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Truncated tau and A β cooperatively impair mitochondria in primary neurons

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Abstract

Mitochondrial dysfunction is likely a significant contributing factor to Alzheimer disease pathogenesis, and both A β and pathological forms of tau may contribute to this impairment. Cleavage of tau at Asp421 occurs early in Alzheimer disease, and Asp421-cleaved tau likely negatively impacts neuronal function. Previously we showed that expression of caspase-cleaved tau in a neuronal cell model resulted in mitochondrial impairment. To extend these findings we expressed either full-length tau or Asp421-cleaved tau (truncated tau) in primary cortical neurons and measured different aspects of mitochondrial function with or without the addition of sub-lethal concentrations of A β . The expression of truncated tau alone induced significant mitochondrial fragmentation in neurons. When truncated tau expression was combined with A β at sub-lethal concentrations, increases in the stationary mitochondrial population and the levels of oxidative stress in cortical neurons were observed. Truncated tau expression also enhanced A β -induced mitochondrial potential loss in primary neurons. These new findings show that Asp421-cleaved tau and A β cooperate to impair mitochondria, which likely contributes to the neuronal dysfunction in Alzheimer disease.

Keywords

tau; Alzheimer; mitochondria; caspase; A β ; oxidative stress

1. Introduction

Tau is a microtubule-associated protein that is the major component of neurofibrillary tangles (NFTs) (Kosik et al, 1986; Grundke-Iqbal et al, 1986). These NFTs are one of the

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Disclosure statement

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primary pathophysiological hallmarks of Alzheimer disease (AD) and were originally suggested to play a major role in neuronal degeneration (Kosik et al, 1986). However studies now suggest that the toxic species may not be the mature tangles but rather a pre-tangle form of tau (Santacruz et al, 2005; Garcia-Sierra et al, 2008). There is increasing evidence that, in addition to aberrant phosphorylation, tau cleaved at Asp421 by caspases may play a role in the oligomerization and formation of pathological pre-tangle tau species in AD (Gamblin et al, 2003; Rissman et al, 2004; de Calignon et al, 2010). Antibodies that recognize Asp421 truncated tau show that tau cleaved at Asp421, active caspase-3 and fibrillar tau pathologies co-localize in AD patient brains (Gamblin et al, 2003; Rissman et al, 2004). In a mouse tauopathy model it was also found that the majority of cells with active caspases also had NFTs (Spires-Jones et al, 2008). Recently, de Calignon and colleagues (2010) showed that caspase activation and the formation of Asp421 cleaved tau precedes tangle formation in an in vivo model, suggesting that truncated tau could play an early and important role in the pathogenesis of AD (de Calignon et al, 2010). Interestingly, Li et al. showed that activation of caspase-3 via mitochondria is required for long-term depression (LTD) and AMPA receptor internalization in hippocampal neurons (Li et al, 2010). This evidence provides new insight in a possible role of mitochondrial function and caspase-3 activation controlling neuronal function.

Studies in cell culture models provide evidence that Asp421-cleaved tau is toxic to neuronal cells (Chung et al, 2001; Matthews-Roberson et al, 2008; Quintanilla et al, 2009). In our previous studies we observed that caspase-cleaved tau expression induces mitochondrial fragmentation in immortalized cortical cells exposed to calcium stress (Quintanilla et al, 2009). These and other findings suggest that mitochondrial impairment could be one of the mechanisms by which Asp421-cleaved tau contributes to neuronal dysfunction.

Increasing evidence suggests that mitochondrial dysfunction is a very early event in the pathogenesis of AD (Blass et al, 2002; Gibson and Shi, 2010). It has also been shown that overexpression of amyloid precursor protein (APP) or treatment with A β -derived diffusible ligands leads to mitochondrial fragmentation and reduces the mitochondrial population present in neuronal processes in hippocampal neurons (Wang et al, 2008). More recent studies from the same group suggest that an altered balance in mitochondrial fission and fusion proteins is an important factor leading to mitochondrial and neuronal dysfunction in AD (Wang et al, 2009). Mitochondria populations were found redistributed away from axons, and dynamin-related proteins 1 (Drp1) were significantly reduced in AD patient samples (Wang et al, 2009). In addition, significant mitochondrial dysfunction was observed in female triple transgenic AD mice (3 $\times$ Tg-AD) at 3 months of age, significantly before the development of amyloid pathology (Yao et al, 2009). Also, proteomic studies using tripleAD mice (P301L tau transgenic mice crossed with APP^{sw}PS2^{N141I} double transgenic mice) showed large changes in protein levels, with a substantial percentage corresponding to mitochondrial proteins related to complex I and IV. Brain mitochondria isolated from these mice showed a reduction of mitochondrial potential, ATP synthesis, and an increase in reactive oxygen species (ROS) production (Rhein et al, 2009). Given the findings that: (1) caspase cleavage of tau and mitochondrial dysfunction are early events in the pathogenesis of AD (Gamblin et al, 2003; Rhein et al, 2009), (2) A β deregulates mitochondrial fission/fusion processes and induces mitochondrial dysfunction in primary neurons (Wang et al, 2009), and (3) our previous findings that Asp421-cleaved tau induces mitochondrial fragmentation (Quintanilla et al, 2009), the focus of this study was on determining how expression of Asp421-cleaved tau in primary cortical neurons affected mitochondrial function in response to A β treatment.

To study the effects of Asp421-cleaved tau on mitochondrial function, we expressed either a full-length form of tau (T4) or tau truncated at Asp421 (T4C3) in rat primary cortical

neurons. The expression of caspase-cleaved tau induced significant mitochondrial fragmentation in comparison with expression of full-length tau or GFP only. Caspase-cleaved tau sensitized neurons to A β -induced impairment of mitochondrial transport and mitochondrial potential decrease in the primary cortical neurons. Truncated tau expression also significantly increased the oxidative stress response in cortical neurons treated with sub-lethal concentrations of A β compared to neurons expressing full-length tau. These observations indicate that presence of caspase-cleaved tau enhances mitochondrial dysfunction and oxidative stress induced by A β in primary neurons.

2. Methods

2.1 Plasmids

Expression cassettes from pcDNA3.1(-)-T4 (CNS tau containing four microtubule repeats and no N-terminal inserts) and pcDNA3.1(+)-T4C3 (tau truncated at Asp421, according to numbering of the longest tau isoform from human brain) (Krishnamurthy and Johnson, 2004; Matthews-Roberson et al, 2008) were subcloned by polymerase chain reaction (PCR), and the resulting products were ligated into the EcoRI/BamHI sites of pEGFP-C1 (Clontech) to make GFP-T4 and GFP-T4C3. Mito-mCherry was constructed by subcloning the mCherry open reading frame from pFH6.II -mCherry (a gift of Dr. K. Nehrke) using PCR. The resulting product was ligated into the AgeI/BsrGI sites of mito-pEGFP (a gift of Dr. Y. Yoon) resulting in the replacement of GFP with mCherry. Therefore, the following constructs were expressed in primary cortical neurons: GFP/Mito-mCherry (control), GFP-T4/Mito-mCherry (full-length tau), and GFP-T4C3/Mito-mCherry (tau truncated at Asp421). The identities of all new plasmids were confirmed by sequencing.

2.2 A β Fibril Formation

Amyloid peptide 1–40 (A β _{1–40}) was obtained from Calbiochem and fibrils were prepared as previously described (Quintanilla et al, 2005; Santos et al, 2005). Briefly, stock solutions were prepared by dissolving freeze-dried aliquots of A β _{1–40} in DMSO to 1M, and further diluting this solution with 0.1 M Tris-HCl (pH 7.4) to a final concentration of 100 μ M A β . The solutions were stirred continuously (210 rpm) at room temperature for 48 h. The formation of amyloid fibrils was verified by electron microscopy (Santos et al, 2005).

2.3 Primary neuron culture, cell culture, and transfection

Primary cortical neuronal cultures from rat embryonic forebrains were prepared as described previously with some modifications (Quintanilla et al, 2005). Briefly, whole brains were removed from fetal rats at day E17–18. The cortices were then dissected, treated with 0.05 % trypsin at 37°C for 30 min, and gently triturated with a fire polished glass Pasteur pipette. Dissociated cells were plated onto poly-D-lysine-coated coverslips or glass-bottom dishes in Minimum Essential Media with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. 5 hr after plating, medium was replaced with Neurobasal medium (NBM) supplemented with 0.4 mM glutamine and B27. Every 3 days, half of the medium was removed and replenished with the fresh NBM supplemented with 0.4 mM glutamine and B27. Materials for cell and neuron cultures were from Invitrogen. All protocols were approved by the University Committee on Animal Resources at the University of Rochester.

Neurons were transiently transfected with 0.8 μ g of plasmids (see methods 2.1) using Lipofectamine 2000 (Invitrogen) at 7 days *in vitro* (DIV) following manufacturer protocols. Imaging experiments were performed at 9 DIV. Immortalized cells were transfected in a similar manner, and cultured for 48 h post-transfection before use in experiments.

2.4 Determination of cell viability

For the determination of viability in primary neurons, neurons were seeded in 12-well dishes and cultured as described. After treatment with A β for the indicated times, neurons were incubated in 400 μ M resazurin for 1h, and fluorescence was measured using a plate reader (BioTek Synergy HT Multi-Detection Microplate Reader) at excitation/emission wavelengths of 530/590 nm.

2.5 Superoxide level determination in live primary neurons

Superoxide levels were determined using DHE (dihydroethidium) (Brennan et al, 2009). Transfected neurons with GFP, GFP-T4, and GFP-T4C3 (control and A β -treated) were incubated with 10 μ M DHE for 20 min in Krebs-Ringer-Hepes (KRH) buffer supplemented with 5 mM glucose at 37 C. Cells were mounted in a microscope chamber with KRH-glucose for further examination. Images were acquired using 520/610 nm configuration with a fluorescence microscope (Axiovert, Zeiss), equipped with a LED source. Estimation of mitochondrial superoxide production was made using Image-Pro Plus 6 software. Results in intensity units were expressed as average of fluorescence signal (F) minus background fluorescence (F0) in every image.

2.6 Mitochondria potential determination in live cells

Mitochondria membrane potential was determined using tetramethyl rhodamine methyl ester (TMRM) (Quintanilla et al, 2009). Prior to A β treatment the cells were loaded for 30 min with TMRM (100 nM) in KRH-glucose buffer containing 0.02% pluronic acid, then washed, and allowed to equilibrate for 20 min. Analyses were carried out using confocal laser scanning microscope (Leica SP2, Germany). TMRM fluorescence was detected by exciting with a 563 nm He-Ne laser attenuated (30% laser power) and the emission was collected at >570 nm. Signal collected from axons and neurites of cortical neurons were compared using identical settings for laser power and detector sensitivity for each separate experiment. The images were collected with LCS Leica confocal software (Germany) and recorded as mean TMRM fluorescence signal per live cell. TMRM fluorescence intensity was calculated as described above and is presented as the pseudoratio ($\Delta F/F_0$) (Quintanilla et al, 2009).

2.7 Estimation of Mitochondrial Length

Estimation of mitochondrial length and frequency analysis was previously described (Quintanilla et al, 2009). Briefly, mitochondrial length was calculated using the measured perimeter of identified objects in axons and neurites of live cortical neurons previously transfected with GFP/Mito-mCherry, GFP-T4/Mito-mCherry, and GFP-T4C3/Mito-mCherry. Alternatively, mitochondrial morphology was observed using Mitotracker Green™ dye (MTG) in untransfected neurons (Quintanilla et al, 2009). Confocal and fluorescence images were taken using either a 40X water immersion objective with 4X digital zoom in a SP1 Leica confocal microscope or a fluorescence microscope (Axiovert, Zeiss). Mitochondrial length quantification was estimated using Image Pro 6 software (MediaCybernetics, MA, USA).

2.8 Mitochondrial Transport Measurements

Mitochondrial transport was evaluated in axons of primary cortical neurons transfected with GFP/Mito-mCherry, GFP-T4/Mito-mCherry, and GFP-T4C3/Mito-mCherry. Transfected neurons were mounted in a microscope chamber filled with 1ml of KRH buffer supplemented with 5 mM glucose. Mitochondrial transport studies were made at room temperature. Mito-mCherry images were taken every 10 sec (520/610 nm) in neurons that were GFP positive. Recordings were made for 5 min in every experiment, and for quantification mitochondria were considered to be mobile if they moved more than 2 μ m

during the 5 min period. The rest of the mitochondria were considered as part of the stationary mitochondrial population. Quantification of mitochondrial transport velocity included both anterograde and retrograde movement. In the A β studies, neurons transfected as described above were pretreated with 0.5 μ M A β for 2h at 37°C in neuronal culture media, prior to mitochondrial transport measures.

2.9 Statistical Analysis

All data is expressed as the mean of at least three independent experiments $\pm$ SE unless otherwise stated. Statistical comparisons between treatment groups were performed using Student's *t* test. *P* values are *P*<0.05 and *P*<0.01, as indicated.

3. Results

3.1 A β at low concentrations significantly affects mitochondrial function and localization in primary neurons

It has been suggested that A β can induce mitochondrial dysfunction in different neuronal cell models (Du et al, 2008; Gibson and Shi, 2010). The majority of these studies have been carried out using relatively high A β concentrations in various conformations (Quintanilla et al, 2005; Santos et al, 2005). However, the effect of low A β concentrations on mitochondrial function in primary neurons has not been fully explored. Therefore we measured mitochondrial function and morphology changes in cortical neurons exposed to 0.5 μ M A β for 24 h (Fig. 1 and 2). Mitochondrial morphology was determined using Mitotracker Green dye (MTG) (Quintanilla et al, 2009), and mitochondrial potential was evaluated using TMRM (Quintanilla et al, 2009). Treatment for 24 h with 0.5 μ M A β significantly decreases mitochondrial potential in cortical neurons (Fig. 1A and B). MTG staining, which is distributed throughout the neuritic network in untreated neurons, was significantly accumulated in the neuronal body 24 h after treatment with A β (Fig. 1A, white arrows). TMRM staining showed a similar aggregation pattern in neurons treated with A β for 24 h (Fig. 1A, white arrows), but fluorescence intensity was significantly lower in the A β -treated neurons, indicating mitochondrial potential had been compromised (Fig. 1B). Interestingly, these changes were independent of a generalized effect on cell health, as treatment with 0.5 μ M A β for 24 h did not attenuate cell viability, although a 5 fold higher concentration (2.5 μ M) did have a negative impact (Fig. 1C).

Previously it has been shown that expression of APP protein induced mitochondrial fragmentation in neuronal cells (Wang et al, 2008). Therefore, we corroborated the changes in mitochondrial morphology and localization after A β treatment in cortical neurons transfected with GFP and Mito-mCherry (Fig. 2). Treatment with A β resulted in a significant reduction in mitochondrial length, and an extensive accumulation of mitochondria in the soma compared to untreated neurons (Fig. 2A, white arrows). These changes were independent of a generalized effect on cell health or morphological damage as the neuritic network remained intact (Fig. 2A, compare GFP images) and the treatment with 0.5 μ M A β did not attenuate cell viability (Fig. 1C). Quantification of four independent experiments showed that A β induced a significant decrease in mitochondrial length (Fig. 2B), and a change in the length distribution of the mitochondrial population (Fig. 2C) in cortical neurons. These observations indicate that chronic treatment (24 h) with low concentrations of A β alters mitochondrial biology, which could be an important contributing factor to the neuronal dysfunction reported in AD.

3.2 Caspase-cleaved tau expression induces mitochondrial fragmentation in primary neuronal cultures

Given the fact that caspase-cleaved tau compromised mitochondrial morphology and function in immortalized cortical cells (Quintanilla et al, 2009), we next wanted to establish that caspase-cleaved tau had similar effects in primary cortical neurons. Primary cortical neurons were transfected with GFP, GFP-T4 or GFP-T4C3 and Mito-mCherry to observe changes in mitochondrial morphology. Expression of full-length tau (GFP-T4) and caspase-cleaved tau (GFP-T4C3) was prominent in the neurites (Fig. 3A). No apparent changes in neuronal development, axonal growth, and branching were observed 48 h after transfection with any of the constructs (data not shown). GFP transfected neurons presented with normal tubular-like mitochondrial morphology and a normal distribution of mitochondria in neurites and axons (Fig. 3A and B). Neurons transfected with full-length tau (GFP-T4) presented similar mitochondrial localization and morphology as neurons transfected with GFP alone (Fig. 3A and B). In contrast, caspase-cleaved tau expressing neurons (GFP-T4C3) showed a significant change in mitochondrial morphology, with a more rounded and fragmented phenotype (Fig. 3A and B). Quantification of four independent experiments showed that GFP-T4C3 expression induced a significant decrease in mitochondrial length (Fig. 3C), and a change in the length distribution of the mitochondrial population (Fig. 3D). These observations indicate that expression of caspase-cleaved tau results in altered mitochondrial morphology in primary neurons.

3.3 Caspase-cleaved tau expression enhances impairment of mitochondrial transport induced by A β in cortical neurons

Mitochondrial transport is critically important for neuronal function (Miller and Sheetz, 2004; Vossel et al, 2010). Previous studies indicate that tau plays a role in regulating mitochondrial axonal transport in primary neurons (Mandelkow et al, 2003; Thies and Mandelkow, 2007; Stoothoff et al, 2009), however the effects of caspase-cleaved tau on mitochondrial transport have not been reported. To evaluate mitochondrial movement, we transfected neurons with GFP, GFP-T4 or GFP-T4C3, and Mito-mCherry to observe mitochondria mobility. Transfected cells were mounted in a microscope chamber at room temperature, and Mito-mCherry signal were measured at 10 sec intervals. Quantification of mitochondrial movement was made considering both anterograde and retrograde transport in the final calculations, and then results were expressed in terms of total mitochondrial movement. Neurons transfected with GFP alone and Mito-mCherry showed active mitochondrial transport, evidenced by numerous mitochondria moving in both directions. In contrast, in GFP-T4 and GFP-T4C3 positive neurons there was a significant decrease in the number of actively moving mitochondria in untreated cells (Fig. 4A). However, within the subpopulation of moving mitochondria, no major changes in mitochondrial transport velocity were observed in untreated neurons transfected with GFP, GFP-T4, and GFP-T4C3 (Fig. 4B). In addition, we evaluated mitochondrial transport in GFP, GFP-T4, and GFP-T4C3 expressing neurons after treatment with 0.5 μ M A β for 2 h. A β treatment for 2 h did not change the percentage of actively moving mitochondria population in GFP and GFP-T4 cells compared to untreated cells, however in neurons expressing GFP-T4C3, we observed a significant decrease in the percentage of moving mitochondria in GFP-T4C3 neurons treated with A β (Fig. 4A). In addition, within the moving mitochondria subpopulation, A β treatment decreased mitochondrial transport velocity in all neurons (Fig. 4B), but the magnitude of this decrease was more significant in neurons expressing GFP-T4C3 compared to neurons transfected with GFP-T4 (Fig. 4B). Altogether these findings indicate that presence of caspase-cleaved tau enhances the impairment of mitochondrial transport induced by A β .

3.4 Caspase-cleaved tau expression enhances mitochondrial dysfunction induced by A β treatment in primary neuronal cultures

Given that mitochondria dysfunction likely plays an important role in the pathogenesis of AD (Du et al, 2008; Gibson and Shi, 2010), and that A β and tau co-operatively or synergistically impair neuronal function (Small and Duff, 2008), we determined the effects of A β on mitochondrial function in neurons expressing full-length or caspase-cleaved tau (Fig. 5). Primary neurons were transfected with GFP, GFP-T4, or GFP-T4C3 and mitochondrial potential changes in response to 0.5 μ M A β treatment were measured (Fig. 5). Untransfected neurons or cortical neurons expressing GFP or GFP-T4 showed a minor decrease in mitochondrial potential after 30 min A β treatment (Fig. 5A and B). In contrast, A β induced a significant decrease in mitochondrial potential in neurons expressing GFP-T4C3 compared to neurons that expressed GFP-T4 treated with A β (Fig. 5A and B). Altogether these observations indicate that expression of caspase-cleaved tau enhances mitochondrial dysfunction induced by A β in cortical neurons.

3.5 Caspase-cleaved tau expression enhances oxidative stress induced by A β treatment in primary neuronal cultures

It has been shown that A β affects mitochondrial function and increases levels of ROS in different mouse and neuronal models (Santos et al, 2005; Yao et al, 2009). To establish a possible connection between tau modifications, A β toxicity and mitochondrial dysfunction, we evaluated ROS levels in primary cortical neurons that were expressing truncated tau. We measured superoxide levels using DHE (Brennan et al, 2009) in neurons expressing GFP, GFP-T4 or GFP-T4C3 in the absence or presence of 0.5 μ M A β . Figure 6A shows representative fluorescent images of cortical neurons transfected with GFP, GFP-T4, and GFP-T4C3 treated with A β for 1 h. These images show that superoxide levels in GFP and GFP-T4 neurons are very similar but in neurons transfected with GFP-T4C3, treatment with A β induced a significant increase in superoxide levels. Quantification of the data revealed that A β treatment significantly increased superoxide production only in cell expressing caspase-cleaved tau (Fig. 6B). Superoxide levels in neurons transfected with GFP, GFP-T4, and GFP-T4C3, but without A β treatment were not significantly different (see Fig. S1).

Altogether these results suggest that caspase-cleaved tau acts cooperatively with A β to compromise mitochondrial function, an event that could be playing a role in neuronal dysfunction present in AD.

4. Discussion

Pathological forms of tau likely play an important role in the pathogenesis of AD (Hernandez and Avila, 2008; Hanger et al, 2009). Asp421-cleaved tau is present in AD patient brains and appears to occur early in the process (Gamblin et al, 2003; Rissman et al, 2004). Recently, Hyman's group showed that caspase activation is a transient and early event, as is the production of Asp421-cleaved tau, which precedes tangle formation (de Calignon et al, 2010). They propose a model where generation of caspase-cleaved tau may initiate tangle formation with this truncated tau recruiting normal tau into a misfolded state (de Calignon et al, 2010). These observations are very important, and suggest that the formation of Asp421-cleaved tau occurs in the early stages of AD and contributes to disease progression. The new evidence presented here is in agreement with these studies and suggests that caspase-cleaved tau could be playing an important part in the pathogenesis of AD perhaps by facilitating A β -mediated mitochondrial impairment.

Mitochondrial dysfunction has been suggested as an important player in the pathogenesis of AD (Dun et al, 2008; Rhein et al, 2009). Dysfunctional mitochondria could compromise

regular neuronal tasks and decrease their capacity to communicate and defend against different stressors (Dong-Hyung et al, 2009; Gibson and Shi, 2010; Vossel et al, 2010). Our findings presented in this paper support the concept that truncated tau contributes to neuronal toxicity by altering mitochondrial dynamics and cooperating with A β to impair mitochondrial biology. Caspase-cleaved tau expression induced mitochondrial fragmentation in immortalized cells (Quintanilla et al, 2009) and primary cortical neurons, and both cell types showed enhanced mitochondrial dysfunction when treated with low concentrations of A β . Mitochondrial potential loss and oxidative stress levels in response to A β were significantly increased in neurons expressing caspase-cleaved tau in comparison with neurons that were expressing full-length tau. In addition, we observed that the velocity of moving mitochondria was reduced in cortical neurons exposed to A β , but this decrease was only significant in neurons expressing GFP-T4C3. Further, although both GFP-T4 and GFP-T4C3 decreased the number of moving mitochondria as expected (Mandelkow et al, 2003; Thies and Mandelkow, 2007), only in the GFP-T4C3 expressing neurons was there a further decrease in moving mitochondria in response to A β . Previous studies showed that A β induced axonal and dendritic swelling, with a concomitant decrease in axonal transport in hippocampal neurons (Shah et al, 2009). Recently, Vossel et al showed that treatment with A β affects mitochondrial transport, and decreasing tau levels prevented these defects in hippocampal neurons (Vossel, et al, 2010). Therefore, the presence of caspase-cleaved tau could act synergistically with A β compromising mitochondrial dynamics and function and eventually neuronal viability.

Previously it has been shown that expression of APP or treatment with A β species induced mitochondrial fragmentation in neuronal cells (Wang et al, 2008; 2009). In addition, nitric oxide produced in response to A β induced mitochondrial fission, synaptic loss, and neuronal damage, by oxidative modification of Drp1 (Dong-Hyung et al, 2009). Given these effects of A β and the findings presented in this study it can be speculated that tau truncated at Asp421, as well as perhaps other pathological forms of tau, may cooperate to impair mitochondrial biology in AD. Further studies are needed to determine the mechanisms by which truncated tau and A β negatively impact mitochondrial dynamics, localization and function.

It has been suggested that the expression of full-length tau can negatively impact mitochondria biology (Stamer et al, 2002; Thies and Mandelkow, 2007). Expression of full-length tau reduced mitochondrial density in axons and neuronal processes in hippocampal neurons (Stamer et al, 2002; Thies and Mandelkow, 2007). However in this present study mitochondria were observed at presumably normal levels in axons and neuronal processes in cortical neurons that were expressing full-length tau. We believe that differences between our findings and these previous studies may be related in part to the detection methods used. For instance, Stamer et al used Mitotracker Red to detect the mitochondrial population, and an anti-tau antibody to observe tau expression in neurons transfected with full-length tau (Stamer et al, 2002). Loading of Mitotracker red depends on the mitochondrial functional state (membrane potential). Therefore, it is possible that this protocol may not be entirely representative of the entire mitochondrial population present in the neuronal processes. Nonetheless our results were similar to the findings of Stamer et al (2002) in terms of the effects of full-length tau expression on the moving mitochondrial population in neurons. Additionally, the same group showed that transfection with full-length tau resulted in increases in oxidative stress and decreased ATP levels in primary neurons (Stamer et al, 2002; Thies and Mandelkow, 2007). Interestingly, in our studies cortical neurons expressing GFP-T4C3, but not GFP-T4, showed mitochondrial depolarization and elevated levels of superoxide after exposure to A β . Therefore it can be hypothesized that presence of truncated tau could enhance oxidative stress levels in cells treated with A β , which negatively impacts mitochondrial function.

Treatment of primary neurons with 0.5 μM A β for 24 h resulted in a significant reduction in relative mitochondrial potential levels, increased mitochondrial fragmentation, and an accumulation of the mitochondrial population at the soma. These changes were observed without any apparent compromise to cell viability. Previously, studies suggest that mitochondrial potential levels may regulate mitochondrial transport as the mitochondria population with a higher potential (healthy mitochondria) was transported to the growth cone, while mitochondria with low potential (impaired mitochondria) were transport towards the cell body (Miller and Sheetz, 2004). Further, Falzone et al reported that reductions in axonal transport could exacerbate tau hyperphosphorylation, formation of insoluble aggregates and tau-dependent neurodegeneration in a genetic model that combined a genetic reduction of kinesin-1 with expression of human tau in *Drosophila* (Falzone et al, 2010). Therefore, stressors that modify axonal transport (A β , oxidative stress) may induced or accelerate abnormal tau behavior reported in AD (Falzone et al, 2010). Taking together, the evidence presented above supports our findings, in where the treatment with low concentrations of A β may induce neuronal dysfunction, by impairing mitochondrial transport (Fig. 4), mitochondrial localization (Fig. 2), and mitochondrial potential, events that may result in pathological changes in tau that may then exacerbate the A β -induced deficits. Our observations suggest that low concentrations of A β together with pathologically modified tau detrimentally affect mitochondrial transport and function without seriously compromising neuronal viability. This is particularly important, because in early stages of AD, A β in concert with caspase-cleaved tau could affect mitochondrial function and mitochondrial dynamics, and subsequently impair neuronal viability.

In summary we suggest that the presence of caspase-cleaved tau compromises mitochondrial biology and thus decreases the ability of the neuron to protect itself against A β toxicity. Indeed, low concentrations of A β acutely impaired mitochondrial function in neurons when caspase-cleaved tau is present. If mitochondria function is impaired, mitochondrial transport is reduced and oxidative stress levels increased and cumulatively these events are likely to result in significant compromise in neuronal function. Overall these observations suggest an important role for caspase-cleaved tau and A β in the mitochondrial dysfunction in AD pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

B

C

Figure 1. A low concentration of Aβ affects mitochondrial function in primary neurons
(A) Representative confocal images from primary cortical neurons that were loaded with Mitotracker Green (MTG) and TMRM to determine mitochondrial localization and membrane potential changes after exposure to 0.5 μM Aβ for 24 h. Aβ-treatment altered mitochondrial localization (increasing mitochondria accumulation, see white arrows) and significantly affected mitochondrial potential levels (TMRM fluorescence levels were decreased after treatment). Bar scale represents 10 μm. **(B)** Quantification of mitochondrial potential levels from cortical neurons treated with Aβ for 24 h. Graph represents quantification of mitochondrial potential fluorescence intensities as relative units, which shows that neurons treated with Aβ for 24 h exhibit a pronounced loss of mitochondrial potential. Data are mean ± S.E. (bars) from three separate experiments. **p* < 0.05, compared to untreated cortical neurons. **(C)** Cell viability loss was evaluated using resazurin assay in primary cortical neurons treated with 0.5 or 2.5 μM Aβ for 24 or 48 h. No loss of viability was detected with 0.5 μM Aβ, however 2.5 μM Aβ significantly reduced neuronal viability compared to untreated cells. Data are mean ± S.E. (bars). *, *p* < 0.01 compared to untreated neurons. **p* < 0.01 by unpaired Student's *t* test.

A

Figure 2. A low concentration of A β induces mitochondrial fragmentation in primary neurons (A) Cortical neurons were transiently transfected with GFP/Mito-mCherry (Mito-mCh), and mitochondrial morphology and localization was observed prior to (control) and after 24 h treatment with 0.5 μ M A β . Representative fluorescent images of untreated neurons showed elongated mitochondrial morphology with evident mitochondrial presence at all neuronal processes (see white arrows). In contrast, neurons treated with A β showed a significant accumulation of mitochondria in the neuronal soma (white arrows). Bar scale represents 10 μ m. (B) Quantification of 3 different experiments revealed that mitochondria in A β -treated cells showed a significant reduction in average mitochondrial length as compared to mitochondria in untreated neurons. Data are mean $\pm$ S.E. (bars)*, $p < 0.05$ compare to untreated cells. * $p < 0.05$ was estimated by unpaired Student's t test. (C) Mitochondrial population was represented in terms of frequency of mitochondrial length present in cortical neurons positive transfected with GFP/Mito-mCherry and incubated in control conditions or with 0.5 μ M A β for 24 h. Around 70% of mitochondria in neurons expressing GFP and treated with A β were less than 2 μ m in length in comparison with untreated GFP positive neurons that the majority of mitochondria ranged between 2–4 μ m.

Figure 3. Effects of caspase-cleaved tau expression on mitochondrial morphology in primary neurons

(A) Primary cortical neurons were doubly transfected with GFP/Mito-mCherry (Mito-mCh), GFP-T4/Mito-mCh or GFP-T4C3/Mito-mCh and mitochondrial morphology was observed. Representative fluorescent images taken 48 h after transfection show mostly long and elongated mitochondrial morphology in neurons expressing GFP or GFP-T4. In contrast, expression of GFP-T4C3 resulted in significant mitochondrial fragmentation and reduced mitochondrial presence in the neuronal processes. Bar scale represents 10 μm. (B) Magnification of boxed regions from A to emphasize differences of mitochondrial morphology in neurites. Bar scale represents 5 μm. (C) Quantification of 4 independent experiments revealed that mitochondria in neurons expressing GFP-T4C3 showed more than a two-fold decrease in average mitochondrial length as compared to mitochondria in neurons expressing GFP-T4. Data are mean ± S.E. (bars)*, $p < 0.05$ compare to GFP-T4 untreated neurons. * $p < 0.05$ was estimated by unpaired Student's *t* test. (D) Mitochondrial population was represented in terms of frequency of mitochondrial length present in cortical neurons transfected with GFP, T4-GFP or GFP-T4C3. More than 60% of mitochondria in neurons expressing GFP-T4C3 were less than 2 μm in length while the majority of mitochondria in GFP and GFP-T4 neurons ranged between 2–6 μm. Quantification is from 4 independent experiments.

Figure 4. Caspase-cleaved tau expression enhances impairment of mitochondrial transport induced by $\text{A}\beta$ in cortical neurons

(A) Primary cortical neurons were doubly transfected with GFP/Mito-mCh, GFP-T4/Mito-mCh, or GFP-T4C3/Mito-mCh to evaluate mitochondrial movement. Transfection with GFP-T4 and GFP-T4C3 significantly decreased the percentage of moving mitochondria in comparison with GFP-expressing neurons. (A) Quantification of mitochondrial moving population in neurons expressing GFP, GFP-T4 or GFP-T4C3 that were untreated (black bars) or treated with 0.5 μM $\text{A}\beta$ (grey bars) for 2 h prior to measuring movement. Bars graph shows quantification of four independent experiments. Data are mean $\pm$ S.E. (bars). *, $p < 0.05$. $p < 0.05$ was estimated by unpaired Student's t test. (B) Mitochondrial transport velocity was evaluated in the moving mitochondria population in neurons transfected with GFP, GFP-T4 or GFP-T4C3 without (grey bars) or with pre-treatment with 0.5 μM $\text{A}\beta$ bars) for 2 h. Untreated neurons showed similar mitochondrial transport rates. Interestingly, $\text{A}\beta$ -treatment decreased mitochondrial transport velocity in GFP and GFP-T4 positive neurons, at the same manner. However, pretreatment with $\text{A}\beta$ induced a more pronounced decrease in mitochondrial velocity in GFP-T4C3 expressing neurons. Bars graph shows quantification of four independent experiments. Data are mean $\pm$ S.E. (bars). *, $p < 0.05$. $p < 0.05$ was estimated by unpaired Student's t test.

Figure 5. Caspase-cleaved tau expression enhances mitochondrial impairment induced by A β treatment in primary neuronal cultures

(A) Primary cortical neurons were transfected with GFP, GFP-T4 or GFP-T4C3 and mitochondrial membrane potential was determined using TMRM. Treatment with 0.5 μ M A β induced a significant mitochondrial potential loss in GFP-T4C3 cells. Untransfected, GFP, and GFP-T4 neurons showed a similar mild decrease in mitochondrial potential levels. The graph trends represent quantification of TMRM fluorescence intensities as relative units, from four independent experiments. (B) Quantification of mitochondrial potential levels after 30 min A β exposure. Data are mean $\pm$ S.E. (bars) from four separate experiments. * $p < 0.01$ compare to GFP-T4 cells treated with 0.5 μ M A β . $p < 0.01$ was estimated by unpaired Student's t test.

Figure 6. Caspase-cleaved tau expression and A β treatment enhance superoxide production in primary neuronal cultures

(A) Primary cortical neurons were transfected with GFP, GFP-T4 or GFP-T4C3 and superoxide levels were determined using dihydroethidium (DHE) after treatment with 0.5 μ M A β for 1 h. White arrows in the DHE panels indicate neurons that were transfected. Images show that superoxide levels in GFP and GFP-T4 neurons are very similar but in neurons transfected with GFP-T4C3, treatment with A β induced a significant increase in levels of superoxide. Bar scale represents 10 μ m. (B) Quantification of three separate experiments revealed that caspase-cleaved tau expression in primary neurons significantly increased superoxide production induced by A β treatment. Data are mean $\pm$ S.E. (bars). * p < 0.01 compare to GFP-T4 cells treated with 0.5 μ M A β . p < 0.01 was estimated by unpaired Student's t test.