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Mitochondrial Movement and Number Deficits in Embryonic Cortical Neurons from 3xTg-AD Mice

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Abstract

Mitochondrial dysfunction is often found in Alzheimer's disease (AD) patients and animal models. Clinical severity of AD is linked to early deficiencies in cognitive function and brain metabolism, indicating that pathological changes may begin early in life. Previous studies showed decreased mitochondrial function in primary hippocampal neurons from triple-transgenic Alzheimer's disease (3xTg-AD) mice and mitochondrial movement and structure deficits in primary neurons exposed to amyloid- β oligomers. The present study characterized mitochondrial movement, number, and structure in 3xTg-AD primary cortical neurons and non-transgenic (nonTg) controls. We found a significant reduction in mitochondrial number and movement in 3xTg-AD primary cortical neurons with modest structural changes. Additionally, application of the sigma-1 receptor agonist, (+)SKF-10,047, markedly increased mitochondrial movement in both 3xTg-AD and nonTg primary cortical cultures after one hour of treatment. (+)SKF-10,047 also led to a trend of increased mitochondrial number in 3xTg-AD cultures. Embryonic mitochondrial movement and number deficits could be among the key steps in the early pathogenesis of AD that compromise cognitive or metabolic reserve, and amelioration of these deficits could be a promising area for further preclinical and clinical study.

Keywords

Alzheimer's disease; mitochondrial dynamics; mitochondrial size; sigma receptors

INTRODUCTION

Alzheimer's disease (AD) is characterized by a progressive decline in cognition, atrophy of cerebral cortex and hippocampus, loss of synapses, and disruption of neuromodulatory

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SUPPLEMENTARY MATERIAL

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systems [1, 2]. Histopathological hallmarks of AD include amyloid- β (A β) peptide aggregates and hyperphosphorylated tau-related neurofibrillary tangles, but the contribution of these neuropathological features to the emergence of AD symptoms, especially cognitive decline, is still unclear [3]. Growing evidence suggests that mitochondrial dysfunction is a critical step in the pathological cascade leading to the AD phenotype [4, 5]. AD patients and at-risk individuals show early regional hypometabolism throughout the cerebral cortex and hippocampus [6]. Mitochondrial structural abnormalities including reduced mitochondrial size have also been reported in brains of AD patients [7, 8] and mouse models [9]. Reduced levels and activity of tricarboxylic acid cycle enzymes in AD brain tissue have been reported [10], and there is a reduction in mitochondrial number in pyramidal neurons in AD patients, corresponding to an increase in mitochondrial DNA within autophagocytic cytoplasmic granules [11]. Reduced mitochondrial number was also seen in M17 cells overexpressing wild-type or mutant APP [12]. Further, AD patients often harbor mitochondrial DNA mutations or deletions [13].

The triple transgenic AD (3xTg-AD) mouse has three mutations (PS1_{M146V}/APP_{Swe}/tau_{P301L}) associated with early onset AD in humans [14]. This mouse mimics the human AD phenotype, with progressive A β production, tau hyperphosphorylation, and neurofibrillary tangle formation across the cortex and hippocampus as well as accelerated cognitive decline [15]. Like human patients, 3xTg-AD mice exhibit early bioenergetic deficits, including reduced ATP production in embryonic hippocampal neurons [16]. We and others previously showed that exogenously applied A β oligomers can impair mitochondrial movement and cause a reduction in mitochondrial size in primary hippocampal neurons [17–19].

3xTg-AD primary cortical neurons express detectable levels of A β as early as 7 days *in vitro* (DIV), and A β increases with time in culture [20]. Prior to this study, it was not known whether the early expression of A β in 3xTg-AD neurons would cause the same mitochondrial phenotypes seen in primary neurons exposed to exogenous A β . To probe this question, we measured mitochondrial structure, number, and movement in primary cortical neurons from 3xTg-AD mice and non-transgenic (nonTg) controls. We hypothesized that 3xTg-AD neurons would exhibit impairment in mitochondrial movement along neurites, reduced mitochondrial size, and increased roundness compared to nonTg mice. We also hypothesized that there would be decreased mitochondrial number in 3xTg-AD neurons similar to the reduction observed in human AD patients [11].

Compounds which act to prevent or counteract mitochondrial deficits could be useful tools to protect neurons from the irreversible damage that occurs in the progression of AD. Sigma-1 receptor (σ_1 R) agonists are a promising drug class to achieve enhanced mitochondrial function among other neuroprotective effects [21]. The σ_1 R is a chaperone protein present in neurons within mitochondrial, nuclear, and plasma membranes. In the inactive state, σ_1 Rs are found in the mitochondrial associated membrane (MAM) of the endoplasmic reticulum (ER) bound to the heat shock 70 protein BiP [22]. Once activated, σ_1 Rs dissociate from BiP and increase Ca²⁺ efflux from the ER through IP3 receptors at the MAM [23, 24]. Mitochondria then take up Ca²⁺ leading to activation of tricarboxylic acid cycle enzymes and enhancement of oxidative phosphorylation. Although exogenous σ_1 R ligands are not related structurally, several have been identified as selective, high affinity

σ_1 R agonists, such as (+)SKF-10,047 [(+)-N-Allylnormetazocine hydrochloride], a member of the benzomorphan drug class. σ_1 R agonists have shown promise for treatment of AD in preclinical studies [25–27], but whether they exert these effects through mitochondrial mechanisms remains to be fully elucidated.

To gain more insight into this question, we studied the effects of σ_1 R activation on mitochondrial structure, number, and movement in primary cortical neurons from 3xTg-AD and nonTg mice using (+)SKF-10,047. Movement of mitochondria along microtubules is carried out through kinesin and dynein proteins, which require ATP for their function [28]. Decreased ATP production, as seen in AD neurons, could impair mitochondrial movement. σ_1 R activation can increase oxidative phosphorylation, which could provide added energy for mitochondrial movement. Therefore, we hypothesized that (+)SKF-10,047 would increase mitochondrial movement in primary cortical neurons. Additionally, σ_1 R agonists can protect against the intracellular stressors that lead to mitochondrial fragmentation and reduced mitochondrial number through multiple downstream effects of σ_1 R activation [29]. We hypothesized that (+)SKF-10,047 would increase mitochondrial size and number in primary cortical neurons while reducing mitochondrial roundness.

MATERIALS AND METHODS

Mice

Animal protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of West Virginia University. Homozygous 3xTg-AD and nonTg controls (C57BL6/129S) were obtained from The Jackson Laboratory, and colonies were bred and maintained at West Virginia University. Animals were housed in cages of up to five animals on a 12 h light cycle with *ad libitum* access to food and water.

Primary cortical culture

Pregnant mice were anesthetized with isoflurane and euthanized by cervical dislocation at E18.5. Brains of pups were removed and placed in ice-cold Hank's Balanced Salt Solution (HBSS). Cortices were dissected and meninges were removed with fine forceps before transferring to neurobasal culture medium (Gibco) supplemented with 2% B-27 (Gibco), 1% pen-strep (HyClone), and 1% Glutamax (Gibco). Cortices were triturated 10–15 times with a 5 mL pipette followed by a series of three flame-polished Pasteur pipettes with increasingly narrow tip diameter before being filtered through a 70 μ m nylon cell strainer (Falcon). Cells were plated on poly-L-lysine (Sigma) coated 35 mm glass-bottom dishes (glass diameter 23 mm, thickness 0.17 mm; World Precision Instruments) at a density of 7.5×10^5 cells in 2 mL of medium and maintained in a 95% humidity, 5% CO₂, 37°C incubator for up to two weeks. At 2 DIV, 1-beta-D-arabinofuranosylcytosine (AraC; Sigma) was added at a final concentration of 4 μ mol/L to inhibit glial growth. Subsequently, half of the culture medium was replaced every three days.

Mitochondrial labeling

Mitotracker® Red CMXRos—Immediately prior to imaging, media was replaced with 80 nmol/L Mitotracker® Red CMXRos (MT-Red; Thermo Fisher Scientific) in complete neurobasal media and incubated for 15 min at 37°C. MT-Red solution was then removed and replaced with the culture-conditioned media in which cells had been growing prior to staining.

pDsRed2-Mito vector—pDsRed2-Mito vector (Clontech) was amplified using GT115 competent *E. coli* cells (InvivoGen) and isolated with a Midiprep kit (Thermo Fisher Scientific). For each transfection, 8 µg of plasmid DNA was diluted in 100 µL serum-free Opti-MEM I (Thermo Fisher Scientific). 10 µL Lipofectamine-2000 (Thermo Fisher Scientific) was diluted in 100 µL serum-free Opti MEM I and allowed to stand for 10 min at room temperature. The plasmid and lipofectamine solutions were combined, gently mixed, and allowed to stand for 20 min at room temperature before application to cultures. Cells were transfected at 12.5 DIV. Cultures were imaged 36 h post-transfection.

Preparation of (+)SKF-10,047—(+SKF-10,047 (Tocris) was dissolved in sterile milliQ water to create a 10 mM stock solution which was aliquoted and stored at –20°C. Just prior to application to cells, a 2 µmol/L working solution was made by diluting the stock solution 1:5000 in pre-warmed complete neurobasal media. Vehicle controls (Veh) were made by diluting sterile milliQ water 1:5000 in pre-warmed complete neurobasal media.

Live-cell imaging—The investigator performing imaging experiments was blinded to treatment group (Veh or SKF). Wide-field imaging was performed on a Nikon Eclipse TE2000-E microscope with an oil immersion 60× Apochromat DIC objective (Nikon) with 1.49 numerical aperture. Neither a polarizer nor an analyzer was used. The microscope's 1.5× magnification lens was used to give a final magnification of 90×. A halogen light source was used with an ND4 neutral density filter, an ET555/25× excitation filter, and an ET605/52 m emission filter (Chroma Technology Corp.) to image mitochondria stained with MT-Red or pDsRed2-Mito vector. For comparisons of 3xTg-AD and nonTg cultures at 12–14 DIV, fluorescence images were captured with an exposure time of 60 ms (MT-Red-stained cultures) or 300 ms (pDsRed2-Mito-stained cultures) on a CoolSNAP monochrome CCD camera (Photometrics) with 2 × 2 binning and 1024 × 1024 resolution giving a pixel size of 141 nm. For experiments at 7–9 DIV, fluorescence images were captured with an exposure time of 60 ms on an ORCA CMOS camera (Hamamatsu) with 2 × 2 binning and 2048 × 2048 resolution giving a pixel size of 72 nm. During imaging, cultures were maintained in a humidified digital stage-top incubator (OKO Labs) at 37°C and 5% CO₂.

Imaging of DIV12–14 cultures—For structural imaging of MT-Red-stained DIV12–14 cultures, eight single-plane images of 143.36 µm × 143.36 µm were captured at equidistant points along a circle midway between the center of the culture and the culture edge. For DIV14 pDsRed2-Mito-transfected cultures, an individual transfected neuron was selected. Time-lapse images from five non-overlapping fields of view containing separate proximal neurites at varying distances within 10–100 µm of the cell body were acquired every 15 s for 1 h.

Imaging of DIV7–9 cultures and treatment with (+)SKF-10,047—For DIV7–9 cultures, time-lapse images were acquired at five non-overlapping fields of view approximately 250 μm apart every 30 s for 30 min starting at a point halfway between the center of the culture and the culture edge. Fifteen single-plane images were then captured along a circle halfway between the edge and center of culture. Half of the culture medium was then replaced with vehicle or (+)SKF-10,047-containing medium to achieve a final concentration of 1 $\mu\text{mol/L}$. We chose this concentration based on the EC_{50} value of 0.8 $\mu\text{mol/L}$ (+)SKF-10,047 previously reported for *in vitro* studies of neuroprotection [30]. One-hour post-treatment, fifteen more single-plane images were captured halfway between the center of the culture and the culture edge at points that had not previously been imaged. Finally, a 30 min post-treatment time-lapse series was captured in the same way as pre-treatment comprising five fields of view that had not been previously imaged separated by approximately 250 μm .

Image analysis

Investigators performing data analysis were blinded to treatment group and genotype.

Mitochondrial structure and number—Images were exported from their native ND2 formats to 16-bit TIFF files using NIS elements (Nikon). Mitochondrial size, width, and number were calculated using an automated Matlab program generously provided by P.M. McClatchey which has been previously described [31] (Supplementary Figure 1A). Mitochondrial roundness (inverse of aspect ratio) was calculated by dividing width by height, with a value of 1 corresponding to a perfect circle and 0 corresponding to an infinitely long polygon in 1 dimension. A total of four fields of view per culture were used for DIV12–14 cultures and eight fields of view were used for DIV7–9 cultures. Odd numbered images were selected for analysis unless they contained putative glial cells, as identified by lack of neurites and a reticular network of mitochondria (Supplementary Figure 1B). If a glial cell was present, the closest even-numbered image was used for analysis. Individual mitochondrial measures from all four (DIV12–14) or eight (DIV7–9) fields of view of a culture were combined and averaged to give average length, width, and roundness for that culture. The total number of mitochondria was divided by the combined imaging area to yield number of mitochondria per square micron. Each culture was treated as an independent n.

Mitochondrial movement analysis in pDsRed2-Mito-transfected neurons—

Analysis was carried out in NIS elements (Nikon). Time-lapse images were aligned, and the advanced de-noising function was used with de-noising power of 50 followed by rolling ball background subtraction (radius = 1 μm). Five 1.1 μm by 6 μm regions of interest (ROIs) were placed orthogonal to proximal neurites between 10 μm and 100 μm from the cell body. The ROI width was chosen based on a previously described method for tracking mitochondrial occupancy along neurites [32]. A time measurement of maximum fluorescence intensity within ROIs was then performed. Only the first 15 min of imaging were included to minimize potential phototoxicity effects. Mitochondrial movement events were defined as an increase or decrease of maximum fluorescence intensity of at least 200 fluorescence units over 30 s or less, corresponding to mitochondrial movement into or out of

the ROI (Supplementary Figure 2A). Mitochondrial transit events, defined as a movement event in which a mitochondrion passed completely from one side of the ROI to the other, were manually counted (Supplementary Figure 2B).

Mitochondrial movement analysis in Mitotracker® Red CMXRos-stained

neurons—Time-lapse images were aligned in NIS elements and exported as stacks of 16-bit TIFF files. Despeckling, background subtraction (rolling ball method; radius = 20 pixels), and auto-thresholding using the Otsu method were performed in FIJI (NIH) to create binary masks. One 300 pixel by 300 pixel (21.6 $\mu\text{m} \times 21.6 \mu\text{m}$) subregion was randomly generated within each image using a random number generator in Excel (Microsoft) for x and y coordinates. If the subregion contained part of a cell body or out-of-focus material, a new region was randomly generated until only in-focus neurites were found within the region. The subregion was cropped and imported into Imaris (Bitplane). Manual spot tracking of every mitochondrion present within the subregion for at least two frames (1 min) was performed. Tracking consisted of placing a dot at the object's center of mass starting at the first frame where it was visible until it 1) disappeared from the edge of the field of view; 2) disappeared for more than 3 consecutive frames without reappearing in the same location during that time; or 3) fused with another object for at least 3 consecutive frames. In the case of fusions, the larger of the two objects continued to be tracked while the smaller object stopped being tracked at the time of fusion. If an object split from another object for at least 3 frames, it was counted as a new object and tracked from the first frame of clear separation from the other object. After all objects were tracked, measurements were exported and average velocity of each spot was calculated by dividing total displacement by track duration. One subregion was tracked in each field of view, and three fields of view were analyzed for each culture. All objects' velocities from the three fields of view were pooled and averaged to give an average mitochondrial velocity for each culture.

Statistical analysis

All statistical analyses were performed using Prism version 7.04 (GraphPad). Two-tailed unpaired *t*-tests were used to assess the effect of genotype on mean mitochondrial length, width, roundness, number/ μm^2 , movement events/min, transits/min, and velocity between nonTg and 3xTg-AD cultures with a significance level of $p < 0.05$. For structure and number measurements in DIV12–14 cultures, $n = 11$ nonTg and $n = 11$ 3xTg-AD cultures spread across three separate pregnancies each were compared. For movement measures in DIV14 cultures, $n = 8$ nonTg and $n = 7$ 3xTg-AD cultures (one neuron per culture) spread across three pregnancies each were compared. For structure, number, and movement measures in DIV7–9 cultures, pre-treatment values from $n = 6$ nonTg and $n = 6$ 3xTg-AD cultures from one pregnancy each were compared. Two-way analysis of variance (ANOVA) was used to assess effect of genotype (nonTg versus 3xTg-AD) and treatment (Vehicle or (+)SKF-10,047) on mitochondrial structure, number, and velocity 1 h post-treatment in DIV7–9 cultures with a significance level of $p < 0.05$. Two-tailed *t*-tests were used to probe effects of treatment within genotypes if significant interaction between genotype and treatment were observed with ANOVA. $n = 3$ cultures per group were used for each treatment and genotype and were derived from one pregnancy for each genotype.

RESULTS

Mitochondrial structure and number in 3xTg-AD cortical neurons at 12–14 DIV

Within individual cultures, mitochondria ranged widely in shapes and sizes including small spheres, larger ovoid shapes, rods of varying length, and branching filaments (Fig. 1A,B). However, average mitochondrial size, number, and roundness remained fairly consistent across different cultures within each group as long as a sufficient number of fields of view were sampled. At 12–14 DIV, mitochondrial length was significantly reduced in 3xTg-AD cultures ($t(20) = 5.108$, $p < 0.0001$) (Fig. 1C). Mitochondrial width showed a marginal tendency toward being increased in 3xTg-AD cultures ($t(20) = 2.024$, $p = 0.0565$) (Fig. 1D). Consistent with these observations, mitochondrial roundness was significantly increased in 3xTg-AD cultures compared to nonTg cultures ($t(20) = 5.568$, $p < 0.0001$) (Fig. 1E). The most pronounced difference between cultures was a decrease in mitochondrial number in 3xTg-AD cultures ($t(20) = 4.226$, $p = 0.0004$) (Fig. 1F).

Mitochondrial movement in neurites of 3xTg-AD cortical neurons at 14 DIV

Mitochondrial movement was assessed in neurites of pDsRed2-mito transfected neurons at 14 DIV using ROIs placed 10–100 μm from the cell body (Fig. 2A, B). Movement events were significantly reduced in 3xTg-AD neurites compared to nonTg controls ($t(13) = 2.942$, $p = 0.0115$) (Fig. 2C). There was also a reduction in number of passing mitochondria per minute in 3xTg-AD neurites ($t(13) = 3.069$, $p = 0.0090$) (Fig. 2D).

Mitochondrial structure and number in 3xTgAD cortical neurons at 7–9 DIV

Mitochondrial number and structure were compared in nonTg and 3xTg-AD neurons at 7–9 DIV (Fig. 3A, B). There was no significant difference in mitochondrial length between nonTg and 3xTg-AD cultures (Fig. 3C). Mitochondrial width was significantly increased in 3xTg-AD neurites at 7–9 DIV ($t(10) = 3.962$, $p = 0.0027$) (Fig. 3D). Mitochondrial roundness was slightly but significantly decreased in 3xTg-AD cultures ($t(10) = 3.609$, $p = 0.0048$) (Fig. 3E). Similar to DIV12–14 cultures, 3xTg-AD cultures at 7–9 DIV had significantly fewer mitochondria per square micron than nonTg cultures ($t(10) = 9.316$, $p < 0.0001$) (Fig. 3F). The percent decrease in mitochondrial number in 3xTg-AD cultures at 7–9 DIV was similar to the change at 12–14 DIV.

Mitochondrial movement in neurites of 3xTg-AD cortical neurons at 7–9 DIV and effect of (+)SKF-10,047

Mitochondrial velocity was measured before and 1 h post-treatment with vehicle or 1 $\mu\text{mol/L}$ (+)SKF-10,047 in nonTg and 3xTg-AD neurons at 7–9 DIV (Fig. 4A). The pre-treatment velocity of mitochondria in 3xTg-AD cultures was significantly lower than nonTg cultures ($t(10) = 12.9$, $p < 0.0001$) (Fig. 4B). Two-way ANOVA revealed a significant effect of both genotype ($F(1,8) = 72.8$, $p < 0.0001$) and (+)SKF-10,047 treatment ($F(1,8) = 20.67$, $p = 0.0019$) on mitochondrial velocity in cultured cortical neurons (Fig. 4C). There was no significant interaction between the effects. NonTg cultures exhibited markedly higher velocities of mitochondrial movement compared to 3xTgAD cultures under either treatment condition (Fig. 4C; upper inset). Additionally, (+)SKF-10,047-treated cultures exhibited

markedly higher velocities of mitochondrial movement compared to vehicle-treated cultures (Fig. 4C; lower inset).

Effect of (+)SKF-10,047 on mitochondrial structure and number in cortical neurons at 7–9 DIV

Mitochondrial number and structure measurements were taken 1 h post-treatment with vehicle or 1 $\mu\text{mol/L}$ (+)SKF-10,047 in nonTg and 3xTg-AD cultures (Supplementary Figure 3A). Two-way ANOVA of mitochondrial length showed a significant interaction between genotype and treatment ($F(1,8) = 7.122$, $p = 0.0284$). Probing this significant interaction, (+)SKF-10,047 treatment significantly increased length in nonTg cultures ($t(4) = 3.237$, $p = 0.0318$) but not in 3xTgAD cultures (Supplementary Figure 3B). There was a main effect of genotype on mitochondrial width ($F(1,8) = 30.25$, $p = 0.0006$) such that DIV7–9 3xTg-AD cultures were wider than nonTg cultures regardless of treatment condition (Supplementary Figure 3C). There was a main effect of (+)SKF-10,047 treatment on roundness ($F(1,8) = 24.54$, $p = 0.0011$) such that (+)SKF-10,047 significantly reduced roundness in both nonTg and 3xTg-AD cultures (Supplementary Figure 3D). There was a significant interaction ($F(1,8) = 13.4$, $p = 0.0064$) between genotype and treatment on mitochondrial number. NonTg cultures had no significant change in number after (+)SKF-10,047 while 3xTg-AD cultures trended toward increased number ($t(4) = 2.57$, $p = 0.0620$) at 1 h post-treatment (Supplementary Figure 3E).

DISCUSSION

This study demonstrates that 3xTg-AD primary cortical neurons have reduced mitochondrial number and movement at both 7–9 DIV and 12–14 DIV compared to nonTg controls. This observation adds to growing evidence that mitochondrial abnormalities early in life, perhaps even during development, are a key pathological feature of AD. One complication of traditional A β - and phospho-tau-centric hypotheses of AD pathophysiology is the fact that a subset of people develop AD pathology without cognitive symptoms [33]. It has been proposed that individuals with a more extensive “cognitive reserve” or “metabolic reserve” are resistant to clinical symptoms of AD in the face of AD pathology [34, 35]. These hypotheses focus on environmental influences on setting up cognitive and metabolic reserves, but it is also possible that early developmental factors could be involved.

Reduction in mitochondrial movement was the most pronounced difference between 3xTg-AD primary cortical neurons and nonTg controls. At 12–14 DIV, about half as many mitochondrial movement events occurred in neurites of 3xTg-AD cultures compared to nonTg controls. We also observed a large reduction in average mitochondrial velocity in neurites of 3xTg-AD neurons when examined at 7–9 DIV. Reduction in mitochondrial movement could lead to problems with distribution of mitochondria at proper locations along axons and dendrites, which could negatively impact synaptic transmission [28]. These deficits, over time, could potentially contribute the loss of synapses seen in AD.

Mitochondrial number was also consistently reduced in 3xTg-AD primary cortical neurons, which had about three-fourths the number of mitochondria per square micron of culture compared to nonTg controls. There was not a significant difference in the number of

neuronal cell bodies between 3xTg-AD and nonTg cultures at either 7–9 DIV or 12–14 DIV (data not shown). An early reduction in mitochondrial number and/or function could be detrimental to survival and normal differentiation of neurons. Some of the hallmarks of AD, including loss of synapses and reduction in grey matter volume, would be expected consequences of early mitochondrial dysfunction in neurons. Additional studies of changes in mitochondrial number in both the 3xTg-AD mouse and human patients at various stages of disease could help to determine if reduced mitochondrial number is a factor in the pathogenesis of AD. Additionally, studies which elucidate the mechanisms responsible for reduced mitochondrial number in 3xTg-AD primary neurons are needed.

Mitochondrial structure also differed between nonTg and 3xTg-AD primary cortical neurons, depending on time *in vitro*, but these differences were not as marked as differences in movement or number. 3xTg-AD cultures at 12–14 DIV had significantly shorter and rounder mitochondria. This would be expected given previous observations that exogenous treatment with A β oligomers causes reduced mitochondrial length [17, 18], although the change in the current study was not as marked as the change with exogenous A β application. On the other hand, reduction in mitochondrial length and increased roundness were not present at 7–9 DIV. Because A β expression increases with time in culture for 3xTg-AD neurons [20], it is possible that mitochondrial length reduction is dependent on the amount of intracellular A β present. We speculate that mitochondrial structural changes at 7–9 DIV could have been compensatory for the reduction in mitochondrial number.

Reversing mitochondrial abnormalities early in development could be a promising strategy for preventing the cascade of events that leads to clinical dementia later in life, including loss of synapses. The σ_1 R agonist (+)SKF-10,047 significantly increased mitochondrial movement in both nonTg and 3xTg-AD cortical neurons. In 3xTg-AD neurons there was a more than two-fold increase in mitochondrial velocity, which brought the final velocity closer to the baseline nonTg average velocity. The mechanism of this increase is unclear, but it is possible that the enhancement of mitochondrial function caused by σ_1 R activation [23, 24] could be responsible for the increased movement. Additionally, σ_1 R are known to translocate from the MAM to other membrane compartments of the cell, including mitochondria, upon stimulation with an agonist. σ_1 R could potentially interact with proteins within or adjacent to the mitochondrial membrane involved in mitochondrial translocation including dynein, members of the kinesin-1 family, and mitochondrial rho (MIRO) proteins among others. Of these, MIRO1 and MIRO2, which serve as adaptor proteins linking mitochondria to kinesin, are particularly promising candidates. It has been shown that σ_1 R bind to Rac1 GTPase in isolated brain mitochondria with increased binding after stimulation with sigma-1 agonists [36]. σ_1 R binding to MIRO could enhance movement by stabilizing MIRO-kinesin binding or increasing kinesin activity. Future studies are needed to document this.

(+)SKF-10,047 also led to a trend of increased mitochondrial number in 3xTg-AD neurons at 7–9 DIV after 1 h of treatment. This increase occurred too fast to be explained by mitochondrial biogenesis which relies on gene expression from nuclear DNA and protein production in the cytosol [37]. It is possible that reduced mitophagy triggered by σ_1 R activation could lead to increased mitochondrial number, as inhibition or loss of σ_1 R has

been shown to increase mitophagy [38]. Perhaps a change in fission/fusion dynamics could be occurring, but this would be expected to cause mitochondrial structure changes; (+)SKF-10,047 had minimal effects on mitochondrial structure in both nonTg and 3xTg-AD cortical neurons. One final possibility is that (+)SKF-10,047 increased mitochondrial membrane potential. Because Mitotracker® Red-CMXRos fluorescence intensity depends on mitochondrial membrane potential [39], an increase in mitochondrial membrane potential could cause previously undetected depolarized mitochondria to increase their signal.

The effects of (+)SKF-10,047 on mitochondrial movement and number observed in primary neurons warrant future studies of the mechanisms of action of σ_1 R in relation to mitochondrial movement, biogenesis, and function. The potential mechanisms of action of (+)SKF-10,047 on increasing mitochondrial movement should be evaluated, including effects on mitochondrial ATP production and interaction with mitochondrial motor proteins. The relationship of reduced mitochondrial number and movement to ER stress should also be examined, as ER stress markers are increased in 3xTg-AD mice as early as 2 months of age [40]. σ_1 R attenuate ER stress [41] and could potentially exert their mitochondrial effects through this pathway. It should be determined if the effects observed in the current study are limited to cultured primary cortical neurons or if they are seen in other cell types *in vitro*, such as glia or hippocampal neurons. Further, *in vivo* studies should be carried out to determine if σ_1 R agonists similarly increase mitochondrial number in neurons in healthy or diseased states and inform their potential use as pharmacological agents to counteract mitochondrial damage associated with neurodegenerative diseases.

Mitochondrial number and movement deficits, such as those seen in 3xTg-AD primary cortical neurons, could be an early initiating factor for synaptic dysfunction and neuronal loss later in life. A caveat of this conclusion is that A β PP, A β , and phosphotau accumulation in the 3xTg-AD mouse is driven by transgenes which are not present in the vast majority of sporadic AD cases in humans. Other developmental insults to mitochondria, however, may still potentially hinder the establishment of cognitive and metabolic reserves and increase risk of AD neurodegeneration later in life. For example, germline mitochondrial DNA mutations have been found to cause premature aging and marked cortical and hippocampal abnormalities in adult mice [42]. Our study provides evidence of early mitochondrial abnormalities in a model of familial AD. Our findings suggest that early treatment with σ_1 R agonists could help ameliorate AD-associated mitochondrial movement and number deficits at critical stages of neurogenesis and neuronal differentiation.

One limitation of this study is that we did not differentiate between axons and dendrites. Mitochondria are significantly smaller and occupy a lower percentage of axons compared to dendrites [32], and mitochondria move more often and faster in axons compared to dendrites [32, 43]. We attempted to overcome this limitation by sampling an adequate number of fields of view and sampling consistently between different cultures so that an equal proportion of axons and dendrites were represented among samples. Another limitation is the inability to directly compare the cultures at 7–9 DIV and 12–14 DIV. Mitochondria are longer and move less in cultures at higher DIV [44]. There is also a significantly higher density of mitochondria in culture at 12–14 DIV compared to 7–9 DIV. We were able to use MT-Red to analyze structure at both time points, but we could not do individual tracking of

mitochondria with MT-Red at 12–14 DIV as we did at 7–9 DIV due to the high number of mitochondria. Therefore, we used pDsRed2-Mito-transfected cultures for measurement of mitochondrial movement using ROIs. One caveat of this method is that there is a difference in events/min depending on the distance of the ROI from the cell body. To overcome this, we measured ROIs in the same range of distances from the cell body in nonTg and 3xTg-AD neurons.

Conclusions

Mitochondrial movement and number deficits were observed in primary cortical neurons derived from embryonic 3xTg-AD mice. These, along with previously observed early mitochondrial function deficits [16], could be key initiating factors in the development of AD pathology later in life. Early mitochondrial deficits could also predispose individuals to reduced cognitive or metabolic reserve, which relate to clinical severity of cognitive decline in the face of AD pathology. Pharmacological amelioration of mitochondrial function, number, and movement deficits early in the development of AD could be a promising strategy for prevention and treatment of cognitive impairment. The current study provides evidence that σ_1 R agonists could be useful agents to restore mitochondrial movement and number in AD neurons in addition to their known neuroprotective effects. More preclinical studies are needed to elucidate the impact of early mitochondrial deficits in AD and to develop novel treatment strategies to counteract these deficits.

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Fig. 1. Mitochondrial structure and number in DIV12–14 primary cortical cultures. A) Sample fields of view from nonTg and 3xTg-AD primary cortical cultures stained with Mitotracker® Red CMXRos at 12–14 DIV. Scale bars = 20 microns. B) Higher magnification views of cultures. Scale bars = 20 microns. C) Mitochondrial length. D) Mitochondrial width. E) Mitochondrial roundness (width/length). F) Mitochondrial number per square micron. $n = 11$ nonTg, $n = 11$ 3xTg-AD cultures; bar graphs show means + SEM; # $p < 0.10$; *** $p < 0.001$; **** $p < 0.0001$.

Fig. 2.

Mitochondrial movement in DIV14 primary cortical cultures. A) Representative regions of interest within neurites of pDsRed2-mito-transfected nonTg and 3xTg-AD primary cortical neurons imaged at 14 DIV (Left). Scale bars = 5 microns. Maximum fluorescence intensity within regions of interest over first 15 min of imaging (Right). B) Representative nonTg neuron showing locations of regions of interest for sampling mitochondrial movement. Scale bar = 50 microns. C) Number of mitochondrial movement events / minute in regions of interest. A movement event is defined by an increase or decrease in maximum fluorescence intensity of 200 fluorescence units over 30 s corresponding to movement into or out of a region of interest. D) Number of mitochondrial transits/minute in regions of interest. A mitochondrial transit is defined as an event in which an individual mitochondrion passes completely through a region of interest. $n = 8$ nonTg, $n = 7$ 3xTg-AD cultures; bar graphs show means + SEM; * $p < 0.05$; ** $p < 0.01$.

Fig. 3. Mitochondrial structure and number in DIV7–9 primary cortical cultures. A) Sample fields of view from nonTg and 3xTg-AD primary cortical cultures stained with Mitotracker® Red CMXRos at 7–9 DIV. Scale bars = 20 microns. B) Higher magnification views of cultures. Scale bars = 20 microns. C) Mitochondrial length. D) Mitochondrial width. E) Mitochondrial roundness (width/length). F) Mitochondrial number per square micron. $n = 6$ nonTg, $n = 6$ 3xTg-AD cultures; bar graphs show means + SEM; ** $p < 0.01$; **** $p < 0.0001$.

Fig. 4. Mitochondrial movement in DIV7–9 primary cortical cultures. A) Movement traces of nonTg and 3xTg-AD neurons 1 h post-vehicle (Veh) or 1 $\mu\text{mol/L}$ (+)SKF-10,047 (SKF) treatment. Scale bars = 4 microns. B) Pre-treatment mitochondrial velocity of nonTg and 3xTg-AD cultures. $n = 6$ nonTg, $n = 6$ 3xTg-AD cultures. C) (Left) Mitochondrial velocity in nonTg and 3xTg-AD cultures 1 h post-treatment with vehicle (Veh) or 1 $\mu\text{mol/L}$ (+)SKF-10,047 (SKF). Insets (Right) show main effects of genotype (Top) and treatment (Bottom). $n = 3$ cultures per group; bar graphs show means + SEM; ** $p < 0.01$; **** $p < 0.0001$.