Caspase-cleaved Tau Expression Induces Mitochondrial Dysfunction in Immortalized Cortical Neurons

IMPLICATIONS FOR THE PATHOGENESIS OF ALZHEIMER DISEASE

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In Alzheimer disease (AD) mitochondrial abnormalities occur early in the pathogenic process and likely play a significant role in disease progression. Tau is a microtubule-associated protein that is abnormally processed in AD, and a connection between tau pathology and mitochondrial impairment has been proposed. However, few studies have examined the relationship between pathological forms of tau and mitochondrial dysfunction. We recently demonstrated that inducible expression of tau truncated at Asp-421 to mimic caspase cleavage (T4C3) was toxic to immortalized cortical neurons compared with a full-length tau isoform (T4). In this study we investigated the effects of T4C3 on mitochondrial function. Expression of T4C3 induced mitochondrial fragmentation and elevated oxidative stress levels in comparison with T4-expressing cells. Thapsigargin treatment of T4 or T4C3 cells, which causes an increase in intracellular calcium levels, resulted in a significant decrease in mitochondrial potential and loss of mitochondrial membrane integrity in T4C3 cells when compared with cells expressing T4. The mitochondrial fragmentation and mitochondrial membrane damage were ameliorated in T4C3 cells by pretreatment with cyclosporine A or FK506, implicating the calcium-dependent phosphatase calcineurin in these pathogenic events. Increased calcineurin activity has been reported in AD brain, and thus, inhibition of this phosphatase may provide a therapeutic target for the treatment of AD.

NFTs are one of the primary pathophysiological hallmarks of Alzheimer disease (AD) and were originally suggested to play a major role in facilitating neuronal degeneration (1). However, recent studies now suggest that mature tangles may not be the toxic species (3, 4). For example, in a repressible tau overexpression transgenic mouse model, turning off tau expression attenuated memory impairment and neuronal loss, whereas NFTs continued to accumulate (5). Furthermore, reduction of endogenous wild type tau attenuated behavioral abnormalities in an APP transgenic AD mouse model, in which substantial NFT pathology is absent (6). These and other findings suggest that a form or forms of tau that precede NFT formation may be the toxic species.

There is increasing evidence that, in addition to aberrant phosphorylation, caspase cleavage of tau plays a role in the oligomerization and formation of a pathological tau species in AD (7, 8). Tau is an in vitro substrate for caspase-3 and is readily cleaved at Asp-421, the caspase-3 cleavage site, located on the carboxyl-terminal end of the protein (7–10). This cleavage event results in a highly fibrillogenic tau isoform which in in vitro studies aggregates more readily and to a greater extent than full-length tau and facilitates aggregate formation of full-length tau (7, 8). Antibodies that specifically recognize Asp-421-truncated tau show that tau cleaved at Asp-421, active caspase-3, and fibrillar tau pathologies co-localize in AD patient brains (7, 8). In a mouse tauopathy model it was also found that the majority of cells with active caspases also had NFTs (11). Furthermore, experiments in cell culture models provide evidence that Asp-421-cleaved tau is toxic to neurons (9–10, 12). However, the mechanism(s) underlying this toxicity has not been elucidated.

Increasing evidence suggests that even before the occurrence of AD-related pathologies or any detectable cognitive decline, there are disruptions in mitochondrial function (13, 14). Mitochondria play a major role in regulating neuronal calcium homeostasis (15). Mitochondria have a vast capacity to accumulate calcium; this ability is driven through an electrochemical gradient provided mainly by the mitochondrial potential and a low intramitochondrial free calcium concentration (16). However, in AD and other neurodegenerative diseases the ability of the mitochondria to buffer increases in cytosolic calcium, produce ATP, and regulate oxidative stress is impaired, which could contribute to neuronal degeneration (17).

Given the fact that both caspase cleavage of tau and mitochondrial dysfunction are early events in the pathogenesis of tauopathies, we hypothesized that caspase-cleaved forms of tau might contribute to mitochondrial abnormalities in AD. To test this hypothesis, we investigated the effects of caspase-3–generated caspase-cleaved tau expression on mitochondrial function and morphology in immortalized human cortical neurons. Our data provide evidence suggesting that caspase-cleaved tau may contribute to the mitochondrial abnormalities in AD, independent of NFT formation.
Caspase-cleaved Tau Compromises Mitochondrial Function

AD (7, 14) and the finding that Asp-421-cleaved tau is toxic in our cell culture model (12), the focus of this study was to determine whether and how Asp-421-cleaved tau affected mitochondrial function. To study the role that Asp-421-cleaved tau may play in compromising mitochondrial function, we used immortalized cortical neurons that inducibly express either a full-length form of tau (T4) or a tau isoform that has been truncated at Asp-421 (T4C3). The cells expressing T4C3 showed an increase in cell toxicity, as measured by lactate dehydrogenase release, compared with T4-expressing cells (12). In this study we found that mitochondria in the T4C3-expressing cells presented with an abnormal morphology, characterized by a decrease in mitochondrial length, suggestive of fragmentation. Furthermore, when both cell types were treated with thapsigargin to globally increase intracellular calcium levels, we found that mitochondria in cells expressing T4C3 showed diminished calcium buffering capacity and higher mitochondrial reactive oxygen species (ROS) levels. Mitochondrial fragmentation and mitochondrial membrane integrity damage were completely inhibited in T4C3 cells by pretreating with the calcineurin inhibitors cyclosporine A (CsA) or FK506, suggesting a role for this calcium-dependent phosphatase in these pathogenic processes.

Our results indicate that the presence of Asp-421-cleaved tau in neurons may compromise the ability of mitochondria to function normally and ultimately contribute to the mitochondrial impairment and neuronal death observed in the AD brain.

EXPERIMENTAL PROCEDURES

Cell Culture—Immortalized cortical neurons (18) expressing inducible full-length (T4) or Asp-421-truncated (T4C3) tau were prepared as described previously (19). Cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose (Mediatech) and supplemented with 5% fetal bovine serum, 0.1% gentamicin, 4 mM glutamine, and 10 units/ml penicillin and 100 units/ml streptomycin at 33 °C. In these studies cells were treated with the tetracycline derivative doxycycline at a concentration of 2 μg/ml for 48 h to induce tau expression. After 48 h the induction media was removed from cells and replaced with Dulbecco’s modified Eagle’s medium/high glucose containing only 4 mM glutamine and 1% fetal bovine serum with 2 μg/ml doxycycline and the cells were moved to a 39 °C incubator and maintained under these conditions before treatment.

Cytosolic and Mitochondrial Calcium Measurements—Cells were grown on 35-mm dishes and loaded for 30 min (37 °C) with 5 μM Fluo-3 AM and 10 μM Rhod-2 AM in KRH-glucose buffer containing 0.02% pluronic acid. The fluorescence changes determined by Fluo-3 represent the cytoplasmic calcium changes (20), and Rhod-2 fluorescence indicates calcium changes in the mitochondria (21–23). To estimate Rhod-2 fluorescence pattern in live mitochondria, we used Mitotracker GreenTM (MTG) to mark the mitochondria (22, 23). Cells were washed 3 times and left in KRH-glucose buffer for 10 min until cell fluorescence equilibrated. Fluorescence was imaged with a confocal laser scanning microscope (Leica TCS SP2) using a 40× water immersion lens, as described previously (22, 24). Images were acquired using a 488-nm argon laser to excite Fluo-3 fluorescence and a 563-nm He-Ne laser to excite Rhod-2 fluorescence. The signals were collected at 505–530 nm (Fluo-3) and at 590 nm (Rhod-2). Fluorescence background signal was subtracted from cell fluorescence measurements in every experiment. The fluorescence intensity variation was recorded from 10–20 cells on average per experiment. Estimation of fluorescence intensities were presented as the pseudo-ratio (ΔF/Fo), which was calculated using the formula ΔF/Fo = (F – Fbase)/(Fbase – B), where F is the measured fluorescence intensity of the indicator, Fbase is the fluorescence intensity before the stimulation, and B is the background signal determined from the average of areas adjacent to the cells (22).

Mitochondrial ROS and Superoxide Level Determination in Live Cells—Cells grown on 35-mm dishes were incubated with the fluorescent probe 2.7-DCF (10 μM) and TMRM (100 nM) for 30 min in KRH buffer supplemented with 5 mM glucose. Cells were washed 3 times and left in KRH-glucose buffer for 10 min until cell fluorescence equilibrated. Fluorescence was imaged with a confocal laser scanning microscope (Leica TCS SP2) using a 40× water immersion lens, as described previously (22). Images were acquired using a 488-nm argon laser to excite 2.7-DCF fluorescence and a 563 nm He-Ne laser to excite TMRM fluorescence. Calculating fluorescence levels in a co-localized area of 2.7-DCF and TMRM fluorescent signals was used to estimate mitochondrial ROS production. The fluorescence background signal was subtracted from cell fluorescence measurements in every experiment. Images were quantified using Image-Pro Plus 6 software. Results in intensity units were expressed as the average of fluorescence signal (F) minus background fluorescence (Fbase) in every image (22).

Mitochondrial superoxide levels were determined using MitoSOX Red (25) in conjunction with mitochondrial marker MTG. Cells grown on 35-mm dishes were incubated with 20 μM MTG and 200 nM MitoSOX Red for 20 min in KRH buffer supplemented with 5 mM glucose. Images were acquired using a 488-nm argon laser to excite MTG and a 563-nm He-Ne laser to excite MitoSOX Red fluorescence. Estimation of mitochondrial superoxide production was quantified using Image-Pro Plus 6 software. Results in intensity units were expressed as the average of fluorescence signal (F) minus background fluorescence (Fbase) in every image.

Mitochondrial Membrane Potential Determination in Live Cells—Mitochondrial membrane potential was determined using TMRM (22). Before thapsigargin 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 a confocal laser scanning microscope (Leica SP2). TMRM fluorescence was detected by exciting with a 563-nm He-Ne laser attenuated (30% laser power), and emission was collected at >570 nm. Signals from T4 cells and T4C3 cells treated with thapsigargin 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 the mean TMRM fluorescence signal per live cell. TMRM fluorescence intensity was calculated as described above and is presented as the pseudo-ratio (ΔF/Fo) (22).

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Estimation of Mitochondrial Integrity Damage—To estimate mitochondrial integrity damage in live mitochondria, we used MTG dye (26, 27). MTG accumulates in the lipophilic environment of live mitochondria, and it has been shown that the signal is independent of the mitochondrial membrane potential and oxidant status (22, 27) (see supplemental data). To corroborate these observations, naïve cells were loaded with MTG and TMRM (mitochondrial potential indicator) for 30 min, and MTG/TMRM fluorescence levels were recorded in cells exposed to 10 \( \mu \text{M} \) FCCP for 20 min. Treatment of naïve cells with FCCP resulted in a pronounced decrease in the TMRM fluorescence levels after a few minutes of treatment (see supplemental Fig. 1); in contrast, MTG fluorescence levels remained constant during the entire FCCP treatment period (see supplemental Fig. 1). For our studies, cells were grown on 35-mm dishes and loaded for 30 min (37 °C) with 20 \( \mu \text{M} \) MTG in KRH-glucose buffer containing 0.02% pluronic acid. Fluorescence was imaged with a confocal laser scanning microscope as described previously (22). Images were acquired using a 488-nm argon laser to excite MTG, and signals were collected at 505–530 nm. The fluorescence intensity variation was recorded from 5–10 cells on average per experiment. An estimation of MTG fluorescence intensity was calculated and is presented as a pseudoratio (\( F/F_0 \)).

Estimation of Mitochondrial Length—Estimation of mitochondrial length and frequency analysis is based on Rintoul et al. (28). The mitochondrial length was calculated using the measured perimeter of identified objects, in live cortical cells preloaded with MTG for 30 min. Confocal and fluorescence images were taken in untreated and treated cells using a 40× water immersion objective with a 4× digital zoom on a SP2 Leica confocal microscope and a Zeiss Axiovert fluorescence microscope using a 63× oil objective. Mitochondrial length quantification was estimated using Image Pro 6 software (Media Cybernetics, MA).

Statistical Analysis—All data are expressed as the mean of at least three independent experiments ± S.E. unless otherwise stated. Statistical comparisons between treatment groups were performed using Student’s t test.

**FIGURE 1.** Expression of T4C3 induces mitochondrial fragmentation in immortalized cortical neurons. Mitochondria in naïve, T4, and T4C3 cells were labeled with the mitochondria-specific marker MTG. Mitochondria in cells expressing T4 presented with the expected tubular, rod-like morphology, whereas cells expressing T4C3 exhibited more rounded and fragmented mitochondria. A, representative confocal images of cells labeled with MTG. B, more than 90% of mitochondria in cells expressing T4C3 were less than 2 \( \mu \text{m} \) in length, whereas the majority of mitochondria in naïve and T4 cells ranged between 2 and 6 \( \mu \text{m} \). C, quantitation revealed that mitochondria in T4C3 cells showed more than a 2-fold decrease in average mitochondrial length as compared with mitochondria in cells expressing T4. Data are the mean ± S.E. (bars). *, \( p < 0.01 \) compared with T4C3 untreated cells; **, \( p < 0.05 \) compared with untreated naïve cells. \( p < 0.01 \) (*) and \( p < 0.05 \) (**) were by unpaired Student’s t test. The bar scale represents 10 \( \mu \text{m} \).
RESULTS

Expression of T4C3 Induces Mitochondrial Fragmentation in Immortalized Cortical Cells—Immortalized cortical neurons were induced to express tau by treatment with doxycycline (2 μg/ml) for 48 h (12, 29). In the absence of doxycycline, inducible cells express an almost undetectable amount of tau, as measured by Western blotting (29). However, treatment with doxycycline resulted in a robust increase in tau expression to levels approximately equivalent to concentrations seen in rat primary neuronal cortical cultures from E18 rat cortices (12). Considering that mitochondrial dysfunction occurs in the AD brain (30, 31), we investigated the possibility that T4C3 may sensitize cells to a loss of viability by compromising mitochondrial function. Mitochondria in T4 and T4C3 cells were labeled with the mitochondria-specific marker MTG to examine mitochondrial morphology in situ (Fig. 1). Nontransfected immortalized cortical neurons (referred to in the text as naïve cells) present with a mixture of tubular and rounded mitochondrial morphologies, and the mitochondria tend to be more aggregated (Fig. 1A). In contrast, mitochondria in cells expressing T4 were distributed throughout the cell body, with the expected tubular, rod-like morphology (Fig. 1A), whereas cells expressing T4C3 exhibited more rounded and apparently fragmented mitochondria (Fig. 1A). In fact, greater than 90% of mitochondria in cells expressing T4C3 were less than 2 μm in length (Fig. 1B, see bars). Quantification of the data revealed that mitochondria in cells expressing T4C3 showed more than a 2-fold decrease in mitochondrial length as compared with mitochondria in naïve cells and cells expressing T4 (Fig. 1C).

Caspase-cleaved Tau Expression Exacerbates Mitochondrial ROS Production in Immortalized Cortical Cells—To assess mitochondrial function in these cells, we measured basal mitochondrial ROS production and mitochondrial membrane potential. 2.7-DCF was used to measure ROS production (22, 23). Naïve, T4, and T4C3 cells were loaded with 2.7-DCF and TMRM for 40 min to measure changes in mitochondrial ROS production using confocal microscopy in situ. A, representative confocal images from immortalized cortical neurons loaded with 2.7-DCF/TMRM in basal conditions. Caspase-cleaved tau (T4C3) expression increased mitochondrial ROS production in immortalized cortical neurons. B, quantified mitochondrial potential levels and ROS production data from three independent experiments reveal that mitochondrial ROS levels in T4C3 cells are significantly elevated in comparison with naïve and T4 cells. Mitochondrial ROS levels were estimated by analyzing the areas of co-localization between 2.7-DCF/TMRM signals. Data are the mean ± S.E. (bars). *, p < 0.05 T4C3 cells compared with T4 cells. C, to corroborate the finding presented above, we evaluated mitochondrial superoxide levels in cortical neurons using MitoSOX. Untreated T4C3 cells showed significantly increased mitochondrial superoxide levels in comparison with T4 cells. Data are the mean ± S.E. (bars), and values are from three separate experiments. *, p < 0.05 T4C3 cells compared with T4 cells. The bar scale represents 10 μm.
and TMRM was used to measure mitochondrial potential (22, 24). Cells were loaded simultaneously with 2.7-DCF and TMRM and subsequently mounted in a confocal microscope chamber for imaging. Fig. 2A shows representative confocal images of naïve, T4, and T4C3 cells loaded with 2.7-DCF and TMRM in basal conditions. T4C3 cells showed increased levels of mitochondrial ROS production in comparison with naïve and T4 cells (Fig. 2A). T4 cells showed low mitochondrial ROS levels, indicating that under basal conditions expression of the T4 isoform did not increase oxidative stress levels in these cells (Fig. 2, A and B). Additionally, we measured basal mitochondrial membrane potential levels in the same cells using TMRM. Those studies showed that mitochondrial potential levels are not significantly altered in any cell type (Fig. 2, A and B). Quantiative analysis of mitochondrial potential fluorescence intensities as relative units, which shows that T4C3 cells treated with thapsigargin for 30 min exhibit a pronounced loss of mitochondrial potential. Data are the mean ± S.E. (bars) from three separate experiments. *, p < 0.05, T4C3 cells compared with T4 cells.

MitoSOX Red dye is used for mitochondrial superoxide determinations (25) in conjunction with a mitochondrial marker; in our experiments we used MTG (Fig. 1). T4C3 cells showed higher levels of mitochondrial superoxide as compared with T4 cells (Fig. 2C). Naïve and T4 cells showed similar mitochondrial superoxide levels in basal conditions (data not shown). These results suggest that T4C3 expression increased mitochondrial ROS and superoxide production, events that could impair mitochondrial function in immortalized cortical neurons.

Expression of T4C3 Induces Mitochondrial Membrane Potential Loss in Cortical Cells Treated with Thapsigargin—Due to the aberrant mitochondrial morphology and high levels of ROS observed in cells expressing T4C3, the calcium buffering ability of mitochondria in these cells was examined. Cells were treated with thapsigargin, which results in a global and transient increase in cytosolic calcium levels (22, 33). This treatment allowed us to examine the ability of mitochondria in these cell models to effectively uptake and sequester calcium. We found that there was no significant difference in increases in cytosolic calcium levels in treated versus untreated cells. The graph represents quantification of mitochondrial potential fluorescence intensities as relative units, which shows that T4C3 cells treated with thapsigargin for 30 min exhibit a pronounced loss of mitochondrial potential. Data are the mean ± S.E. (bars) from three separate experiments. *, p < 0.05, T4C3 cells compared with T4 cells.

Expression of T4C3 induces mitochondrial dysfunction in cortical neurons treated with thapsigargin. Mitochondria in cells expressing T4C3 respond differently to increases in intracellular calcium levels in acute experiments. A, Fluo-3 was used to measure cytosolic calcium levels in the cells after thapsigargin (Thap, 1 μM) treatment for 30 min. We found no significant difference in cytosolic calcium increases between cells expressing T4 or T4C3 after treatment. B, mitochondrial calcium levels were measured after treatment using Rhod-2. Mitochondrial calcium levels increased in cells expressing T4, whereas mitochondria in cells expressing T4C3 showed a decrease in calcium levels. C, mitochondrial membrane potential was measured using TMRM. TMRM fluorescence levels decreased significantly in T4C3 cells but not in naïve and T4 cells after thapsigargin treatment. D, the graph represents quantification of mitochondrial potential fluorescence intensities as relative units, which shows that T4C3 cells treated with thapsigargin for 30 min exhibit a pronounced loss of mitochondrial potential. Data are the mean ± S.E. (bars) from three separate experiments. *, p < 0.05, T4C3 cells compared with T4 cells.
calcium levels between cells expressing T4 and T4C3 after 1 μM thapsigargin treatment (Fig. 3A), although the level of cytosolic calcium increase in T4C3 cells did trend higher. When mitochondrial calcium levels were measured, differences between T4 and T4C3 cells were apparent (Fig. 2B). Cells expressing T4 and loaded with Rhod2 AM (a mitochondrial calcium indicator) showed an increase in mitochondrial calcium uptake (Fig. 3B). In addition, studies in naïve cells exposed to 1 μM thapsigargin showed cytosolic and mitochondrial calcium levels similar to that of the T4 cells (data not shown). We also measured mitochondrial membrane potential using TMRM (22). TMRM fluorescence levels decreased significantly in T4C3 cells after thapsigargin treatment for 30 min but not in naïve and T4 cells (Fig. 3C). Quantification of three independent experiments showed that thapsigargin induces a significant mitochondrial potential loss in T4C3 cells in comparison with naïve and T4 cells (Fig. 3D). These observations suggest that cytosolic calcium overload induced mitochondrial potential loss in T4C3 cells, and as a result mitochondrial function is impaired in cortical cells that express T4C3.

Caspase-cleaved Tau Compromises Mitochondrial Function

Calcium levels between cells expressing T4 and T4C3 after 1 μM thapsigargin treatment (Fig. 3A), although the level of cytosolic calcium increase in T4C3 cells did trend higher. When mitochondrial calcium levels were measured, differences between T4 and T4C3 cells were apparent (Fig. 2B). Cells expressing T4 and loaded with Rhod2 AM (a mitochondrial calcium indicator) showed an increase in mitochondrial calcium uptake (Fig. 3B). In addition, studies in naïve cells exposed to 1 μM thapsigargin showed cytosolic and mitochondrial calcium levels similar to that of the T4 cells (data not shown). We also measured mitochondrial membrane potential using TMRM (22). TMRM fluorescence levels decreased significantly in T4C3 cells after thapsigargin treatment for 30 min but not in naïve and T4 cells (Fig. 3C). Quantification of three independent experiments showed that thapsigargin induces a significant mitochondrial potential loss in T4C3 cells in comparison with naïve and T4 cells (Fig. 3D). These observations suggest that cytosolic calcium overload induced mitochondrial potential loss in T4C3 cells, and as a result mitochondrial function is impaired in cortical cells that express T4C3.

Caspase-cleaved Tau Expression Results in Mitochondrial Membrane Damage in Immortalized Cortical Cells—Immortalized cortical neurons expressing T4C3 showed a mitochondrial fragmentation phenotype, an increased sensitivity to calcium overload, and subsequently, mitochondrial dysfunction. To study changes in mitochondrial morphology in live cells, we loaded naïve, T4, and T4C3 cells with MTG dye (22, 26, 27). MTG binds to the mitochondrial lipid membrane, and the fluorescence staining is independent of the status of the mito-
changes in MTG fluorescence are reflective of the compromised integrity of the mitochondrial membrane. Fig. 4 shows representative confocal images of naïve, T4, and T4C3 cells loaded with MTG and treated with 1 μM thapsigargin for 30 min. Mitochondrial fragmentation was evident in untreated T4C3 cells but not in naïve and T4 cells (Figs. 1 and 4 A). Furthermore, treatment with thapsigargin for 30 min did not induce significant changes in MTG fluorescence levels in naïve and T4 cells (Fig. 4A); in contrast, treatment of T4C3 cells with thapsigargin did result in a decrease in MTG fluorescence intensity, suggesting a loss of mitochondrial integrity induced by calcium overload (Fig. 4A). Fig. 4B shows representative trends of MTG fluorescence changes in naïve, T4, and T4C3 cells exposed to thapsigargin over 30 min (Fig. 4B). Thapsigargin treatment for 30 min induced a significant decrease in MTG levels in T4C3 cells in comparison with naïve and T4 cells (Fig. 4B). Quantification of four independent experiments revealed that cytosolic calcium overload induced by thapsigargin selectively compromised mitochondrial integrity in T4C3 cells (Fig. 4C), which is not unexpected given that calcium uptake and mitochondrial membrane potential are both impaired (Figs. 1–3).

CsA Prevents Mitochondrial Fragmentation Induced by Caspase-cleaved Tau Expression in Immortalized Cortical Cells—Calcium-induced opening of the mitochondrial permeability transition pore (mPTP) has been shown to occur in cell culture models of neurodegenerative disorders (34, 35). mPTP is activated in response to different stressors (i.e. cytosolic calcium overload) in diverse cell models (35). Based on the loss of mitochondrial integrity observed in T4C3 cells treated with thapsigargin (Fig. 4, B and C), we investigated whether the opening of the mPTP may contribute to this apparent loss of mitochondrial membrane integrity. Pretreatment with 1 μM CsA for 60 min prevented fragmentation of the mitochondrial in the T4C3 cells (see confocal images in Fig. 5A). In fact, T4C3 cells pretreated with CsA exhibited an increase in the average mitochondrial length and a distribution of mitochondrial length...
that was comparable with that observed in T4 cells (Figs. 5, B and C, respectively).

CsA Prevents Mitochondrial Membrane Integrity Loss in Response to Thapsigargin in Caspase-cleaved Tau-expressing Immortalized Cortical Cells—To determine whether CsA treatment could ameliorate the mitochondrial integrity damage observed in T4C3 cells exposed to thapsigargin, we pretreated T4 and T4C3 with 1 μM CsA for 60 min and then evaluated mitochondrial integrity using MTG (Figs. 6, A and B). Pretreatment with CsA (36, 37) resulted in an increased retention of MTG fluorescence in T4C3 cells in response to thapsigargin treatment, comparable with levels observed in cells expressing T4 (Figs. 6, A and B). Quantitative analysis from three independent experiments is shown in Fig. 6B, demonstrating that CsA pretreatment prevented thapsigargin-induced mitochondrial integrity damage in T4C3 cells (Fig. 6B). These studies suggested that caspase-cleaved tau expression may induce mitochondrial dysfunction in immortalized cortical neurons exposed to calcium overload by a mechanism that involves mPTP opening.

CsA Prevents Mitochondrial Membrane Potential Loss in Response to Thapsigargin in Caspase-cleaved Tau-expressing Immortalized Cortical Cells—CsA treatment prevented thapsigargin-induced mitochondrial potential loss induced by thapsigargin in T4C3 cells. The graph represents quantification of TMRM fluorescence intensities as relative units, which shows that T4C3 cells treated with CsA plus thapsigargin exhibit a significant increase in the relative mitochondrial potential levels. D, data are the mean ± S.E. (bars) from three separate experiments. *, p < 0.05 compared with T4 cells treated with thapsigargin; **, p < 0.01 compared with T4C3 cells treated with thapsigargin.
significantly attenuated mitochondrial potential impairment in T4C3 cells (Fig. 6D).

**Inhibition of Calcineurin Prevents Mitochondrial Fragmentation in Caspase-cleaved Tau-expressing Cells**—Pretreatment with CsA attenuated mitochondrial fragmentation and dysfunction in T4C3 cells. Because CsA inhibits mPTP opening (36, 37), this would suggest a possible role for mPTP opening in mitochondrial dysfunction induced by caspase-cleaved tau expression in cortical neurons. Recently, Cereghetti et al. (38) showed that in HeLa cells mitochondrial fragmentation induced by depolarization agents involved cytosolic calcium overload and activation of the calcium-dependent phosphatase calcineurin. Interestingly, they observed that inhibition of calcineurin using FK506 as well as CsA, which in addition to inhibiting mPTP opening also inhibits calcineurin activity, prevented mitochondrial fragmentation induced by FCCP (38). Considering these data, we next examined the possible role of calcineurin activity in mitochondrial fragmentation and mitochondrial function impairment present in T4C3 cells. T4 and T4C3 cells were pretreated with 0.6 μM FK506 for 2 h and then loaded with MTG to examine mitochondrial morphology. Pretreatment with FK506 prevented fragmentation of the mitochondria in the T4C3 cells (see the representative fluorescence images, Fig. 1A). T4C3 cells pretreated with FK506 exhibited an increase in the mitochondrial length, and the distribution of mitochondrial length was comparable with that observed in T4 cells (Fig. 7A). Quantification of the data revealed that mitochondria in cells expressing T4C3 and pretreated with FK506 showed comparable mitochondrial length as those of mitochondria in cells expressing T4 (Fig. 7B). CsA and FK506 treatment increased average mitochondrial length in T4C3 cells with more than 70% of mitochondria more than 2 μm in length (for comparison see Fig. 1B). Naive cells presented an average mitochondrial length of 2.5 μm, and CsA and FK506 values were expressed as a function of this mitochondrial length. Data are the mean ± S.E. (bars), *, p < 0.01 compared with T4 untreated cells; **, p < 0.05 compared with untreated T4C3 cells. p < 0.01 (*) and p < 0.05 (**) by unpaired Student’s t test. C, CsA and FK506 treatment increased average mitochondrial length in T4C3 cells with more than 70% of mitochondria more than 2 μm in length (for comparison see Fig. 1B). Naive cells presented an average mitochondrial length of 2.5 μm, and CsA and FK506 values were expressed as a function of this mitochondrial length. Data are the mean ± S.E. (bars), *, p < 0.01 compared with T4 untreated cells. The bar scale represents 10 μm.

**FK506 Prevents Mitochondrial Membrane Potential Loss in Response to Thapsigargin in Caspase-cleaved Tau-expressing Cells**

**FIGURE 7. FK506 prevents mitochondrial fragmentation in caspase-cleaved tau-expressing cells.** T4 and T4C3 cells were pretreated with 0.6 μM FK506 for 2 h and then loaded with MTG to evaluate mitochondrial morphology changes. A, representative fluorescence images of cortical cells that indicate pretreatment with FK506 significantly diminishes the mitochondrial fragmentation observed in T4C3 cells (see Fig. 1A for comparison). B, quantification of three independent experiments revealed that the average length of mitochondria in T4C3 cells increased significantly in response to FK506 pretreatment. Data are the mean ± S.E. (bars), *, p < 0.01 compared with T4 untreated cells; **, p < 0.05 compared with untreated T4C3 cells. p < 0.01 (*) and p < 0.05 (**) by unpaired Student’s t test. C, CsA and FK506 treatment increased average mitochondrial length in T4C3 cells with more than 70% of mitochondria more than 2 μm in length (for comparison see Fig. 1B). Naive cells presented an average mitochondrial length of 2.5 μm, and CsA and FK506 values were expressed as a function of this mitochondrial length. Data are the mean ± S.E. (bars), *, p < 0.01 compared with T4 untreated cells. The bar scale represents 10 μm.
Caspase-cleaved Tau Compromises Mitochondrial Function

**DISCUSSION**

The purpose of this study was to elucidate the mechanism(s) by which caspase-cleaved tau might facilitate cell death in neurons and, in particular, how this AD relevant pathological modification of tau may impact mitochondrial function. Previously, we reported that when expressed in immortalized cortical neurons, T4C3 compromised cell viability (12). This is in agreement with previous findings that T4C3 is an effector of cell death when expressed in a number of different cell types (10, 39). In this study we observed that mitochondria in cells expressing T4C3 presented with a fragmented morphology and high levels of oxidative stress, indicative of mitochondrial dysfunction (Figs. 2 and 3). In addition to a primary role in ATP production, mitochondria also function to help maintain intracellular calcium homeostasis (15). When these cells were treated acutely with thapsigargin, which increases cytosolic intracellular calcium levels, the calcium-buffering ability of these mitochondria was found to be severely diminished (Fig. 3B), with a significant decrease in mitochondrial membrane potential and mitochondrial membrane integrity (Figs. 3C and 4). These findings suggest that Asp-421-truncated tau may facilitate neuronal death in AD by negatively affecting normal mitochondrial function and, additionally, sensitizing the cell to other stressors.

Mitochondria assist in taking up and buffering increases in intracellular calcium (15). This function of mitochondria com-
combined with the growing evidence that calcium deregulation is probably playing one of the earliest roles in AD progression (40) led us to ask whether the calcium-buffering ability of mitochondria in T4C3 cells was compromised. Indeed, short term treatment with thapsigargin revealed that cells expressing T4C3 were unable to buffer the increases in cytosolic calcium (Fig. 3B). Additionally, mitochondrial membrane potential and integrity decreased in T4C3 cells (Figs. 3 and 4). Cells expressing T4C3 also present with fragmented mitochondria that are more rounded and shorter in length than mitochondria in cells expressing T4 (Fig. 1). In addition, chronic treatment with thapsigargin induced significant mitochondrial fragmentation in T4 cells, but prolonged treatment of T4C3 cells with thapsigargin resulted in more severe mitochondrial abnormalities with a significant number of swollen and distorted mitochondria in the majority of the cells. Interestingly, naïve cells did not show any significant changes in their mitochondrial morphology in response to prolonged thapsigargin treatment when compared with untreated naïve cells. These observations are of great importance given the fact that naïve cells express extremely low levels of tau protein (12) in comparison to T4 and T4C3 cells (12). Altogether these observations are in agreement with previous evidence that supports a key role for mitochondrial fragmentation in cell death and the pathogenesis of neurodegenerative diseases (41, 42).

There is an increasing appreciation of mitochondrial morphology as an important determinant of mitochondrial function, and abnormal alterations in mitochondrial structure are thought to contribute significantly to neuronal injury and death (43, 44). The processes of fission and fusion regulate the dynamic morphological changes in mitochondria, and it is has been proposed that during times of stress these two processes...
function to regulate the number of independently operating mitochondria in an attempt to enhance their efficiency (44). Changes in mitochondrial morphology in neurons and astrocytes in response to calcium loading have been reported. For example, it was found that mitochondria changed from a rod-like structure to more of a spherical morphology when challenged with increased calcium load (34). In addition, Wang et al. (46) reported that reduction in dynamin-related protein 1 (Drp1), a regulator of mitochondrial fission and distribution, caused mitochondrial morphology abnormalities in AD patient fibroblasts. The same group recently published an elegant report in which they showed that amyloid-β protein overproduction induces an imbalance of mitochondrial fission/fusion mechanisms that result in mitochondrial fragmentation and abnormal distribution (47). These previous studies are in agreement with the observations presented in this paper, as they emphasize the importance of understanding mitochondrial morphology changes in neurodegenerative disorders, which actively contribute to mitochondrial and neuronal dysfunction.

The classic mPTP inhibitor CsA was effective in partially preventing mitochondrial morphological changes and mitochondrial depolarization seen in T4C3 cells (Fig. 4D). Moreover, pretreatment with CsA attenuated mitochondrial membrane integrity loss after thapsigargin treatment (Fig. 6B). This is intriguing given the recent studies published by Du et al. (45), which suggests that interaction of cyclophilin D (an integral protein of the mPTP) with mitochondrial amyloid-β protein potentiates mitochondrial, neuronal, and synaptic stress observed in the pathogenesis of AD.

However, the findings in our study suggest that calcineurin, rather than mPTP opening, is more likely to be involved in regulating T4C3-induced mitochondrial dysfunction. In this paper we report that inhibition of calcineurin activity prevents fragmentation and mitochondrial dysfunction in T4C3 cells. In support of our data, Cereghetti et al. (38) recently reported that inhibition of calcineurin using FK506 and CsA could prevent mitochondrial fragmentation induced by FCCP. In their study it was suggested that both compounds act through the same mechanism given the fact that CsA can indirectly affect calcineurin activity and FK506 can suppress, specifically, calcineurin activity independent of inhibiting mPTP opening (38). Our data are in concordance with these observations, as we observe similar effects for CsA and FK506 in T4C3 cells (Fig. 7C). In fact, co-incubation with CsA and FK506 prevented mitochondrial fragmentation and depolarization induced by thapsigargin to the same extent that each compound did individually (data not shown). Several other studies have also shown that inhibition of calcineurin ameliorates mitochondrial dysfunction. For example, FK506-induced inhibition of calcineurin efficiently prevents the loss of mitochondrial membrane potential in glutamate-treated astrocyte cultures (48). In addition, treatment of hippocampal neurons with glutamate resulted in inhibition of mitochondrial movement and a decrease in mitochondrial length, which were prevented by inhibiting calcineurin either with FK506 or CsA (49).

Although the data indicate that T4C3 induces mitochondrial fragmentation through activation of calcineurin, further studies are required to elucidate the mechanisms. However, it is interesting to note that subunits A and B of calcineurin bind tau, and calmodulin interferes with this association, affecting the dephosphorylation state of tau in vivo (50). Therefore, it can be hypothesized that T4 and T4C3 may differentially interact with calcineurin, and this may contribute to the increased activity toward specific substrates in the T4C3 cells and subsequently increased mitochondrial fragmentation.

Our data support the hypothesis that Asp-421-truncated tau might compromise mitochondrial function in AD brain and that this may be an important factor involved in disease progression, as both Asp-421-cleaved tau (7) and mitochondrial dysfunction (30, 31) occur early in the disease process, indeed, before NFT formation. Furthermore, data showing that neurons containing active caspase-3 do not necessarily undergo acute death (11) coupled with the findings that active caspase-3 and Asp-421-cleaved tau co-localize in AD brain (7, 8) suggest that Asp-421-cleaved tau could result in compromised mitochondrial function early in the process of AD and contribute substantially to the progression of the disease. Understanding the long term ramifications of the negative impact of Asp-421-cleaved tau on mitochondrial function, especially in reference to calcium regulation and how the cell may be sensitized to other common stressors associated with AD, could be of great importance in helping to understand a crucial process in AD progression.

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