divided into three categories according to their lengths: short (< 0.87 µm), medium-sized (0.87–1.60 µm), and elongated (> 1.6 µm).

2.8. Quantitative mitochondrial functional analysis

Mitochondrial superoxide production was determined by the mitochondria-specific superoxide sensor MitoSOX™ Red (Life Technologies) according to the manufacturer's instructions. Briefly, hippocampal neurons were incubated with MitoSOX™ Red dye (2.5 µM) in dark at room temperature for 5 min, followed by washing with pre-warmed Hank's buffered saline solution (HBSS, pH 7.4; Life Technologies) supplemented with glucose (5.6 mM, Sigma-Aldrich). Mitochondrial Ca²⁺ concentrations were measured using the mitochondrial-specific fluorescent Ca²⁺ dye Rhod-2.A.M. (Rhod-2) (Life Technologies). To facilitate mitochondrial localization, hippocampal neurons were loaded with Rhod-2 (0.5 µM) in HBES (20 mM)–buffered Neurobasal™ medium in dark for 30 min on ice. The dye was then washed by pre-warmed glucose-supplemented HBSS. Mitochondrial membrane potential was monitored by the fluorescence intensity of Tetramethylrhodamine, ethyl ester (TMRE, Life Technologies). Briefly, TMRE (20 nM) was loaded to the cells and incubated in dark for 5 min at room temperature, followed by washing with pre-warmed glucose-supplemented HBSS. ADP/ATP ratio was measured using the ADP/ATP ratio assay kit (BioVision) according to the manufacturer's protocol.

2.9. SDS-PAGE and Western blotting

Primary hippocampal neurons were lysed in lysis buffer (10 mM Tris at pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₂HPO₄, 1% Triton X-100, 1% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich) as previously described [40]. The extracted proteins were diluted into indicated amounts and separated on 8–12.5% SDS/PAGE and transferred onto polyvinylidene difluoride (PVDF) blotting membranes (BioRad). The membranes were blocked in 5% non-fat milk (Cell Signaling) dissolved in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 for 1 h at room temperature and probed with different antibodies: anti-DLP1 (1:3000, BD Biosciences), anti-Mfn1 (1:1000, Novus Biologicals), anti-Mfn2 (1:1000, Sigma-Aldrich), anti-VDAC (1:1000, Cell Signaling), anti-cleaved-caspase-3 (Asp175) (1:1000, Cell Signaling), and anti-β-actin (1:10,000, Sigma-Aldrich). ECL (GE Healthcare) and Western Bright ECL (Advanta) were used to develop the film. Quantification analysis was performed on scanned films using Image J (NIH).

2.10. Mitochondrial fractionation

Mitochondrial fractionation was performed as previously described [42]. Briefly, neurons were lysed in mitochondrial fractionation lysis buffer (20 mM HEPES at pH 7.5, 250 mM sucrose, 20 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) on ice. The cell lysates were then centrifuged at 600 × g for 10 min at 4 °C to remove cell debris. Following centrifugation, the supernatant was transferred into a new tube and was centrifuged again at 7000 × g for 10 min at 4 °C. The resulting supernatant and pellet after centrifugation were collected as the cytosolic fraction and mitochondrial fraction, respectively.

2.11. Statistical analysis

All results are represented as mean ± S.E.M. for the indicated number (n) of independent experiments. All experiments were repeated for at least 3 times. One-way ANOVA test and Kruskal-Wallis test were used for statistical analysis when appropriate using the GraphPad Prism software (GraphPad). Tukey's (for one-way ANOVA) and Dunn's (for Kruskal-Wallis) multiple comparison tests were used as post hoc comparisons of individual groups. p values are as indicated in the figure legends.

3. Results

3.1. Aβ induces changes in mitochondrial morphology in cultured hippocampal neurons, which are morphologically distinct from mitochondrial fragmentation

Primary hippocampal neurons were transfected with mitochondria-targeted green fluorescent protein (mito-GFP) to visualize individual mitochondrion using confocal live cell imaging. In control neurons, mitochondria were mostly thread-like in shape, consistent with previous studies [43] (Fig. 1a). As early as 1–2 h following exposure to Aβ, a change in mitochondria morphology was observed as supported by a significant reduction in mitochondrial length when compared with controls (Fig. 1c). To further validate the changes in mitochondrial length, neurons were sub-divided into three groups (short, medium, elongated) according to the mean mitochondrial length [44]. There were significantly higher percentages of transfected neurons with "short" mitochondria following Aβ treatment (Fig. 1d). To investigate if the reduction in length was a result of change in shape or in size, the volume of mitochondria was measured with both total and individual volume of mitochondria, which were found to be stable when compared with control (Fig. 1e). Morphometric analysis revealed an approximate 20% reduction in mitochondrial aspect ratio (major axis: minor axis ratio; a measure of elongation) following exposure to Aβ, suggesting that Aβ-induced granular mitochondria exhibited a rather "compressed" and elliptical shape when compared to controls (Fig. 1f). Furthermore, Aβ-treated mitochondria had a significantly higher score in circularity when compared with controls (Fig. 1g). It is important to note that there was no significant change in the number of mitochondria per neuron when compared with control (Fig. 1h). Western blot analysis also revealed no significant change in the expression level of the mitochondrial marker voltage-dependent anion channel (VDAC) following exposure to Aβ (Fig. S2). These results suggest that there was no mitochondrial loss despite the morphological changes.

To determine whether Aβ-induced granular mitochondria were
distinct from spherical mitochondria found in apoptosis, potent mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) was used to induce fragmentation of the mitochondrial network. In FCCP-treated neurons, mitochondria showed a distinct spherical shape which were significantly shorter in length when compared with control (Fig. 1b-d). Notably, there was also a
reduction in both volume and number of mitochondria in FCCP-induced fragmented mitochondria (Fig. 1e & h; p < 0.001), which was not found to be the case in Aβ−treated neurons. In addition, FCCP-treated fragmented mitochondria displayed significantly lower aspect ratio and higher circularity than mitochondria in neurons treated with control and Aβ (Fig. 1f-g).

It is important to note that although there were significant changes in mitochondrial length, aspect ratio, and circularity in neurons after exposure to Aβ or FCCP, Aβ-induced granular mitochondria was significantly different from spherical mitochondria resulting from FCCP treatment in all shape parameters measured (Fig. 1). While FCCP-induced mitochondrial fragmentation showed a distinct spherical shape accompanied by a reduction in size, Aβ appeared to induce granular mitochondria without affecting mitochondria size (Fig. 1). Hence, it is evident that Aβ-treated granular mitochondria are morphologically distinct from classic mitochondrial fragmentation. Taken together, mitochondria changed from a long and interconnected tubular network to “granular-shaped” which are morphologically distinct from the classic spherical mitochondria in mitochondrial fragmentation (Fig. 1 & S1).

To further characterize if Aβ-induced changes in mitochondrial morphology is a transient process towards fragmentation, neurons were treated with Aβ for a longer period i.e. 48 h to reveal long-term effects of Aβ on mitochondrial morphology (Figs. S2–S3). Consistent with the presence of granular mitochondria at 4 h, changes in mitochondrial morphology in terms of length, aspect ratio and circularity with no significant changes in the total volume, number of mitochondria (Fig. S3) and mitochondrial content (Fig. S2) were observed when compared with the control. More importantly, mitochondria following 48 h of Aβ exposure were also found to be significantly different from FCCP-induced fragmented spherical mitochondria (Fig. S3b-g). These results confirmed that Aβ-induced granular mitochondria at early time points was not a momentary state to fragmentation and indeed persists for a long period of time in the presence of Aβ.

3.2. Granular mitochondria are functionally distinct from mitochondrial fragmentation

Mitochondrial fragmentation has often been linked with apoptosis [26]. Since granular and fragmented mitochondria shared certain degrees of morphological similarities i.e. reduction in aspect ratio and increased circularity, albeit to a different extent, we questioned whether granular mitochondria were also functionally impaired. In response to apoptotic stimuli, a reduction of mitochondrial membrane potential (ΔΨm) causes rupture of the outer mitochondrial membrane, which subsequently initiates the mitochondria-mediated intrinsic pathway of apoptosis [45]. Mitochondrial membrane potential was monitored by the fluorescence intensity of tetramethylrhodamine, ethyl ester (TMRE). While ΔΨm was significantly reduced in FCCP-treated mitochondria, ΔΨm remained intact within 6 h of Aβ treatment (Fig. 2a-b). Apart from changes in membrane potential if apoptosis really occurs, induction of apoptosis promotes the translocation of the proapoptotic Bcl-family protein Bax (B-cell lymphoma 2 (Bcl-2)-associated X protein) from the cytoplasm to mitochondria to form foci with Drp1 and Mfn2 to facilitate fission [46]. In order to investigate if Bax is associated with granular mitochondria, hippocampal neurons were co-transfected with EGFP-Bax and mito-RFP. It was found that following 1–6 h of Aβ treatment, Bax remained in the cytoplasm (Fig. 2d-e). In addition, there was no significant change in the expression levels of the apoptotic marker, cleaved caspase-3, following exposure to Aβ (Fig. 2f-g). These data demonstrated that Aβ-induced granular mitochondria were not associated with the initiation of apoptosis.

Although granular mitochondria were shown to be distinct from fragmented mitochondria found in apoptosis, we also examined other aspects of organelle functions in Aβ-induced granular mitochondria compared with FCCP-treated fragmented mitochondria. Production of ATP is an important indicator of mitochondrial function. While ATP production was disturbed in FCCP-treated fragmented mitochondria, as revealed by a significant increase in the ADP/ATP ratio, the ADP/ATP ratio remained stable after 1–6 h of Aβ treatment (Fig. 2c). This suggests that granular mitochondria maintained their ability to supply energy for neurons despite changes in morphology. The endoplasmic reticulum (ER)-mitochondria interaction is essential for proper function and morphology of the two organelles [47–49]. In addition, our laboratory has previously reported collapse of the ER network following Aβ treatment [40]. Owing to the intimate relationship between the two organelles, we investigated whether early change of mitochondrial morphology could cause dissociation of mitochondria from the ER, thereby affecting mitochondrial function. Primary hippocampal neurons were co-transfected with mito-GFP and ER-targeted RFP (ER-RFP). No significant change in the co-localization level between ER and mitochondria was detected after Aβ treatment, indicating that the interaction between the two organelles remained intact despite drastic changes in mitochondrial morphology (Fig. S4). In contrast, induction of mitochondrial fragmentation with FCCP substantially reduced the co-localization between the ER and mitochondria (Fig. S4). In sum, as opposed to fragmented mitochondria, granular mitochondria maintained functional integrity as well as ER-mitochondria tethering, and are not associated with apoptosis.

3.3. Aβ impairs mitochondrial dynamics without changes in fission/fusion protein expression

With no significant change in mitochondrial function in the presence of morphological changes, we went on to study whether changes in mitochondrial morphology was due to disruptions in fission and fusion, the two opposing processes that tightly regulate mitochondrial morphology [8]. Fission and fusion imbalance has also been implicated in a number of neurodegenerative diseases including AD [50].

In our study, expression levels of various fission/fusion-mediating proteins such as Drp1, OPA1, Mfn1, and Mfn2 all remained stable after 1–6 h of Aβ treatment (Fig. S5). Phosphorylation of Drp1 at Ser616 has been reported to activate Drp1-mediated fission and translocation of Drp1 has also been suggested to be a critical step to initiate fission [51]. However, no significant change in the level of phospho-Drp1Ser616 (Fig. S5) and the mitochondrial Drp1: cytosolic Drp1 ratio (Fig. S6b & d) was detected after Aβ treatment. These data suggest that Aβ-induced formation of granular mitochondria does not involve changes in the expression of common fission and fusion proteins.

Since expression of fission and fusion proteins in neurons was not altered after exposure to Aβ, mitochondrial dynamics was quantified in hippocampal neurons co-transfected with mito-RFP and mitochondria-targeted photoactivatable GFP (mito-PAGFP). The region of interest was simultaneously photobleached and photoactivated by two-photon excitation. Simultaneous photoactivation of mito-PAGFP and fluorescence recovery after photobleaching (FRAP) imaging of mito-RFP (SPA-FRAP) allows for a novel two-way measurement of mitochondrial dynamics. Both the diffusion rate of mito-PAGFP and fluorescence recovery rate of mito-RFP were significantly slower than that of controls (Fig. 3a-c; p = 0.004 and p = 0.0139, respectively), suggesting that mitochondrial dynamics was impaired following Aβ treatments at early time points. Although axonal transport of mitochondria appears to slow down after Aβ treatment, mitochondria speed was not significantly different from control (Fig. 3d).

Therefore, although no changes in the expression of fission/fusion-mediating proteins were found, there was a significant impairment in mitochondria dynamics in Aβ treated neurons.

3.4. Accumulation of reactive oxygen species in mitochondria as the triggering factor of granular mitochondria

Previous studies have implicated the role of oxidative stress in
a. Aβ treatment (h)

1. 0
2. 1
3. 2
4. 4
5. 6
6. FCCP

b.

TMR Fluorescence

% of control

0 1 2 4 6 FCCP

c.

ADP/ATP ratio

% of control

0 1 2 4 6 FCCP

d.

% of neurons with

mitochondria

0 1 2 4 6 FCCP

e. Aβ treatment (h)

0 1 2 4 6 FCCP

f.

| Treatment (h) | Aβ (μM) |
|--------------|---------|
| 4            | 0 10    |
| 48           | 0 10    |

Cleaved Caspase-3

β-actin

(g.)

Relative Cleaved Caspase-3 expression

Fold of control

0 4 48
**Fig. 2.** Aβ-induced granular mitochondria maintain functional integrity and are not associated with apoptosis. (a) Representative confocal images of hippocampal neurons loaded with TMRE, for the determination of mitochondrial membrane potential, and treated with 10 μM Aβ for the indicated periods (in h) or 10 μM FCCP for 2 h (Scale bar: 10 μm). (b) Statistical quantification of mitochondrial membrane potential for each condition, calculated from z-axis confocal stacks. **p < 0.001 compared with control, Kruskal-Wallis test. Data represent mean ± SEM of 4 independent experiments. (c) Statistical quantification of ADP/ATP ratio following 10 μM Aβ for the indicated periods (in h). *p < 0.05 compared with control, Kruskal-Wallis test. Data represent mean ± SEM of 4 independent experiments. (d) Quantification of ADP/ATP ratio following 10 μM Aβ for the indicated periods (in h). *p < 0.05 compared with control, Kruskal-Wallis test. Data represent mean ± SEM of 6 independent experiments. (e) Quantification of the percentage of neurons with mitochondrial Bax in primary hippocampal neurons co-transfected with EGFP-Bax and mito-RFP and treated with 10 μM Aβ for the indicated time (h). (f) Western blot analysis of cleaved caspase-3 in primary hippocampal neurons treated with 10 μM Aβ for 4 and 48 h. Quantification of western blot analysis showed no significant difference in the expression level of cleaved caspase-3 in neurons treated with 10 μM Aβ for 4 and 48 h (p = 0.1073), Kruskal-Wallis test. Data represent mean ± SEM of at least 3 independent experiments.

**Fig. 3.** Aβ-induced increase in mitochondrial superoxide, and impairment in mitochondria dynamics. (a-b) Representative confocal images of hippocampal neurons loaded with MitoSOX™ Red to detect superoxide concentration in mitochondria, and treated with 10 μM Aβ for the indicated periods (in h) (Scale bar: 10 μm). Statistical quantifications of MitoSOX™ Red fluorescence intensity for each condition, calculated from z-axis confocal stacks, respectively. **p < 0.001 compared with control; Kruskal-Wallis test and one way ANOVA test where applicable. Data represent mean ± SEM of 3 independent experiments. (c-e) Quantification of mitochondrial dynamics by simultaneous photoactivation and fluorescence recovery after photobleaching (SPA-FRAP). Representative confocal images of primary hippocampal neurons co-transfected with mito-RFP and mito-PAGFP and treated with 10 μM Aβ for 4 h (Scale bar: 10 μm). The region of interest (as shown by the boxed area) was irradiated with an 800 nm laser using two-photon excitation to achieve photobleaching of mito-RFP and photoactivation of mito-PAGFP simultaneously. The irradiation site is shown in the magnified image. (d-e) Quantification of mitochondrial dynamics using SPA-FRAP by measuring the diffusion rate of mito-PAGFP fluorescence and recovery rate of mito-RFP fluorescence (p = 0.004 and p = 0.0139 respectively). Data represent mean ± SEM of at least 4 independent experiments. (f) Quantification of mitochondria axonal transport following Aβ treatment. No significant change in the speed of mitochondria was detected following 4 h Aβ treatment. p = 0.1658 compared with control, Mann Whitney test. Data represent mean ± SEM of 5 independent experiments.
Fig. 4. Oxidative stress induces changes in mitochondria morphology comparable to Aβ treatment. (a & d) Representative confocal images of hippocampal neurons expressing mito-GFP treated with H$_2$O$_2$ and rotenone at the specified concentrations for 1 h, respectively (Scale bar: 10 µm). Images in the bottom row represent magnification of the boxed areas in the upper row (Scale bar: 2 µm). (b-c & e-f) Quantification of mitochondria volume and aspect ratio in neurons treated with H$_2$O$_2$ and rotenone, respectively. ***p < 0.001 compared with control, Kruskal-Wallis test, Dunn’s multiple comparison post-hoc test. Data represent mean ± SEM of at least 3 independent experiments. (g) Representative confocal images of primary hippocampal neurons co-transfected with mito-GFP and mitochondria-targeted photosensitizer mito-KillerRed. The irradiated area is indicated with a square, which is shown in the magnified image. Representative images from 3 independent experiments are shown.
Interestingly, overexpression of Drp1K38A also inhibited the morphological change of mitochondria into granular shape which are morphologically and functionally distinct from spherical mitochondria seen in classic apoptosis-related mitochondrial fragmentation. Most importantly, we have shown a reciprocal relationship between ROS and mitochondrial dynamics at early stages of neurodegeneration. Mitochondrial ROS is found to disturb mitochondrial dynamics and play an important role in the induction of granular mitochondria by Aβ. MitoTEMPO attenuates the induction of mitochondria following Aβ treatment. Conversely, genetic and pharmacological manipulation of fission also diminishes the level of ROS in mitochondria. The induction of granular mitochondria by Aβ at early time points is likely to be reversible and normal tubular mitochondria morphology can be restored by manipulations of mitochondrial ROS as well as mitochondrial dynamics.

Mitochondria are particularly important in neurons owing to the high-energy demand for neuronal synaptic transmission [58]. Mitochondrial dysfunction, often described as impairment of the electron transport chain, increased production of ROS and altered mitochondrial dynamics, is a prominent feature in neurodegeneration [2,3]. In particular, recent studies have highlighted the crucial role of mitochondrial dynamics in health and diseases [59-61]. In normal conditions, the dynamic feature of mitochondria enables them to travel along the axons and deliver energy to the synapses located at the far ends of a neuron [62,63]. In response to apoptotic stimuli, as seen in neurodegeneration, mitochondria undergo fragmentation in which filamentous mitochondria break into punctate spheres [64].

Traditionally, apoptosis-associated fragmentation of the mitochondrial network has been seen as a sign of mitochondrial dysfunction at late stages of neurodegeneration. However, disturbance in mitochondrial dynamics and oxidative damage have been shown as early events in AD pathogenesis prior to substantial neuronal death [65]. In this study, we have demonstrated that a change in mitochondrial shape at early stages of neurodegeneration does not necessarily result in mitochondrial dysfunction. Notably, we characterized a unique form of granular mitochondria induced by Aβ at early time points which is distinct from Aβ-triggered mitochondrial fragmentation as previously described (Fig. 1). Despite the change in morphology, ΔΨm and ATP production remained stable at early time points following Aβ treatment, implying that granular mitochondria are not functionally defective (Fig. 2). Furthermore, ER-mitochondria co-localization remained unchanged (Fig. S4). Thus, Aβ-induced granular mitochondria are morphologically and functionally distinct from classic mitochondrial fragmentation in apoptosis. Previous studies have emphasized on the relationship between mitochondrial fragmentation and organelle dysfunction in neurodegeneration. However, here we show that mitochondria maintain their normal functioning at early stages of disease despite a change in morphology. Indeed, this unique granular shape was also found following prolonged Aβ treatment and is not a mere transition state toward fragmentation (Fig. S3).

The purpose of mitochondrial remodelling into granular shape remains elusive. Recent reports have shown that in stress conditions and starvation-induced macroautophagy, mitochondria elongate to promote cell survival and protect themselves from autophagosomal degradation [20,44]. Furthermore, mitochondria have been shown to display a “donut-like” toroidal shape during hypoxia-reoxygenation stress [21]. It is possible that different cellular stressors at different stages of disease progression induce different changes in mitochondria morphology as a protective mechanism. Interestingly, in primary cultured neurons under the same treatment condition, those exhibit mitochondrial Bax showed a significantly lower mitochondrial aspect ratio than those with cytosolic Bax (Fig. S9). This implies that the transition of mitochondrial morphology to granular shape might be a possible protective mechanism for neurons. Mitochondrial network remodelling to
irreversible fragmentation as observed in neurodegeneration may not be a straightforward process.

The morphology of mitochondria is tightly regulated by the balance between fission and fusion. Although we found no change in the expression level of common fission and fusion proteins (Fig. 3a), Aβ was confirmed to disrupt mitochondrial dynamics by imaging experiments (Fig. 3b-d). Traditionally, mitochondrial dynamics are quantified using FRAP imaging or photoactivable GFP, independently [20,66,67]. To the

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mito-RFP and treated with 10 nM mitoTEMPO or mitochondria-targeted superoxide scavenger mitoTEMPOL or mitochondrial division inhibitor 1 (mdivi-1) for 1 h as indicated (Scale bar: 10 μm). Images in the bottom row represent magnification of the boxed areas in the upper row (Scale bar: 2 μm). (d) Quantification of mitochondrial aspect ratio with or without 10 μM Aβ (4 h) treatment and mdivi-1 (1 h) or mitoTEMPO (1 h) pre-treatment as indicated. p < 0.05, ***p < 0.001, Kruskal-Wallis test and Dunn’s multiple comparison post-hoc test where applicable. Data represent mean ± SEM of at least 4 independent experiments. (e) Representative confocal images of hippocampal neurons co-transfected with ECFP-Drp1K38A and mito-RFP and treated with 10 μM Aβ (4 h) (Scale bar: 10 μm). X axis is magnified in the image below. (f) Quantification of mitochondrial aspect ratio in primary hippocampal neurons co-transfected with ECFP-Drp1K38A and mito-RFP and treated with 10 μM Aβ (4 h). Data represent mean ± SEM of at least 3 independent experiments. (g) Representative confocal images of MitoSOX Red loaded hippocampal neurons with and without transfection with ECFP-Drp1 or after treatment with 10 μM Aβ (4 h) (Scale bar: 10 μm). (h) Quantification of MitoSOX Red fluorescence intensity with or without 10 μM Aβ, mdivi-1, or mitoTEMPO treatment as indicated. p < 0.05, ***p < 0.001, one way ANOVA test and Tukey’s multiple comparison post-hoc test where applicable. Data represent mean ± SEM of at least 4 independent experiments. (i) Representative confocal images of hippocampal neurons co-transfected with ECFP-Drp1K38A and mito-RFP and treated with 10 μM Aβ (4 h) (Scale bar: 10 μm). Boxed area is magnified in the image below. (j) Quantification of mitochondrial aspect ratio in primary hippocampal neurons co-transfected with ECFP-Drp1K38A and mito-RFP and treated with 10 μM Aβ (4 h) (10 μM). Data represent mean ± SEM of at least 3 independent experiments. (k) Representative confocal images of MitoSOX Red loaded hippocampal neurons with and without transfection with ECFP-Drp1 or after treatment with 10 μM Aβ (4 h) (Scale bar: 10 μm). (l) Quantification of MitoSOX Red intensity in 10 μM Aβ (4 h) treated primary hippocampal neurons with and without ECFP-Drp1 expression. ***p < 0.001, Kruskal-Wallis test and Dunn’s multiple comparison post-hoc test where applicable. Data represent mean ± SEM of at least 3 independent experiments.

Fig. 5. Aβ-induced ROS accumulation and change in mitochondria morphology can be restored with mitochondria-targeted superoxide scavenger mitoTEMPO and pharmacological and genetic manipulations of Drp1 activity. (a) Representative confocal images of primary hippocampal neurons, expressing MitoSOX™ Red, treated with 10 μM Aβ for 4 h and pre-treated with 2.5 μM mitochondria-targeted superoxide scavenger mitoTEMPO or 5 μM mitochondrial division inhibitor 1 mdivi-1 for 1 h as indicated. (b) Quantification of MitoSOX™ Red fluorescence intensity with or without 10 μM Aβ, mdivi-1, or mitoTEMPO treatment as indicated. p < 0.05, ***p < 0.001, Kruskal-Wallis test and Dunn’s multiple comparison post-hoc test where applicable. Data represent mean ± SEM of at least 4 independent experiments (c) Representative confocal images of primary hippocampal neurons, expressing mito-GFP, treated with 10 μM Aβ for 4 h and pre-treated with mitochondria-targeted superoxide scavenger mitoTEMPO or mitochondrial division inhibitor 1 (mdivi-1) for 1 h as indicated (Scale bar: 10 μm). Images in the bottom row represent magnification of the boxed areas in the upper row (Scale bar: 2 μm). (d) Quantification of mitochondrial aspect ratio with or without 10 μM Aβ (4 h) treatment and mdivi-1 (1 h) or mitoTEMPO (1 h) pre-treatment as indicated. p < 0.05, ***p < 0.001, one way ANOVA test and Tukey’s multiple comparison post-hoc test where applicable. Data represent mean ± SEM of at least 4 independent experiments. (e) Representative confocal images of hippocampal neurons co-transfected with EFPG-Drp1K38A and mito-RFP and treated with 10 μM Aβ (4 h) (Scale bar: 10 μm). X axis is magnified in the image below. (f) Quantification of mitochondrial aspect ratio in primary hippocampal neurons co-transfected with ECFP-Drp1K38A and mito-RFP and treated with 10 μM Aβ (4 h). Data represent mean ± SEM of at least 3 independent experiments. (g) Representative confocal images of MitoSOX™ Red loaded hippocampal neurons with and without transfection with ECFP-Drp1K38A after treatment with 10 μM Aβ (4 h) (Scale bar: 10 μm). (h) Quantification of MitoSOX™ Red intensity in 10 μM Aβ (4 h) treated primary hippocampal neurons with and without ECFP-Drp1K38A expression. ***p < 0.001, Kruskal-Wallis test and Dunn’s multiple comparison post-hoc test where applicable. Data represent mean ± SEM of at least 3 independent experiments. (i) Representative confocal images of MitoSOX™ Red loaded hippocampal neurons with and without transfection with ECFP-Drp1 or after treatment with 10 μM Aβ (4 h) (Scale bar: 10 μm). (j) Quantification of MitoSOX™ Red fluorescence intensity with or without 10 μM Aβ, mdivi-1, or mitoTEMPO treatment as indicated. p < 0.05, ***p < 0.001, one way ANOVA test and Tukey’s multiple comparison post-hoc test where applicable. Data represent mean ± SEM of at least 3 independent experiments.
unique reciprocal relationship between mitochondrial dynamics and reactive oxygen species and provides a new possible therapeutic target at early stages of neurodegenerative disease pathogenesis.

Author's contributions

CHLH and RCCC conceived the study and designed the experiments. CHLH performed the experiments and data analysis. CHLH and YTC. performed the mitochondrial morphometric analysis. CHLH and SSYC participated in data interpretation. CHLH, SSYC and RCCC wrote the manuscript with help from all authors. YSH, SMYL and RCCC obtained funding to support the study.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.08.010.

References

[1] S.J. Balyasny, Mitochondrial alterations in Alzheimer’s disease, J. Alzheimer's Dis. 9 (2006) 119–126.
[2] M.F. Beal, Mitochondria take center stage in aging and neurodegeneration, Ann. Neurol. 58 (2005) 495–505.
[3] M.T. Lin, M.F. Beal, Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases, Nature 443 (2006) 787–795.
[4] F.A. Cabezas-Opazo, K. Vergara-Pulgar, M.J. Perez, C. Jara, C. Osorio-Fuentealba, R.A. Quintanilla, Mitochondrial dysfunction contributes to the pathogenesis of Alzheimer’s disease. Oxid. Med. Cell. Longev. 15 (2015) 509654.
[5] X. Zhu, G. Perry, M.A. Smith, X. Wang, Abnormal mitochondrial dynamics in the pathogenesis of Alzheimer’s disease. J. Alzheimer's Dis. 33 (Suppl. 1) (2013) S253–S262.
[6] M.H. Yan, X. Wang, X. Zhu, Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease, Free Radic. Biol. Med. 62 (2013) 90–101.
[7] M. Amiri, P.J. Hollenbeck, Mitochondrial biogenesis in the axons of vertebrate neurons, Curr. Opin. Neurobiol. 19 (2009) 502–509.
[8] J. Bereiter-Hahn, M. Voth, Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria, Microsc. Res Tech. 27 (1994) 198–219.
[9] G. Tieg, A. Elorza, A.J. Molina, H. Mohamed, J.D. Wikstrom, G. Walser, L. Stiles, S.E. Haigh, S. Katz, G. Las, et al., Fission and selective fusion govern mitochondrial segregation and elimination by autophagy, EMBO J. 27 (2008) 433–446.
[10] H. Chen, S.A. Detmer, A.J. Ewald, E.E. Grippo, D.C. Chan, Mitofilins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development, J. Cell. Biol. 160 (2003) 189–200.
[11] S. Gopala, O. Martinis de Brito, B. Dal Zilio, L. Scorrano, OPA1 requires mitofusin 1 to promote mitochondrial fusion, Proc. Natl. Acad. Sci. USA 101 (2004) 15907–15912.
[12] F. Legros, A. Lombes, P. Frachon, M. Rojo, Mitochondrial fusion in human cells is efficient, requires the inner membrane potential, and is mediated by mitofusins, Mol. Biol. Cell 13 (2002) 4343–4354.
[13] M. Rojo, F. Legros, D. Chateau, A. Lombes, Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo, J. Cell. Sci. 115 (2002) 1663–1674.
[14] A. Santel, M.T. Fuller, Control of mitochondrial morphology by a human mitofusin, J. Cell. Sci. 114 (2001) 867–874.
[15] S. Gandre-Babek, A.M. van der Bliik, The novel tail-anchored membrane protein Mfn controls mitochondrial and peroxisomal fission in mammalian cells, Mol. Biol. Cell 19 (2008) 2402–2412.
[16] D.I. James, P.A. Parone, Y. Mattenberger, J.C. Martinou, hFis1, a novel component of the mammalian mitochondrial fission machinery, J. Biol. Chem. 278 (2003) 36373–36379.
[17] H. Otera, C. Wang, M.M. Cleland, K. Setoguchi, S. Yokota, R.J. Youle, Mitochondria are spared from degradation and sustain cell viability, Nat. Cell. Biol. 13 (2011) 589–598.
[18] X. Liu, G. Hajnoczy, Altered fusion dynamics underlie unique morphological changes in mitochondria during hypoxia-reoxygenation stress, Cell Death Differ. 18 (2011) 1561–1572.
[19] J. Bereiter-Hahn, M. Voth, Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria, J. Biol. Chem. 284 (2009) 13843–13855.
[20] L. Wang, L. Guo, L. Lu, H. Sun, M. Shao, S.J. Beck, L. Li, J. Ramachandran, Y. Du, Mitochondrial dynamics changes with age in an APPswe/PS1dE9 mouse model of Alzheimer’s disease, Neureport 28 (2017) 222–228.
[21] M.J. Calkins, P.H. Reddy, Amyloid beta impairs mitochondrial anterograde transport and degrades synapses in Alzheimer’s disease neurons, Biochim. Biophys. Acta 1812 (2011) 507–513.
[22] L.I. Xu, Y. Shen, X. Wang, W. Wei, P. Wang, H. Yang, C.F. Wang, Z.H. Xie, J.Z. Bi, Mitochondrial dynamics changes with age in an APPswe/PS1dE9 mouse model of Alzheimer’s disease, Neureport 28 (2017) 4515–4529.
