Mitochondrial Membrane Potential Influences A β Production and Amyloid Precursor Protein localization

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Abstract

Beta amyloid (A β), which derives from the amyloid precursor protein (APP), forms plaques and serves as a fluid biomarker in Alzheimer's disease (AD). How A β forms from APP is known, but questions relating to APP and A β biology remain unanswered. AD patients show mitochondrial dysfunction and an A β / APP/ mitochondria relationship exists. Here, we considered how mitochondrial biology may impact APP and A β biology. We showed that mitochondrial depolarization routes APP to, while hyperpolarization routes APP away from, the organelle. Mitochondrial APP and cell A β secretion inversely correlate, as cells with more mitochondrial APP secrete less A β , and cells with less mitochondrial APP secrete more A β . Overall, our findings indicate mitochondrial function alters APP localization and suggest enhanced mitochondrial activity or factors associated with enhanced mitochondrial activity promote A β secretion while depressed mitochondrial activity or factors associated with depressed mitochondrial activity minimize A β secretion. Our data complement other studies that indicate a mitochondrial, APP, and A β nexus, and could help explain why cerebrospinal fluid A β is lower in those with AD. Our data further suggest A β secretion could serve as a biomarker of cell or tissue mitochondrial function.

Introduction

Amyloid beta (A β) plaques are a hallmark of Alzheimer's disease (AD). Decades of research define how A β is derived from amyloid precursor protein (APP) and how A β forms various insoluble species [1]. However, a major knowledge gap in the AD field is what drives APP metabolism. A clear relationship between APP, A β , and mitochondria exists but the exact mechanism has remained elusive[2; 3].

APP and A β are localized to mitochondria [4]. The trafficking of APP to mitochondria results in its transmembrane arrest where it blocks mitochondrial translocases and inhibits the import of nuclear DNA encoded mitochondrial proteins [5]. A β could be produced locally within mitochondria by a resident γ -secretase [6]. This is important, because A β interacts with mitochondrial enzymes causing mitochondrial dysfunction [7; 8]. A β interacts with the mitochondrial enzyme, A β binding alcohol dehydrogenase (ABAD), which results in inhibition of NAD⁺ binding [4]. A β inhibits cytochrome oxidase (COX) activity, reduces ATP production, increases reactive oxygen species (ROS), and depolarizes the mitochondrial membrane potential [5; 9; 10].

A relationship between A β production and mitochondrial dynamics has been demonstrated. Inhibition of COX with mitochondrial uncoupling increased the production of an 11.5 kDa APP C-terminal fragment (CTF) containing the A β sequence [3]. The non-amyloidogenic APP derivative, sAPP α , is reduced through glycolysis inhibition, a phenomenon reversed with antioxidants. Amyloidogenic APP processing is also enhanced through complex I inhibition *in vitro* and *in vivo* [11].

Consistent with *in vitro* and *in vivo* studies, changes in bioenergetics and mitochondrial function are observed in the brain and periphery of AD subjects. Brain glucose metabolism is reduced in AD subjects. COX enzyme maximum velocity (V_{max}) activity is lower in AD subject platelets, brain tissue, and

fibroblasts [12; 13; 14; 15]. Given the relationship between mitochondria, APP, and A β we examined the relationship between mitochondrial functional states and A β secretion. We found a strong correlation between A β secretion, mitochondrial membrane potential, and APP mitochondrial localization.

A β fluid biomarker findings show that AD subjects have reduced cerebrospinal fluid (CSF) A β [16]. This finding to some extent has led to confusion in the field. One hypothesis is that reduced A β in CSF is indicative of increased plaque burden in the brain and less release into CSF [17]. However, plaques accumulate decades before AD conversion, so this hypothesis does not provide a complete explanation of the disease course [18]. Our data indicate that mitochondrial function likely dictates A β release and therefore CSF A β fluid biomarkers could reflect cell and mitochondrial health.

Materials And Methods

Cell Culture. Human SH-SY5Y neuroblastoma cells were cultured in high glucose DMEM with 5% FBS and penicillin/streptomycin. SH-SY5Y p0 cells were cultured in high glucose DMEM with 5% FBS, penicillin/streptomycin, 50 μ g/mL uridine and 100 μ g/mL pyruvate. When SH-SY5Y cells were compared to SH-SY5Y p0 cells media for both cell types contained uridine and pyruvate as indicated above. Cultivation of cells was completed approximately every 48 hours using 0.25% trypsin with EDTA. Cells were plated for experiments in appropriate vessels and allowed to reach 80% confluence before experiments were completed.

The iPSC line DF6-9-9T.B (referred to as DF6) was purchased from WiCell. DF6 were maintained on a feeder free system using Matrigel (Corning) and mTESR1 medium (StemCell Technologies). Cells were passaged with ReLeSR (StemCell Technologies) when colonies became large and confluent to avoid spontaneous differentiation as outlined in manufacturer protocols.

Neuronal Differentiation. iPSCs were differentiated into neural progenitor cells (NPCs) using STEMDiff Neural Induction Medium (NIM) from StemCell Technologies. iPSCs were placed into a single cell suspension in NIM with SMADi/ROCKi (SMAD inhibitor and ROCK Inhibitor) in an AggreWell 800 plate. Embryoid bodies were cultured in the AggreWell plate for 5 days with NIM/SMADi partial medium changes daily. Embryoid bodies were plated onto Matrigel (Corning) coated plates and fed daily with NIM/SMADi medium until day 12 to allow neural rosette formation. Neural rosettes were selected using Neural Rosette Selection Reagent (StemCell Technologies) and plated onto Matrigel coated dishes with NIM/SMADi. Medium was changed daily for 7 days, after which neural progenitor cells were cryopreserved and split into defined Neural Progenitor Medium (StemCell Technologies). NPCs were plated on PLO/Laminin (Sigma) coated dishes in Neural Progenitor Medium. The following day media was changed to StemDiff Forebrain Neural Differentiation Medium (StemCell Technologies) and medium was changed daily for 7 days [19; 20]. Cells were then plated onto PLO/laminin coated dishes in defined Brain Phys Medium (with N2A, SM1, BDNF, GDNF, cAMP, and ascorbic acid) for neuronal maturation. Neurons were matured for 7 days for downstream experiments.

Astrocyte Differentiation. NPCs were plated onto Matrigel coated dishes in Neural Progenitor Medium. The following day cells were placed in astrocyte differentiation medium consisting of DMEM, B27, 1% FBS, glutamine, bFGF, CNTF, BMP8, Activin A, heregulin 1b, and IGF1. Medium was changed every other day and cells will be passaged as needed [21]. After 30 days or approximately 5–6 passages astrocytes were used for western blotting controls.

3M APP. Wild-type (WT) APP 695 construct was obtained from Addgene (30137). This plasmid is on the pCAX vector backbone. Three point mutations were engineered using site directed mutagenesis as previously described [5]. Site directed mutagenesis and sequencing was completed at Genewiz. The point mutations included Lys to Asp at position + 51 (AAG to AAC), His to Asp at position + 44 (CAC to AAC) and at position + 40 Arg to Asp (required two mutations AGA to AAA and AAA to AAC).

Transfection. Transfections with WT or 3M APP were completed using Lipofectamine 2000 (ThermoFisher). We followed the manufacturer's protocol and using 5 µg DNA for 6-well dishes or 100 ng DNA for 96 well dishes.

Cell Treatments. Cells were treated with FCCP, Oligomycin, or starvation. All treatments were completed for 4 hours. Doses for FCCP and oligomycin ranged from 5 µM to 20 µM and are indicated in the figure legends. Starvation treatment was completed using HBSS with Ca^{2+} and Mg^{2+} . Ethidium bromide treatment of iPSC derived neurons was completed at a dose of 100 ng/mL for seven days.

Aβ ELISA Assays. Media protein was concentrated using ice-cold 100% acetone at a 2:1 ratio. Media samples were incubated in acetone for a minimum of 30 minutes at -20°C. Samples were sedimented by centrifugation at 5,000 x g for 5 minutes followed by a 70% ethanol wash. Samples were resuspended in 8M urea and used in downstream ELISA assays. Aβ₄₀ and Aβ₄₂ were measured using human ELISA assays from ThermoFisher with samples at a dilution of 1:5. All data were normalized to protein content of the samples from a BCA protein assay (ThermoFisher).

Membrane Potential. Mitochondrial membrane potential was determined using Tetramethylrhodamine, ethyl ester (TMRE from ThermoFisher). 1 mM TMRE was diluted into cell culture medium with indicated treatments to a final concentration of 200 nM. Hoechst was added to a final concentration of 10 µg/mL. Cells were incubated with TMRE/Hoechst for 30 minutes and washed two times with Hank's Balanced Buffer Solution (HBSS with Ca^{2+} and Mg^{2+}). Cells were imaged using the Cytation 1 Cell Imaging MultiMode Reader from BioTek. TMRE intensity was normalized to total cell counts for each image.

Plasma membrane potential was determined using (Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol) (DiBAC4(3) from ThermoFisher). 1 mM DiBAC4(3) was diluted into cell culture medium with indicated treatments to a final concentration of 200 nM. Hoechst was added to a final concentration of 10 µg/mL. Cells were incubated with DiBAC4(3)/Hoechst for 30 minutes and washed two times with Hank's Balanced Buffer Solution (HBSS with Ca^{2+} and Mg^{2+}). Fluorescence intensity was determined using a Tecan infinite M200 plate reader. DiBAC4(3) intensity was normalized to total cell counts for each image.

For correlation of mitochondrial and plasma membrane potential both dyes were used simultaneously with Hoescht. Fluorescence intensity was then determined using the Tecan Infinite M200 plate reader.

Mitochondrial Isolation. Mitochondria were isolated as previously described with moderate changes [22]. Briefly, SH-SY5Y cells were resuspended in ice-cold MSHE buffer (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4). The suspended cells were disrupted via nitrogen cavitation for 15 minutes on ice with 900 PSI chamber pressure. Enriched mitochondrial pellets were sedimented by centrifugation (1000 g for 5 min at 4°C, followed by two 10 min spins at 20,000 g and 4°C). The heavy membrane fraction was collected after the first centrifugation (the pellet). Mitochondria were re-suspended in MSHE and treated with 50 µg/mL trypsin for 15 minutes at room temperature. Mitochondria were sedimented at 20,000 x g for 5 minutes and washed with MSHE. Protein concentration was determined using a BCA protein assay for downstream western blotting.

Western Blotting. Western blotting was completed as previously described [22]. Whole cell lysates were generated using RIPA buffer with protease and phosphatase inhibitors (Sigma). Briefly, equal amount of protein were resolved via SDS-PAGE on Criterion TGX gels 4–15% (BioRad). Gels were transferred to PVDF membrane and blocked with 5% BSA in PBST. Primary antibodies were incubated overnight at 4°C followed by three washes with PBST. Secondary antibodies (ThermoFisher) were incubated at room temperature for one hour. Gels were imaged using WestFemto ECL (ThermoFisher) and the BioRad ChemiDoc XRS imaging system. Loading controls included either Actin (Cell Signaling) or AmidoBlack (Sigma) total protein stain. Primary antibodies include APP 6E10 (Biolegend), COX41I (Cell Signaling), Calreticulin (Cell Signaling), GAPDH (Cell Signaling), GFAP (Abcam), HDAC (Cell Signaling), MAP2 (Abcam), Nestin (ThermoFisher), S100β (Abcam), and Synaptophysin (Abcam).

qPCR. RNA was isolated using Trizol and phenol/chloroform extraction. cDNA synthesis was performed using 1 µg of RNA and iScript cDNA synthesis kit (BioRad). qPCR was performed using Taqman assays and reagents against ActB, GAD1, GFAP, MAP2, NES, PAX6, and SOX1 on the QuantStudio 5 platform (ThermoFisher). For DNA samples, DNA was isolated using a phenol/chloroform extraction. Taqman assays and reagents against ND1, mtCO3, and 18S were completed on the QuantStudio 5 platform (ThermoFisher).

Immunohistochemistry. Cells were fixed by adding 4% paraformaldehyde to the culture medium for a final concentration of 1% and incubated on ice for 30 minutes. Cells were washed one time with PBS and placed in blocking/permeabilization buffer (5% BSA in PBS with 0.01% TritonX-100) on ice for 30 minutes. Cells were incubated with primary antibody overnight 4°C (GAD67, MAP2, and Synaptophysin all Abcam) at the manufacturer's recommended dilution. The following day cells were washed three times with PBS and incubated with secondary antibody and Hoechst (Either Texas Red or FITC from ThermoFisher; 1:500 dilution) for 1 hour on ice. Cells were washed three times with PBS and imaged using the Cytation 1 Cell Imaging MultiMode Reader from BioTek.

Electron Microscopy. SH-SY5Y cell monolayers on Thermanox coverslips were fixed on ice with 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1M sodium cacodylate, pH 7.4 for 30 minutes. Cells

were washed one time with PBS and placed in blocking/permeabilization buffer (5% BSA in PBS with 0.01% TritonX-100) on ice for 30 minutes. Cells were incubated with primary antibody overnight 4°C (APP 6E10 from Biolegend) at 1:250. The following day cells were washed three times with PBS and incubated with secondary antibody (Gold 10 nm from Abcam 1:250) for 1 hour on ice. Cells were washed three times with PBS. Cells were then fixed again with 2% glutaraldehyde in 0.1M sodium cacodylate, pH 7.4 for 2 hours and washed twice with 0.1M sodium cacodylate buffer. Samples were post-fixed in 1% osmium tetroxide with 1.5% potassium ferrocyanide in 0.1M sodium cacodylate for 30 minutes at room temp and rinsed three times with distilled water. Samples were dehydrated in a graded series of ethanol as follows: 50%, 70%, 80%, 95%, 100%, 100%. A drop of Embed 812 resin (Electron Microscopy Sciences, Hatfield, PA) was added to each coverslip and samples embedded cell side down on Thompson embedding molds then polymerized overnight at 60°C. Coverslips were peeled from molds, blocks trimmed, thin-sectioned at 80 nm thickness, and picked up on 250 mesh Cu grids. Ultrathin sections were contrasted with 3% uranyl acetate for 5 min and 3% Reynolds lead citrate for 5 min then viewed using a JEOL JEM-1400 TEM at 100KV and digital images acquired with an AMT digital camera.

Statistics. Data were summarized by means and standard errors. To compare means between two groups we used a student's T-test. For correlation analysis a Pearson test was used. *p*-values less than 0.05 were considered statistically significant. All experiments were completed with a minimum of six replicates unless indicated in the figure legend.

Results

Chronic depletion of mtDNA (p0) depolarizes mitochondrial membrane potential in SH-SY5Y neuroblastoma cells (Fig. 1A). The p0 cells are devoid of detectable mtDNA and have no mitochondrial oxygen consumption as previously described [23]. We examined A β secretion from SH-SY5Y control cells and SH-SY5Y p0 cells. p0 cells secrete significantly less A β_{40} and A β_{42} when compared to control cells (Fig. 1B). A significant inverse correlation between mitochondrial membrane potential and plasma membrane potential was observed (Fig. 1C and D). As a separate model of mitochondrial membrane potential depolarization we examined cells treated with FCCP. FCCP treated SH-SY5Y cells showed significant depolarization of mitochondrial membrane potential and significantly reduced levels of secreted A β_{40} and A β_{42} (Fig. 1E-F). As observed in the SH-SY5Y p0 cells, FCCP mitochondrial membrane depolarization negatively correlated with plasma membrane hyperpolarization (Fig. 1H). The effects on the A $\beta_{42}/_{40}$ ratio were disparate between SH-SY5Y p0 cells and FCCP treated cells. In mtDNA depleted cells the ratio was increased while in FCCP cells the ratio was decreased (Fig. 1B and F).

Oligomycin, an ATP synthase inhibitor, is known to induce hyperpolarization of mitochondrial membrane potential. We observed significant hyperpolarization of mitochondrial membrane potential with oligomycin treatment in SH-SY5Y cells (Fig. 2A). Oligomycin treated SH-SY5Y cells showed increased A β_{42} secretion and decreased A β_{40} secretion (Fig. 2B). Oligomycin mitochondrial membrane hyperpolarization induced a depolarization of the plasma membrane (Fig. 2C,D). Similar results were observed with starvation of SH-SY5Y cells. Starvation induced hyperpolarization of mitochondrial

membrane potential (Fig. 2E) and increased the secretion of $A\beta_{40}$ and $A\beta_{42}$ (Fig. 2F). Consistent with other models, the starvation induced mitochondrial membrane potential hyperpolarization was associated with depolarization of the plasma membrane (Fig. 2G,H).

APP localizes to mitochondria (Fig. 3A) and mitochondrial membrane potential is associated with APP mitochondrial localization. SH-SY5Y cells with depolarized mitochondrial membrane potential due to either FCCP or chronic mtDNA depletion had increased APP localized to mitochondria (Fig. 3B,C). SH-SY5Y cells with hyperpolarized mitochondrial membrane potential due to either oligomycin or starvation, had decreased APP mitochondrial localization (Fig. 3D,E). An inverse relationship between mitochondrial localized APP and $A\beta_{42}$ and $A\beta_{40}$ secretion was observed (Fig. 3F,G). As mitochondrial APP levels are reduced higher levels of $A\beta_{42}$ and $A\beta_{40}$ are secreted. The relationship between mitochondrial APP localization and secreted $A\beta_{42}/A\beta_{40}$ ratio was negative. As more mitochondrial APP was present the ratio of secreted $A\beta_{42}/A\beta_{40}$ was lower (Fig. 3H).

Based on these data we examined the effects of APP mitochondrial localization on $A\beta$ production and mitochondrial membrane potential. Expression of a mitochondrial localization incompetent APP construct, 3M APP did not alter $A\beta_{40}$ or $A\beta_{42}$ production but did change the ratio of $A\beta_{42}/A\beta_{40}$ (Figure S1). Data show reduced localization of APP at mitochondria through western blotting and electron microscopy in SH-SY5Y cells expressing 3M APP versus WT APP (Figure S1). SH-SY5Y cells expressing either WT or 3M APP were treated with varying doses of FCCP or oligomycin. FCCP depolarized mitochondrial membrane potential in SH-SY5Y cells expressing either WT or 3M APP (Fig. 4A). As observed in Fig. 1, FCCP reduced $A\beta_{42}$ secretion in WT APP expressing SH-SY5Y cells (Fig. 4B). In 3M APP expressing SH-SY5Y cells, FCCP did not reduce $A\beta_{42}$ secretion (Fig. 4B). In SH-SY5Y cells expressing WT APP, a significant correlation between mitochondrial membrane potential and $A\beta_{42}$ secretion was observed with FCCP. This correlation was absent in SH-SY5Y cells expressing 3M APP (Fig. 4C) treated with FCCP.

Oligomycin hyperpolarized mitochondrial membrane potential in SH-SY5Y cells expressing either WT or 3M APP (Fig. 4D). As observed in Fig. 2, oligomycin increased $A\beta_{42}$ secretion in WT APP expressing SH-SY5Y cells (Fig. 4E). However, 3M APP expressing SH-SY5Y cells treated with oligomycin did not increase $A\beta_{42}$ secretion (Fig. 4E). In SH-SY5Y cells expressing WT APP, a significant correlation between mitochondrial membrane potential and $A\beta_{42}$ secretion was observed with oligomycin treatment. No correlation between mitochondrial membrane potential and $A\beta_{42}$ secretion was observed in SH-SY5Y cells expressing 3M APP (Fig. 4F) treated with oligomycin.

To verify our findings, we differentiated iPSCs into forebrain neurons. The identity and purity of the iPSC derived neurons is shown in Figure S2. The iPSC derived neurons have robust protein expression of neuronal and synaptic markers (GAD67, MAP2, Synaptophysin, Nestin) and relatively no detectable expression of glial markers (S100 β) and GFAP (Glial Fibrillary Associated Protein) (Figure S2 A,C). Gene expression analysis shows similar results. Our iPSC derived neurons have no detectable expression of

GFAP, an astrocytic marker, but very high gene expression levels of neuronal markers MAP2, NES (Nestin) [24], PAX6 (Paired Box 6) [25], GAD1 (glutamate decarboxylase 1) [26], and SOX1 [27; 28] (Figure S2 B). The gene expression pattern observed in our differentiated neurons from iPSCs is consistent with other published work [20].

iPSC derived neurons treated with FCCP showed depolarized mitochondrial membrane potential and reduced A β ₄₂ secretion (Fig. 5A,B). iPSC derived neurons treated with oligomycin showed hyperpolarized mitochondrial membrane potential and increased A β ₄₂ secretion (Fig. 5C,D). mtDNA depletion over seven days of the iPSC neurons depolarized mitochondrial membrane potential (Fig. 5E,F). mtDNA depleted iPSC neurons had reduced A β ₄₀ and A β ₄₂ secretion (Fig. 5G). A positive correlation between mitochondrial membrane potential and A β ₄₂ secretion was observed in this model (Figs. 5H).

Conclusions

Prior studies have found a strong association with mitochondrial function and APP processing flux. These studies when examined as a whole provide a conflicting picture regarding what is driving the interaction between mitochondrial function, APP, and A β . Models which have altered mtDNA in mice either through targeting an endonuclease to the mitochondria or expressing a mutant version of polymerase γ have shown disparate effects on A β production. Targeting an endonuclease to mitochondria, which effectively cleaves mtDNA and reduces intact mtDNA molecules led to a reduction in brain A β plaques [29]. Conversely, expression of the D257A polymerase γ mutation in mice, which drives high accumulation of mtDNA mutations and enhanced aging, increased brain A β burden [30]. In a separate mouse model, knockout of *COX10*, a farnesyltransferase required for COX assembly and function, also reduced brain A β burden [31]. Based on these findings and other *in vitro* work we sought to determine which facet of mitochondrial function could best predict A β levels.

Using SH-SY5Y and iPSC derived neuronal cell models we show that mitochondrial membrane potential predictably modulates A β secretion. Upon mitochondrial membrane depolarization A β secretion is reduced. Conversely upon mitochondrial membrane hyperpolarization A β secretion is increased.

Mitochondrial membrane potential also predictably affected APP localization to mitochondria. When mitochondrial membrane is depolarized more APP localized to mitochondrial membranes and mitochondrial membrane hyperpolarization reduced APP localized to mitochondrial membranes. Our mitochondrial isolation protocols use trypsin to digest any proteins which are not within mitochondrial membranes, so our findings are unlikely due to contamination by non-mitochondrial proteins. An inverse correlation between APP mitochondrial localization and A β secretion was evident. These findings are of interest because other studies have shown an increase in APP localization at mitochondria in postmortem human AD brain [5; 32; 33; 34].

We also found an inverse correlation between mitochondrial membrane potential and plasma membrane potential. These data show that altering mitochondrial membrane potential also affects potentiation at

plasma membranes. This is important especially with regards to neuronal cell function. Mitochondrial membrane potential could affect synapse function and ultimately A β secretion. This requires further study.

We further found that APP localization to mitochondria directly affected the relationship between mitochondrial membrane potential and A β production. Using a mitochondrial targeting incompetent construct, 3M APP, we found that altering mitochondrial membrane potential had either no or minimal effects on A β secretion. Overall, APP localization appears to be critical for the relationship between mitochondrial membrane potential and A β secretion.

Overall, our findings show a strong correlation between mitochondrial membrane potential, APP mitochondrial localization, and A β secretion. These findings likely explain CSF A β biomarkers in AD. As more APP is localized to mitochondrial membranes (as observed in postmortem AD brain) less A β is secreted. CSF A β levels may reflect brain mitochondrial function.

The importance of understanding the biology of APP and A β are imperative. Gaining a better understanding of the biological function and purpose of APP processing to A β will be the next big leap in the AD field.

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Declarations

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The authors declare no conflicts of interest

Figures

Figure 1

Mitochondrial membrane depolarization reduces Aβ₄₀ and Aβ₄₂ secretion. For A through D SH-SY5Y or SH-SY5Y p0 cells expressing WT APP were used. A. Cells stained with TMRE and Hoechst for 30 minutes. Fluorescent images were captured, and intensity was normalized to total cell number. Numbers indicate average and standard error. B. Media was collected, and protein extracted. Samples were incubated in either a Aβ₄₀ or Aβ₄₂ ELISA. Data were normalized to protein content and control samples. Data indicate

average and standard error. C. Cells stained with DiBAC4(3) and Hoechst for 30 minutes. Fluorescent intensity was normalized to Hoechst intensity. Data indicate average and standard error. D. Correlation of mitochondrial membrane potential and plasma membrane potential in SH-SY5Y and SH-SY5Y p0 cells. For E through H SH-SY5Y cells expressing WT APP were treated with 20 μ M FCCP for four hours. E. Cells stained with TMRE and Hoechst for 30 minutes. Fluorescent images were captured, and intensity was normalized to total cell number. Numbers indicate average and standard error. F. Media was collected, and protein extracted. Samples were incubated in either a A β 40 or A β 42 ELISA. Data were normalized to protein content and control samples. Data indicate average and standard error. G. Cells stained with DiBAC4(3) and Hoechst for 30 minutes. Fluorescent intensity was normalized to Hoechst intensity. Data indicate average and standard error. H. Correlation of mitochondrial membrane potential and plasma membrane potential in control and FCCP treated cells. **p<0.005, *** p<0.0005, ****p<0.0001.

Figure 2

Mitochondrial membrane hyperpolarization increases Aβ₄₂ secretion. For A through D SH-SY5Y cells expressing WT APP were treated with 20 μM oligomycin for four hours. A. Cells stained with TMRE and Hoechst for 30 minutes. Fluorescent images were captured, and intensity was normalized to total cell number. Numbers indicate average and standard error. B. Media was collected, and protein extracted. Samples were incubated in either a Aβ₄₀ or Aβ₄₂ ELISA. Data were normalized to protein content and

control samples. Data indicate average and standard error. C. Cells stained with DiBAC4(3) and Hoechst for 30 minutes. Fluorescent intensity was normalized to Hoechst intensity. Data indicate average and standard error. D. Correlation of mitochondrial membrane potential and plasma membrane potential in control and oligomycin treated cells. For E through H SH-SY5Y cells expressing WT APP were starved in HBSS for four hours E. Cells stained with TMRE and Hoechst for 30 minutes. Fluorescent images were captured, and intensity was normalized to total cell number. Numbers indicate average and standard error. F. Media was collected, and protein extracted. Samples were incubated in either a A β 40 or A β 42 ELISA. Data were normalized to protein content and control samples. Data indicate average and standard error. G. Cells stained with DiBAC4(3) and Hoechst for 30 minutes. Fluorescent intensity was normalized to Hoechst intensity. Data indicate average and standard error. H. Correlation of mitochondrial membrane potential and plasma membrane potential in control and starved cells. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$.

Figure 3

Mitochondrial membrane potential alters APP localization. SH-SY5Y cells expressing WT APP were treated as indicated and mitochondria were isolated using nitrogen cavitation and trypsin treatment. A. SH-SY5Y cell mitochondrial or whole cell lysate (WCL) fractions. B. SY5Y (S) versus SY5Y p0 (p) cells. Data indicate average and standard error. Representative western blot images. C. SH-SY5Y cells treated with vehicle (C) or 20 μ M FCCP (F) for four hours. Data indicate average and standard error.

Representative western blot images. D. SH-SY5Y cells treated with vehicle (C) or 20 μ M oligomycin (O) for four hours. Data indicate average and standard error. Representative western blot images. E. SH-SY5Y cells starved (S) using HBSS for four hours or kept in regular medium (C). Data indicate average and standard error. F. Correlation of mitochondrial localized APP and secreted A β 42. G. Correlation of mitochondrial localized APP and secreted A β 40. H. F. Correlation of mitochondrial localized APP and secreted A β 42/A β 40. *p<0.05, **p<0.005, *** p<0.0005.

Figure 4

APP localization affects the relationship between mitochondrial membrane potential and A β 42 secretion. For A through C SH-SY5Y cells expressing WT or 3M APP were treated with 5 μ M, 10 μ M, or 20 μ M FCCP for four hours. A. Cells stained with TMRE and Hoechst for 30 minutes. Fluorescent images were captured, and intensity was normalized to total cell number. Data indicate average and standard error. B. Media was collected, and protein extracted. Samples were incubated in an A β 42 ELISA. Data were normalized to protein content and control samples. Data indicate average and standard error. C. Correlation of mitochondrial membrane potential with secreted A β 42 in cells expressing either WT or 3M APP and treated with FCCP. For D through F SH-SY5Y cells expressing WT or 3M APP were treated with 5 μ M, 10 μ M, or 20 μ M oligomycin for four hours. D. Cells stained with TMRE and Hoechst for 30 minutes. Fluorescent images were captured, and intensity was normalized to total cell number. Data indicate average and standard error. E. Media was collected, and protein extracted. Samples were incubated in an A β 42 ELISA. Data were normalized to protein content and control samples. Data indicate average and standard error. F. Correlation of mitochondrial membrane potential with secreted A β 42 in cells expressing either WT or 3M APP and treated with oligomycin. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$.

Figure 5

Mitochondrial membrane potential affects Aβ₄₀ and Aβ₄₂ secretion in iPSC derived neurons. For A and B iPSC derived neurons were treated with 20 μM FCCP for four hours. A. Cells stained with TMRE and Hoechst for 30 minutes. Fluorescent images were captured, and intensity was normalized to total cell number. Numbers indicate average and standard error. B. Media was collected, and protein extracted. Samples were incubated in either Aβ₄₀ or Aβ₄₂ ELISAs. Data were normalized to protein content and

control samples. Data indicate average and standard error. For C and D iPSC derived neurons were treated with 20 μ M oligomycin for four hours. C. Cells stained with TMRE and Hoechst for 30 minutes. Fluorescent images were captured, and intensity was normalized to total cell number. Data indicate average and standard error. D. Media was collected, and protein extracted. Samples were incubated in either A β 40 or A β 42 ELISAs. Data were normalized to protein content and control samples. Data indicate average and standard error. For E through G iPSC derived neurons were treated with 100 ng/mL EtBr for seven days. E. DNA was isolated, and qPCR completed against 18S, ND1, and mtCO3. Data indicate average and standard error. F. Cells stained with TMRE and Hoechst for 30 minutes. Fluorescent images were captured, and intensity was normalized to total cell number. Data indicate average and standard error. G. Media was collected, and protein extracted. Samples were incubated in either an A β 40 or A β 42 ELISA. Data were normalized to protein content and control samples. Data indicate average and standard error. H. Correlation of mitochondrial membrane potential and A β 42 secretion in iPSC neurons. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Supplementary Files

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