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Tau facilitates Aβ-induced loss of mitochondrial membrane potential independent of cytosolic calcium fluxes in mouse cortical neurons

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Abstract

Alzheimer's disease (AD) is defined by presence of two pathological hallmarks, the intraneuronal neurofibrillary tangle (NFT) formed by abnormally processed tau, and the extracellular amyloid plaques formed primarily by the amyloid beta peptide (Aβ). In AD it is likely that these two proteins act in concert to impair neuronal function, and there is evidence to suggest that one of the key targets on which they converge is the mitochondria. For example, overexpression of a pathologic form of tau in rat primary cortical neurons exacerbates Aβ-induced mitochondrial membrane potential (ΔΨm) loss due to impairment of the calcium (Ca^{2+}) buffering capability of mitochondria. However the role of physiological levels of tau in mediating Aβ-induced mitochondrial dysfunction was not examined. Therefore in this present study we used primary neurons from wild type (WT) and tau knockout ($tau^{-/-}$) mice to investigate whether endogenous tau facilitates Aβ–induced ΔΨm loss and alterations in cytosolic calcium (Ca^{2+}_{cyt}). Knocking out tau significantly protected mouse primary cortical neurons from loss of ΔΨm caused by low concentrations of $Aβ_{42}$, which supports our previous findings. However, the absence of tau resulted in significantly greater increases in Ca^{2+}_{cyt} in response to Aβ treatment when compared to those observed in WT mouse primary cortical neurons. This unexpected outcome may be explained by findings that suggest $tau^{-/-}$ neurons display certain phenotypic abnormalities associated with alterations in Ca^{2+}_{cyt}. Overall, data indicate that tau facilitates Aβ-induced mitochondrial dysfunction and this effect is independent of Aβ-induced alterations in Ca^{2+}_{cyt}.[1]

Keywords

Alzheimer's disease; tau; amyloid beta; mitochondrial dysfunction; calcium dyshomeostasis

[1]Aβ – amyloid beta, AD – Alzheimer's disease, APP – amyloid precursor protein, Ca^{2+}_{cyt} – cytosolic calcium, Ca^{2+}_{mito} – mitochondrial calcium, ΔΨm – mitochondrial membrane potential, ER – endoplasmic reticulum, FTDP – frontotemporal dementia with parkinsonism, hAPP – human amyloid precursor protein, mPTP – mitochondrial permeability transition pore, OxPHOS – oxidative phosphorylation, PS2 – presenilin 2, ROS – reactive oxygen species, TCA – tricarboxylic acid, WT – wild type, 3xTgAD – triple transgenic AD mouse model

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Introduction

Tau and Aβ are both essential elements in the pathogenesis of AD. Initially Aβ was considered to be the primary effector of AD, with tau playing a minor secondary role [14]. However, it is now clear that tau is an essential player in the progression of AD that can have effects independent of, parallel to, or in concert with Aβ [34]. This first became apparent when it was demonstrated that $tau^{-/-}$ mouse primary neurons were protected from Aβ-induced neurotoxicity compared to neurons derived from WT mice [28]. There are now numerous studies indicating that tau and Aβ act in concert to elicit damage to neurons [6, 7, 25, 29]. Further, studies in mouse models have shown that reduction or absence of endogenous murine tau attenuates spatial memory deficits in mice overexpressing a familial AD form of human amyloid precursor protein (hAPP), as well as preventing excitotoxin induced epileptic activity on a hAPP or non-transgenic background [31]. These studies indicate that expression of tau is required for Aβ to elicit certain negative effects in neurons supporting the idea that tau and Aβ work cooperatively. Evidence for this has also been shown in humans. In a longitudinal study, cerebral spinal fluid (CSF) from cognitively intact elderly adults was analyzed for a pathological form of tau phosphorylated at threonine 181 (ptau-181) and $Aβ_{42}$ upon entry into the study. Participants who tested positive for $Aβ_{42}$ were more likely to experience cognitive decline over the course of the study, but only if ptau-181 was also present in their CSF [6].

The conceptual framework for tau and Aβ acting cooperatively to cause neuronal dysfunction originated with a study that compared patterns of protein deregulation in mouse models that develop neurofibrillary tangles of tau and/or plaques of Aβ. Rhein, et al. [29], conducted a proteomic analysis of vesicular preparations from non-transgenic mice, mice carrying familial AD mutations in APP and presenilin 2 (PS2) that develop Aβ pathology ($APP^{sw}PS2^{N141I}$), pR5 mice that express tau with the P301L frontotemporal dementia with parkinsonism (FTDP) mutation causing tangle formation, and triple transgenic (3xTgAD) mice that carry genes for mutant APP, mutant PS1 and P301L tau that exhibit both Aβ and tau pathology. One third of the deregulated proteins in these mice were mitochondrial proteins involved in oxidative phosphorylation (OxPHOS). Mice expressing mutant tau showed deregulation of OxPHOS complex I proteins , while mice expressing genes with Aβ-forming mutations showed deregulation of complex IV proteins. The 3xTgAD mice showed deregulation of proteins related to both complex I and IV and had the earliest and worst mitochondrial dysfunction compared to mice exhibiting either Aβ or tau pathology alone. Therefore, tau and Aβ can damage mitochondria separately, but are able to cooperate when both are present and enhance the pathological defects that they each cause [8, 29].

The role of tau and Aβ in mitochondrial dysfunction is particularly interesting due to evidence suggesting mitochondrial damage occurs early in AD and is central to its etiology [3, 11, 35]. Evidence of impaired tricarboxylic acid (TCA) cycle protein activity and deregulation of mitochondrial genes encoding OxPHOS proteins has been shown in postmortem brains of AD patients, which likely compromised metabolism [1, 19]. Female 3xTgAD mice that develop both Aβ and tau pathologies mimick these disease characteristics very early. At 3 months of age, these mice display decreased mitochondrial respiration and protein levels of pyruvate dehydrogenase, a key TCA cycle protein [37]. Thy-1 $APP^{SW/L}$

mice also show decreased ATP production, ΔΨm, and OxPHOS complex IV activity, with increased reactive oxygen species (ROS) production at 3 months of age. This coincides with detection of intracellular Aβ, but occurs several months prior to extracellular Aβ deposition suggesting mitochondrial deficits precede Aβ pathology and are a key pathogenic element in AD [15].

Calcium deregulation also contributes to AD etiology and has been associated with mitochondrial dysfunction [2, 30, 32]. Cybrids produced by transforming AD patients' platelet mitochondria into SH-SY5Y human neuroblastoma cells show increased basal Ca^{2+}_{cyt}, and inositol triphosphate (IP^3)-induced Ca^{2+} release from the endoplasmic reticulum (ER), compared with cybrids from healthy controls [33]. There is also evidence that total cellular Ca^{2+} levels are elevated in cultured fibroblasts from AD patients as compared to aged and young controls [23]. Elevated Ca^{2+}_{cyt} is known to induce rapid Ca^{2+} uptake by mitochondria in microdomains of high Ca^{2+}_{cyt} that exist near Ca^{2+} channels on the plasma membrane and ryanodine receptors on the endoplasmic reticulum (ER) [22, 30]. The resultant mitochondrial Ca^{2+} (Ca^{2+}_{mito}) overload can cause depolarization of ΔΨm, increased ROS production, opening of the mitochondrial permeability transition pore (mPTP), and mitochondrial swelling - especially in synaptic mitochondria, which are more likely to exist in high Ca^{2+}_{cyt} microdomains [13, 38]. Furthermore, a recent study found $Aβ_{42}$ oligomers cause Ca^{2+} influx into primary rat neurons from cortex, hippocampus, and cerebellum, which leads to Ca^{2+}_{mito} overload, mPTP opening, and cell death [32]. In contrast, Ma et. al., argue that mitochondrial malfunction underlies Ca^{2+} dyshomeostasis as dissipation of ΔΨm and mPTP opening in N2A cells stably expressing hAPP695 leads to impairment of store-operated Ca^{2+} entry [18]. It is unclear whether mitochondrial dysfunction precedes Ca^{2+}_{cyt} disruption or vice versa.

Recently we showed that treatment of primary neurons with sub-lethal concentrations of $Aβ_{40}$ fibrils caused ΔΨm in the presence of endogenous tau. Overexpression of a pathological form of tau truncated at D421 also caused mitochondrial dysfunction on its own and exacerbated mitochondrial dysfunction induced by $Aβ_{40}$ fibrils in rat primary neurons and in a cortical neuronal cell line [25, 26]. We also showed that tau phosphorylated at Ser396/404 (PHF-1 epitope) enhances Aβ-induced mitochondrial injury [27]. This suggested that tau and Aβ act in cooperation to cause mitochondrial damage. However, these previous studies in primary neurons were carried out using exogenously expressed tau. To more clearly define the role of tau mediating the effect of Aβ on mitochondrial function in this current study we examined the effects of Aβ on ΔΨm in neurons from $tau^{-/-}$ mice. As expected, we found that neurons from $tau^{-/-}$ mice were resistant to $Aβ_{42}$-induced ΔΨm loss compared to neurons from WT mice. However we unexpectedly found that $tau^{-/-}$ neurons showed a more pronounced Ca^{2+}_{cyt} elevation in response to $Aβ_{42}$, suggesting that tau facilitates Aβ induced ΔΨm loss in a manner that is independent from Ca^{2+}_{cyt} dyshomeostasis. The fact that $Aβ_{42}$ induced a greater increase in Ca^{2+}_{cyt} in $tau^{-/-}$ neurons may indicate that the lack of tau causes neuronal functional changes that are not universally beneficial, a factor that needs to be considered when using these mice to understand AD pathogenesis [4, 5].

Materials and Methods

Aβ Oligomer Formation

Synthetic human $A\beta_{42}$ peptide was obtained from Calbiochem (PP69, NJ, USA) in 250μg aliquots. Entire aliquots were reconstituted to 250μM in sterile PBS and incubated at 37°C without agitation for 3 days. The presence of fibrils and oligomers was confirmed via electron microscopy. Working solutions of 0.5μM $A\beta_{42}$ were diluted from the 250μM stock in sterile PBS.

Animals

Animals were housed and euthanized in accordance with guidelines of the University of Rochester committee on animal resources. Wild type C57Bl/6 (000664) and $tau^{-/-}$ (B6.129X1-*Mapt*^{tm1Hnd}/J, 007251) mice used in this study were obtained from Jackson Laboratories. $Tau^{-/-}$ mice were originally made and characterized by Dawson et al. [5]. These mice carry a homozygous deletion of the Mapt gene and have been backcrossed at least ten times onto on a C57BL/6 background.

Western Blotting

Lysates were prepared from WT and $tau^{-/-}$ mouse cerebral cortex, protein concentrations determined and 15μg of protein were separated by electrophoresis and immunoblotted with a polyclonal antibody to tau (Dako, CA, USA) and a monoclonal antibody to mouse β-actin (Millipore, MA, USA).

Mouse Primary Cortical Neuron Culture

Multi-well tissue culture plates containing 15mm #1 thickness glass coverslips were coated with 0.2% polyethylenimine (PEI) in borate buffer (pH 8.5) at room temperature in the dark for 6 hours. Plates were then rinsed with sterile distilled water and dried overnight in the dark. Mouse primary cortical neurons were isolated from C57Bl/6 or $tau^{-/-}$ mouse embryos at embryonic day (E18). Pregnant dams were euthanized with CO^2 followed by decapitation and embryos were extracted immediately. Cortices were aseptically dissected from embryos and were chemically dissociated in pre-warmed 0.25% trypsin/EDTA for 20 minutes at room temperature. Cortices were rinsed with warm DMEM before mechanical dissociation via trituration in neurobasal media supplemented with 0.5mM L-glutamine, 25uM glutamate and B27 supplement. Neurons were then plated at a density of $8x10^5$ cells/mL and were maintained at 37°C in a 5% CO^2 atmosphere. Every 4 days, the neurons received a half volume media change.

Mitochondrial Membrane Potential and Cytosolic Calcium Imaging

Between DIV7 and DIV9, neurons were loaded with 100nM Mitotracker Red CMXRos (M-7521, Life Technologies, NY, USA) and 1μM Fluo4 AM (F-14201, Life Technologies, NY, USA) in KREBS ringer buffer (136mM NaCl, 10mM HEPES, 4.7mM KCl, 1.25 mM $MgSO^4$, 1.25mM $CaCl^2$, and 25mM glucose, pH 7.4) for 30 min at 37°C to track $\Delta\Psi m$ and Ca^{2+}_{cyt}, respectively. After dye loading, neurons were rinsed with KREBS ringer buffer and imaged on a heated stage with a 40x oil immersion objective on a Zeiss Axio Observer D1

inverted fluorescent microscope. Using AxioVision software, time-lapse images were collected once a minute for thirty minutes at 595nm (Mitotracker Red CMXRos) and 488nm (fluo4 AM). Fluorescent excitation was attenuated to 25% with a Colibri light-emitting diode system. $A\beta_{42}$ was added at 5 min, after a stable baseline was established. Data is presented as an average change in fluorescence (ΔF) from the baseline reading and total difference in ΔF between the first image and the last.

Statistical Analysis

A linear mixed model generalized estimating equation was used to study the effects of $A\beta_{42}$ concentration and time on ΔΨm and Ca^{2+}_{cyt} outcomes by comparing the slopes of the full time-lapse traces. Non-parametric ANOVA was used to compare the endpoints (mean change from baseline at the thirty-minute time point) between genotypes and $A\beta_{42}$ concentrations for ΔΨm and Ca^{2+}_{cyt} experiments. Non-parametric tests were used in this case due to the non-normal data distribution.

Results

Tau$^{-/-}$ cortical neurons are protected from Aβ_{42}-induced ΔΨm loss

Time-lapse imaging of tau$^{-/-}$ and WT mouse primary cortical neurons was performed using the ΔΨm indicator MitoTracker Red CMXRos. WT primary cortical neurons showed large, decreases in ΔΨm in response to acute treatment with both 0.25μM (Fig 1, A, top) and 5μM $A\beta_{42}$ (Fig 1, B, top). In contrast, tau$^{-/-}$ neurons maintained baseline ΔΨm in response to 0.25μM $A\beta_{42}$ (Fig 1 A, bottom) and exhibited ΔΨm loss in response to 5μM $A\beta_{42}$ that was significantly less severe than that of WT (Fig 1, B, bottom) according to linear mixed model statistical analysis comparing the slopes of the quantified 30-minute time-lapse experiments (Fig 1, C). Quantitation of the total change in ΔΨm between baseline and the last imaging time point also showed that tau$^{-/-}$ neurons were significantly protected at both concentrations of $A\beta_{42}$ compared to WT mouse neurons (Fig 1 D). We also confirmed the absence of tau expression in the tau$^{-/-}$ model (Fig 1 E). These data suggest that tau plays a role in facilitating mitochondrial damage induced by low levels of $A\beta_{42}$. However, sufficiently high concentrations of $A\beta_{42}$ overcome the necessity of tau's facilitation.

Tau$^{-/-}$ cortical neurons are more susceptible to Aβ_{42}-induced Ca^{2+}_{cyt} increases than WT neurons

Tau$^{-/-}$ and WT primary cortical neurons were loaded with the Ca^{2+}_{cyt} indicator, Fluo4, prior to treatment with a range of $A\beta_{42}$ concentrations during time-lapse imaging. Contrary to our expectations, tau$^{-/-}$ neurons were more susceptible to $A\beta_{42}$-induced Ca^{2+}_{cyt} increase than WT neurons (Fig 2). Comparison of the slope of time-lapse traces revealed that both 0.25μM and 5μM $A\beta_{42}$ induced a greater increase in Ca^{2+}_{cyt} in tau$^{-/-}$ neurons than in WT neurons (Fig 2, C). In addition, the total change in Ca^{2+}_{cyt} over the entire experiment (comparing fluo4 fluorescence at baseline to that at the last time point) was also significantly higher in tau$^{-/-}$ neurons than in WT, but no significant difference was found between Aβ concentrations within genotypes suggesting the effect of $A\beta_{42}$ on Ca^{2+}_{cyt} increase is not dose-dependent (Fig 2, D).

Discussion

Tau and Aβ can cause mitochondrial dysfunction independently, but recent studies suggest they may act cooperatively to amplify their effects [7, 8, 26, 29, 37]. Our lab has previously shown that overexpression of pathologic forms of tau exacerbates Aβ-induced mitochondrial dysfunction in primary rat neurons and immortalized cortical neurons derived from mouse embryos [25, 27]. This result was intriguing, but came with the caveat that overexpression of proteins may cause off-target effects in cellular models. Therefore, we used neurons from tau−/− mice to verify that endogenous tau facilitates $A\beta_{42}$-induced mitochondrial dysfunction.

The results of this study clearly demonstrate that endogenous tau facilitates $A\beta_{42}$-induced ΔΨm. Low concentrations of $A\beta_{42}$ caused significantly greater loss of ΔΨm in primary mouse neurons that expressed endogenous tau compared to those in which tau expression was ablated. It is worth noting that the ΔΨm loss we observed in this study occurred within a much smaller scale than what was observed in our overexpression model [25]. Nonetheless, these findings clearly support the conclusion that tau and $A\beta_{42}$ act cooperatively to cause mitochondrial damage in AD.

Previous studies suggest Ca^{2+} homeostasis is impaired in AD based on evidence of enhanced total cellular Ca^{2+} concentrations and IP^3-mediated mobilization of Ca^{2+} stores in cultured fibroblasts from AD patients [16, 23]. More recent reports have implied that Ca^{2+}_{cyt} dyshomeostasis underlies Aβ toxicity in neurons [32] and neural cell lines [17, 18, 32]. The mechanism by which Aβ causes enhanced Ca^{2+} levels in these cells is a matter of some debate. For example it has been suggested that Aβ may enhance activity of N-type voltage gated Ca^{2+} channels in rat cerebellar granule neurons [24], prolong depolarization of rat hippocampal neurons by physically blocking fast-inactivating potassium channels [12] or inhibiting the sodium/potassium-ATPase that usually maintains resting membrane potential [20, 21], or by creating Ca^{2+} permeable pores in the plasma membrane of GT1-7 cells [17].

Other studies have suggested that mitochondria play an integral role in Aβ-induced Ca^{2+} dyshomeostasis and toxicity via a mechanism involving Ca^{2+}_{mito} overload, dissipation of ΔΨm, mPTP opening, and impairment of store-operated Ca^{2+} entry to replenish depleted internal Ca^{2+} stores [18, 23, 32, 33, 38]. Since approximately half of all mitochondria in cells are localized to microdomains of high Ca^{2+}_{cyt}, they are highly susceptible to Ca^{2+}-induced injury [22, 30, 38]. Because mitochondrial dysfunction and Ca^{2+} dyshomeostasis are inextricably intertwined in AD, we investigated the contribution of tau and Aβ to these pathologic phenomena.

Given that $tau^{-/-}$ mouse primary neurons were protected from $A\beta_{42}$-induced ΔΨm, we predicted their mitochondrial Ca^{2+} handling mechanisms would be intact, allowing them to effectively buffer $A\beta_{42}$-induced Ca^{2+}_{cyt} fluxes. Surprisingly, we observed just the opposite. $Tau^{-/-}$ neurons showed significantly larger increases in Ca^{2+}_{cyt} produced by both low and high concentrations of $A\beta_{42}$ compared to WT neurons. The exacerbation of $A\beta_{42}$-induced Ca^{2+}_{cyt} increases in $tau^{-/-}$ neurons may be due to alterations in neuronal function due to the absence of tau. While the $tau^{-/-}$ mouse is a well-established model [28, 31], neurons from

these mice show abnormal rates of neurite outgrowth [5, 28, 31] and the mice are more susceptible to Aβ-induced neurodegeneration at 12 months of age [4]. Axon outgrowth is governed by Ca^{2+} transients and large, frequent L-type Ca^{2+} channel transients cause growth cones to pause, effectively slowing neurite outgrowth [36]. Given that $tau^{-/-}$ mice exhibit slowed neurite outgrowth, they likely exhibit larger Ca^{2+} transients than their WT counterparts. A study by Furukawa et. al. [10], suggests that this may happen in any model in which tau's role in microtubule homeostasis is impaired. They found that expression of a FTDP mutant form of tau whose microtubule binding function is impaired causes increased L-type Ca^{2+} channel activity in a manner that requires MT depolymerization [9]. This suggests that the $tau^{-/-}$ mouse neuron cultures may have aberrant Ca^{2+} signaling mediated by microtubule destabilization, which makes them more susceptible to secondary insults, such as Aβ.

Despite previous work linking mitochondrial damage to Ca^{2+} deregulation, the results of the current study suggest that there may be a disconnect between $A\beta_{42}$ induced mitochondrial damage and Ca^{2+}_{cyt} dyshomeostasis. In t $tau^{-/-}$ primary cortical neurons large Ca^{2+}_{cyt} increases did not coincide with loss of ΔΨm suggesting that impairment of mitochondrial Ca^{2+} buffering was not the cause of the Ca^{2+}_{cyt} increase and that high Ca^{2+}_{cyt} does not always lead to mitochondrial damage.

Conclusion

Tau facilitates loss of ΔΨm induced by low concentrations of $A\beta_{42}$ independent of Ca^{2+}_{cyt} deregulation. In contrast, the *absence* of tau in primary cortical neurons facilitated $A\beta_{42}$-induced Ca^{2+}_{cyt} increases, which may indicate Ca^{2+} dyshomeostasis previously linked to microtubule destabilization [9] and developmental abnormalities in tau−/− neurons [5, 36]. Taken together, these outcomes suggest that decreasing tau - but not ablating it - may be beneficial to ameliorate AD-related mitochondrial dysfunction.

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Highlights

- $Tau^{-/-}$ mouse neurons are protected from $A\beta_{42}$-induced $\Delta\Psi m$ loss
- $Tau^{-/-}$ mouse neurons are sensitized to $A\beta_{42}$-induced Ca^{2+}_{cyt} increases
- Tau facilitates Aβ-induced mitochondrial dysfunction independent of Ca^{2+} alterations
- Reduction of tau may prevent AD-related mitochondrial dysfunction
- Complete ablation of tau may impair Ca^{2+} homeostasis

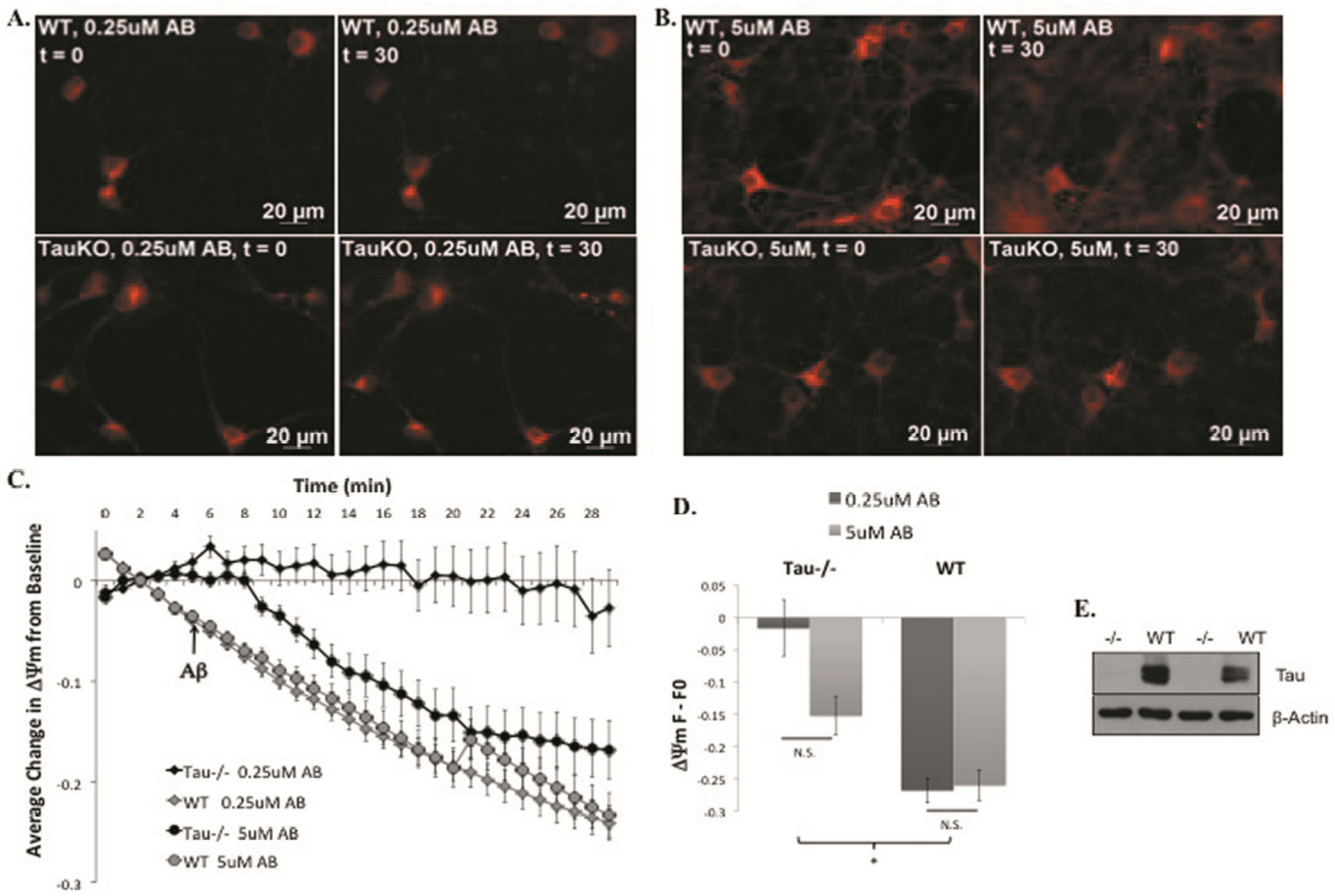

Figure 1. Tau$^{-/-}$ cortical neurons are protected from ΔΨm loss induced by low concentrations of Aβ$_{42}$

Representative images from t=0 (left) and t=30 (right) of WT (top) and tau$^{-/-}$ (bottom) primary cortical neurons loaded with ΔΨm indicator, MitoRed, and treated with 0.25μM (A) or 5μM Aβ$_{42}$ (B). Quantification of full time-lapse experiments (C) and the change from t=0 to t=30 (D). Ablation of tau was confirmed via western blot analysis of cortical lysates from tau$^{-/-}$ and WT mouse brains using a tau polyclonal antibody and β-actin monoclonal antibody (E). *=P<0.05

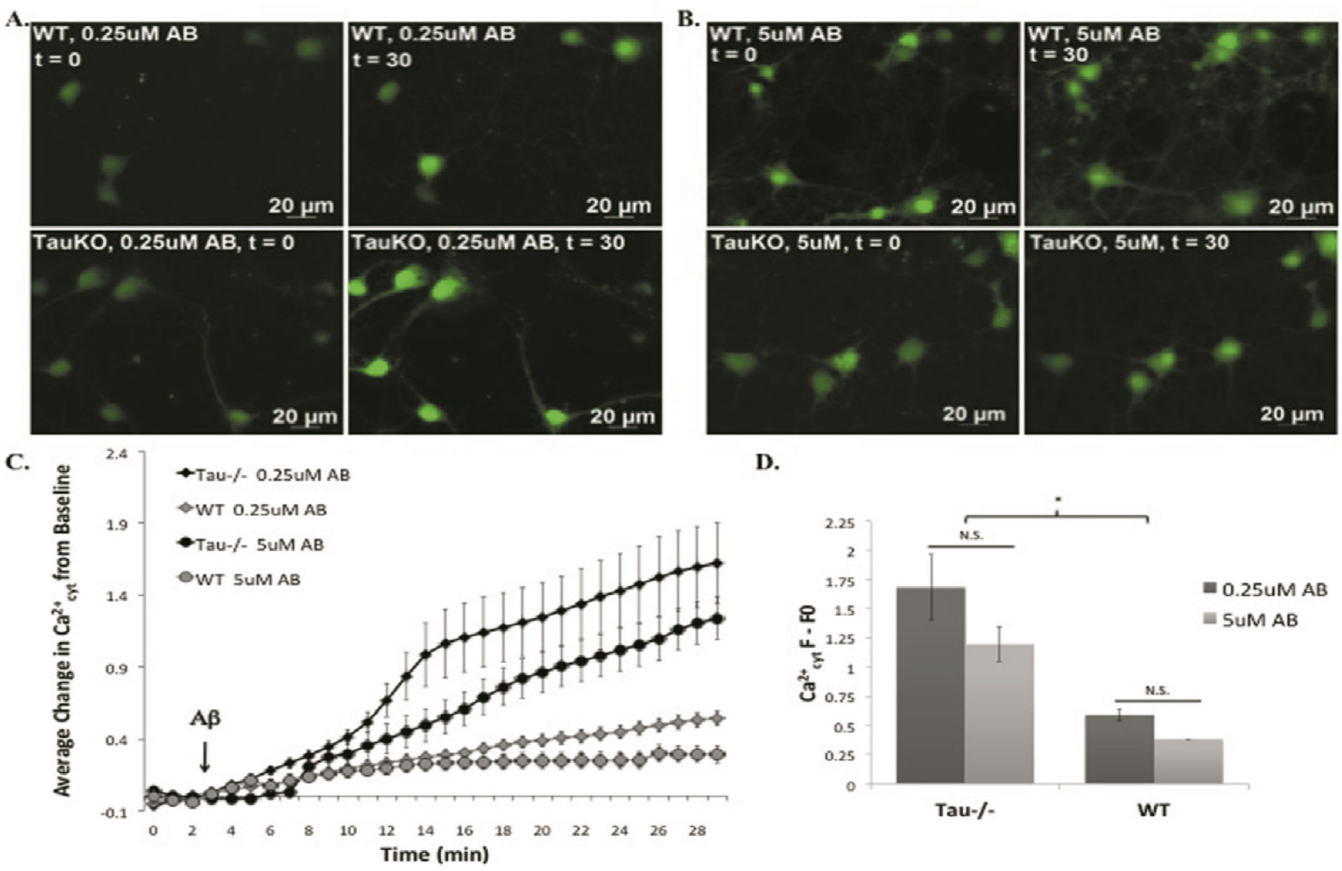

Figure 2. Tau$^{-/-}$ cortical neurons are more susceptible to Aβ_{42} induced Ca^{2+}_{cyt} increase than WT neurons

Representative images of t=0 (left) and t=30 (right) for WT (top) and tau$^{-/-}$ (bottom) mouse primary cortical neurons loaded with the Ca^{2+}_{cyt} indicator, fluo4, and treated with 0.25µM (A) and 5µM Aβ_{42} (B). Full time-lapse experiments (C) and the change from t=0 to t=30 (D) were quantified. *=P<0.05