Regulation of tau by mTor

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Background: Perturbations of the mammalian target of rapamycin (mTor) signaling pathway are implicated in Alzheimer disease (AD).

Results: The activated mTor alters the activity of major tau kinases contributing to the formation of tau dyshomeostasis.

Conclusions: We established a cellular system using genetic activation of mTor developing authentic AD like changes.

Significance: The study provides potential tools for identifying tau-based therapeutics.

SUMMARY

Previous evidence from post-mortem Alzheimer disease (AD) brains and drug (especially rapamycin)-oriented in vitro and in vivo models implicated an aberrant accumulation of the mammalian target of rapamycin (mTor) signaling in tangle bearing neurons in AD brains and its role in the formation of abnormally hyperphosphorylated tau. Compelling evidence indicated that the sequential molecular events such as the synthesis and phosphorylation of tau can be regulated through p70S6 kinase, the well-characterized immediate down-stream target of mTor. In the present study, we further identified that active form of mTor per se accumulates in tangle-bearing neurons, in particular those at early stages in AD brains.

By using mass spectrometry and Western blotting we identified 3 phospho-epitopes of tau directly phosphorylated by mTor. We have developed a variety of stable cell lines with genetic modification of mTor activity using SH-SY5Y neuroblastoma cells as background. In these cellular systems, we not only confirmed the tau phosphorylation...
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sites found in vitro, but also found that mTor mediates the synthesis and aggregation of tau, resulting in compromised microtubule stability. Changes of mTor activity cause fluctuation of the level of a battery of tau kinases such as protein kinase A (PKA), v-Akt Murine Thymoma Viral Oncogene Homolog-1 (Akt), glycogen synthase kinase 3β (GSK-3β), cyclin-dependent kinase 5 (Cdk5) and tau protein phosphatase 2A (PP2A). These results implicate mTor in promoting an imbalance of tau homeostasis— a condition required for neurons to maintain physiological function.

Alzheimer disease (AD), the single major cause of dementia in middle and old aged individuals, is histopathologically characterized by filamentous lesions, such as those composed of the 39–43 amino acid β-amyloid peptide (Aβ) and hyperphosphorylated tau (1). AD is multifactorial and heterogeneous. The vast majority of AD cases represent the so called sporadic form of the disease which is not associated with any known genetic mutation (2). The sporadic form of AD itself probably involves several different etiopathogenic mechanisms (2). Aging, neuroinflammation, head trauma, and diabetes have been implicated as risk factors for AD. The neurofibrillary degeneration is a slow and progressive retrograde neuronal degeneration, it is observed as neurofibrillary tangles (NFTs) of paired helical filaments (PHF)/straight filaments (SF) in the cell soma, and in dystrophic neurites surrounding the β-amyloid plaque core, and in neuropil threads (3). Although associations per se cannot prove cause-effect relationships, tau- forming inclusion: NFT, is widely thought to contribute to AD pathogenesis as NFT formation correlates with the duration and progression of AD (4). Both insoluble and soluble forms of abnormally hyperphosphorylated tau exist in AD brains, and they do not interact with tubulin (5,6). Furthermore, when soluble form of abnormally hyperphosphorylated tau is present, it would sequester the normal tau and microtubule-associated proteins 1 and 2 (7), accelerating disruption of the microtubule network.

It was demonstrated in transgenic mouse brains that the abnormal hyperphosphorylation of tau precedes the formation of NFTs and neuronal loss (8,9). The expression of tau pseudophosphorylated in vitro at T212, T231, and S262 triggers apoptosis (10), which is accompanied by tau aggregation and breakdown of the microtubule network (10,11). On the other hand, the expression of wild type tau in vivo exhibits synaptic loss, whereas deletion of tau rescues Aβ-induced toxicity on synapse (12-16). These evidences suggest that dysregulated production, phosphorylation, and aggregation of tau might be the key events that trigger neuronal degeneration in AD. However, little is known about the upstream intracellular effectors that account for these molecular events in the process of tau deposition, resulting in changes of neuronal function and cognitive decline, although activation of the crucial integrator of multiple signal pathways, mammalian target of rapamycin (mTor) has been proposed (17-21).

mTor is an evolutionarily conserved 289-kDa S/T kinase. Depending on the associ-
ation patterns with other proteins, two distinct complexes: the mTor complex (mTorC) 1 that controls a balance between protein synthesis and degradation and the mTorC2 that controls cellular shape by modulating actin function and promotes cell survival (22,23), can be distinguished. Both mTorC1 and mTorC2 share an identical regulatory catalytic core: Deptor, MIST8, and mTor. Raptor and PRAS40, Rictor, mSN1 and Protor are the regulatory or scaffolding components for mTorC1 and mTorC2, respectively. mTorC2 is less sensitive to rapamycin compared to mTorC1. mTorC1 is activated by growth factors, nutrients (amino acids and glucose) and stress via phosphoinositide 3-kinase (PI3K) /v-Akt Murine Thymoma Viral Oncogene Homolog-1 (Akt) and Ras /extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathways, and inhibited by deficient energy via 5’ adenosine monophosphate-activated protein kinase (AMPK) and glycogen synthase kinase -3β (GSK-3β). cAMP-dependent protein kinase (PKA) up-regulates mTorC1 via activating ERK1/2 (24), while cyclin-dependent protein kinases 5 (Cdk5) is a downstream substrate of PI3K-mTorC1 (25). mTorC1 regulates protein homeostasis via activating p70 S6 kinase (S6K), mTorC2 as a core component of PI3K pathway-phosphoinositide-dependent kinase (PDK) 2, phosphorylates Akt, and stimulates cell survival (26). Immunohistochemical and biochemical studies using postmortem human AD brains indicate a correlation between an aberrant up-regulation of mTor and the above-mentioned up- or downstream protein interactors with the occurrence and progress of tau neuropathology (20,21,27-33). Moreover, besides mTor, all of these kinases have been shown to phosphorylate tau in sites hyperphosphorylated in PHFs (27,29-36). Protein phosphatase 2A (PP2A) seems to be the major phosphatase that counterparts kinases to maintain the balance of tau phosphorylation (37). PP2A activity is down-regulated in AD brains (38,39). Blocking mTor activity with rapamycin and metformin in primary neurons and mice resulted in increased PP2A activity and reduced tau phosphorylation at three PP2A dependent epitopes: S202, S356, and S262 (40,41).

Gene sequence comparison identified that tau mRNA belongs to 5’ top mRNA group. It has been established that mTor activation via downstream S6K increases the translation of tau mRNA (19,36). It is plausible that activated mTor may facilitate tau deposition simply by increasing its translation in AD brains. Supporting this view, it has been shown in vitro that rapamycin suppresses tau translation, whereas constitutively active S6K increases tau translation (42). The levels of soluble p-Tau were significantly reduced in the brains of rapamycin-treated 3 xTg- AD mice compared to non-treated controls (43).

In view of the evidence linking mTor to tau deposition, we reasoned that up-regulation of mTor in AD brains could be a key process of AD pathogenesis. However, the manner in which mTor interacts with tau, and consequently leads to tau deposition has yet to be modeled. Here we first report that active form of mTor aberrantly accumulates in NFT-bearing neurons, especially in those at early
stage, and mTor phosphorylates tau in AD epitopes \textit{in vitro}. To further study the link between mTor and tau, based on human SH-SY5Y neuroblastom cells, we have created a series of cell lines with over-expression of wild-type mTor, rapamycin resistant mTor, mTor kinase dead, S6K kinase dead, and with suppression of mTor expression. We found that up-regulated mTor promotes tau dyshomeostasis by mediating the synthesis, phosphorylation, and aggregation of tau.

\textbf{EXPERIMENTAL PROCEDURES}

\textit{Antibodies, plasmids, reagents and materials} - For the detailed information of the antibodies used in the study, please refer to Table 1. pcDNA3.0 plasmids with constructs human flag-mTor wild type (m-WT), human flag-mTor-S2035T (rapamycin resistant site, m-S), and human flag-mTor-S2035T/D2357E (both rapamycin resistant and kinase dead sites, m-SD) were gifts from Dr. Jie Chen (University of Illinois at Urbana-Champaign, Urbana, Illinois). EECMV plasmids pp968 carrying human S6K wild type (WT), pp1971 carrying human S6K kinase dead (KD), and pp1631 carrying empty vector were kindly given by Dr. D.J. Templeton (University of Virginia Medical School, Charlottesville, VA). pLko.1 plasmids with human mTor small hairpins (sh) RNAs, packaging plasmid pCMV-dR8.2 dvpr, and envelope plasmid pCMV VSVG were purchased from Addgene (Cambridge, MA). The recombinant tau (2N4R) protein was a generous gift from Dr. M. Novak (Bratislava, Slovak Republic). Rapamycin, okadaic acid, retinoic acid (RA), brain derived neurotrophic factor (BDNF) and protease inhibitor cocktail were purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden), and culture media from Invitrogen (Stockholm, Sweden). Cytosolic abnormally hyperphosphorylated tau (AD p-tau) and PHF-tau isolated from an AD brain were described previously (21). Homogenates from undifferentiated and differentiated SH-SY5Y cells with selection pcDNA3.0 empty control vector, murine neuroblastoma N2a cells, the medial temporal cortex of a female, 91 years AD patient, and the cortical cortex of Hsp27 transgenic mouse were prepared by ourselves (44-47).

\textit{Immunohistochemistry & immunofluorescence} - Brain tissues from 6 AD cases and 6 age-matched non-neurological controls from The Netherlands Brain Bank were used in the study (Table 2). Paraffin sections (6 \(\mu\)M) of the hippocampus and adjacent temporal cortex were deparaffinized in xylene, rehydrated, and incubated with primary antibodies. The bound rabbit anti-p-mTor (S2448) or mTor (S2481) antibodies were incubated with secondary anti-rabbit IgG (1:200), detected using the avidin biotin system from Vectastain (BioNordika AB, Stockholm, Sweden), and visualized with 3, 3’-diaminogenizidine (DAB) (Sigma-Aldrich Sweden AB, Stockholm, Sweden) as brown color as previously described (28). The sections were subsequently incubated by mouse monoclonal anti-tau PHF-1 or rabbit polyclonal anti-p-Tau S422, anti-mouse/rabbit IgGs (1:200), and visualized by Vector® SG (BioNordika AB, Stockholm, Sweden) as dark grey /blue color. At least 10 contiguous microscopic fields were examined using 20 x objec-
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tive, and the enzyme positive neurons that had clearly the perikaryon were counted (48).

For immunofluorescent staining, we followed the method previously described (21) with minor modification. After dewaxing, the sections were incubated with mixture of primary antibodies against p-mTor (S2448) and anti-p-tau (PHF-1 and AT8) overnight at 4°C. Unbound antibodies were removed by washing. Bound antibodies were detected by incubation for 1h with Dylight 488 conjugated goat anti-mouse IgGs or Dylight 594 conjugated goat anti rabbit IgGs (1:200 for both, JACKSON ImmunoResearch). After staining the nuclei with DAPI, fluorescent signals were assessed using confocal microscopy (Zeiss, Oberkochen, Germany). The number of pretangles and tangles including classic tangles and ghost tangles at a relatively early stage were distinguished following the criteria described previously (32), and examined in contiguous fluorescent microscopic fields using 63x objective.

In vitro phosphorylation and Mass spectrometry - 60 ng mTor (1362-end) active kinase (having specific activity of 413 U/mg, 1 unit defined as 1 nmol of phosphate incorporated into 2 mg/ml substrate/minute), or 60 ng S6K (having specific activity of 197 U/mg, 1 unit defined as 1 nmol of phosphate incorporated into 100 µM substrate/minute) (MILLIPORE AB, Solna, Sweden) was used to phosphorylate 10 µg purified tau (2N4R) protein in a buffer containing 450 mM HEPES pH7.5, 9 mM EGTA, 0.09% Tween-20 in the presence of 0.5 mM ATP at 30°C for 2h. Reaction was stopped by adding 3% formic acid, and monitored by Western blotting with specific polyclonal antibody to p-Serine. Samples that reacted positively were digested by trypsin (1:20 trypsin/tau ratio) at 37°C for 20 h, and p-sites on tau were analyzed by strong cat ion exchange (SCX) and TiO(2)-based fractionation followed by nano-LC and mass spectrometry analysis (49). The identified phosphorylation sites of tau were further validated by western blots.

Overexpression of mTor and S6K in SH-SY5Y cells - SH-SY5Y cells were maintained in Neurobasl medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, and incubated in a humidified 5% CO₂ atmosphere at 37°C. For stable expression, SH-SY5Y cells were grown on 6-well plates until 80–90% confluence and transected with 3 µg of plasmids with various cDNAs of mTor, and empty pcDNA3.0 vector per well using Fugene HD (1:10 DNA) (Roche, Stockholm, Sweden). To make stable cells, after 48 h the transfected cells were trypsinized and re-plated into 10-cm plates at various densities in culture medium containing 0.5 mg/ml G418. For S6K, SH-SY5Y cells were co-transfected with EECMV plasmids and pcDNA3.0 (1:10 ratio) in the presence of the 0.5 mg/ml G418 in culture media.

After 7–10 days, the resistant cells were plated on 96 well plates (0.5 cell /well). The single clones that survived after G418 suppression were expanded. The expression efficiency was analyzed by western blots using antibodies against the expressed proteins of targeted genes.

Silencing the expression of S6K and mTor in SH-SY5Y cells - S6K shRNAs (SR)
were cloned into pSilencer 1.0-U6 plasmids and co-transfected with pCDNA3.0 vector in SH-SY5Y cells in this study. Briefly, S6K-SRs were designed based on the sequence of rat S6K DNA exons, and the most specific two complementary pairs of oligos 1A, 1B, 2A and 2B, with 21nt sequences started with GG or GGG, were selected. They are: S6K-SR1A: GA AGC TTC AGC GCC ACT TCA A; S6K-SR1B: AGCTT AGT GGC GCT GAA GCT TC; S6K-SR2A: AGCTT AGT GCC GCT GAA GCT TCC TTTTG; S6K-SR2B: AATTCAAAAA GGA AGC TTC AGC GCC ACT TCA A. The Oligo double helixes (S6K-SR1 and S6K-SR2) were made by mixing and incubating the complementary oligo pairs: 1A and 1B; 2A and 2B (10 mM each, 10 ml) at 95°C for 5’, 65°C for 10’, and 37°C for 10’. The annealed duplexes S6K SR1 and S6K SR2 were cloned into pSilencer 1.0-U6. Following the protocol from Ambition, the duplexes were cut from the recombinant plasmids, the correct size of plasmid and inserts were checked by 2.5% agarose gel filtration and the inserts were further confirmed by sequencing. SH-SY5Y cells were co-transfected with pSilencer 1.0-U6 plasmids for S6K-SR1 or S6K-SR2 and pcDNA3.0 (1:10 ratio) in the presence of the 0.5 mg/ml G418.

mTor shRNAs were cloned into pLko.1 vector. Following the instruction from Addgene, plasmids 1855 carrying mTor_1 shRNA (pLko.1 mTor SR1, m-SR1), 1856 carrying mTor_2 shRNA (pLko.1 mTor SR2, m-SR2) and 8453 carrying empty vector (pLko.1 puro, pLko.1) were propagated in and purified from Stbl2 bacterial cells (Invitrogen, Stockholm, Sweden), and co-transfected together with the pCMV-dR8.2 dvpr and pCMV VSVG plasmids (Addgene, Cambridge, MA) into actively growing HEK293T cells using Fugene HD (Roche, Stockholm, Sweden) as described (50). Virus-containing supernatants were collected at 60 h after transfection and concentrated by ultracentrifugation at 23,000 rpm using an SW28 rotor at 4°C for 1.5h. Pellets were re-suspended overnight at 4°C in 1/600 of the original volume. SH-SY5Y cells were transfected twice in the presence of 6 μg/ml protamine sulfate (Sigma-Aldrich Sweden AB, Stockholm, Sweden), and the SH-SY5Y cells carrying pLko.1 vector were selected in the presence of 0.5 μg/ml puromycin.

The resistant cells for S6K-SRs and mTor-SRs were expanded and plated on 96 well plates (0.5 cell /well). The single clones that survived after suppression of G418 (pcDNA3.0) and puromycin (pLko.1) were expanded. The silence efficiency was checked by western blots.

Cell cultures and sample preparations- To control the expression levels of the targeted genes (S6K and mTor), after transfection, the cells were washed with ice-cold phosphate-buffered saline (PBS), harvested and suspended in lysis buffer containing 40 mM Hepes, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanagnate, 1% Triton X-100 and 1% protease inhibitor cocktail on ice for 20 min. The supernatant samples were obtained after centrifuging at 13,000 × g for 20 min.

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For the majority of the experiments, the stable transfected cells were grown to 70–80% confluence in 100 mm culture dishes, employing Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (1:1) supplemented with 10% fetal bovine serum (FBS). The cells were then cultured in 1% FBS media for 24h, and experiments were performed in serum deprived condition from 30 min to 8h before cells were harvested.

For experiments treated with the physiological (100 μM) and pathophysiological (300 μM) dosages of zinc that have been characterized in our previous studies (21,44,51), cell lysates were sonicated on ice and centrifuged at 1 000-12 000 × g, 4°C for 10-20 min to collect supernatants free of nuclei and big cell debris.

For preparation of differentiated SH-SY5Y cells homogenates, SH-SY5Y cells with selection pcDNA3.0 empty control vector were plated in 100mm culture dishes at a density of 10^5 cells per cm^2. After 24 h, the culture medium was changed to the serum-free employing DMEM/F12 medium supplemented with 10 μM RA for 5 days and 0.5-5 ng / ml BDNF 2 days. The cells were deprived of BDNF 1 days before samples were collected. After deprivation of both serum and BDNF, cells were washed with PBS and suspended in Triton lysis buffer containing 3% SDS.

Isolation of soluble and insoluble fractions- Cell lysates in Triton lysis buffer were sonicated on ice and centrifuged at 100,000 × g, 4°C for 1h for separating supernatant (cytosolic fraction) from pellet (insoluble fraction) that was re-suspended in 1% Triton X-100 lysis buffer containing 3% SDS.

In situ microtubule binding ability assay of tau- The samples were prepared as previously described with minor changes (52). Briefly 4.5 h after serum removal, cells were rinsed with warm PBS and suspended in warm microtubule-stabilizing buffer (80 mM PIPES/KOH, pH 6.8, 1 mM GTP, 1 mM MgCl_2, 1 mM EGTA, 0.5% Triton X-100, and 30% glycerol) containing 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 μM okadaic acid, and 10 μM Taxol. The samples were then centrifuged at 5 000 × g at room temperature for 10 min to remove nuclei. The postnuclear lysates were further centrifuged at 100,000 × g at room temperature for 1h. The supernatant (S) was collected, while the pellet (P) was rinsed twice, re-suspended in microtubule-stabilizing buffer, and briefly sonicated.

Protein measurement and Western blotting- After cell culture, protein concentration of samples prepared from the stable cell lines was determined by a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Equal amounts (20-80 μg/lane) of protein were loaded onto 8-10% (w/v) SDS-polyacrylamide gels. Separated proteins were blotted onto PVDF mats.
membranes (MILLIPORE AB, Solna, Sweden), and blocked in 5% (w/v) nonfat milk for 1h diluted in Tris-buffered saline supplemented with 0.1% (v/v) Tween-20 (TBST). The membranes were incubated with primary antibodies (Table 1) at 4ºC, overnight, and then with secondary peroxidase coupled anti-mouse or anti-rabbit antibodies (1:2000, GE Healthcare AB, Solna, Sweden) at room temperature for 1h. After exposure to Hyperfilm MP (Amersham Biosciences, Piscataway, NJ), bound antibody intensity was analyzed using Image J software. Probed filters were stripped by using stripping buffer (100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM, Tris–HCl pH 6.8) at 50ºC for 30 min with occasional agitation.

Statistical analysis-For data from western blotting, statistical comparisons between different experimental groups were performed by one-way ANOVA followed by Bonferroni post-hoc test analyses. A value of p ≤0.05 was considered as significant.

RESULTS

Increase of mTor immunoreactivity in tangle bearing neurons in AD brains- By double immunostaining, the mottled immunopositive aggregates of phosphorylated (p-) mTor (S2448) was observed to be increased in CA1 pyramidal neurons that accumulate hyper-p-Tau pS422 (Fig.1A) and PHF-1 (Fig.1B), the classic NFTs indicated by fat white arrow. The increase of p-mTor (S2448) was also found in the pretangle neurons that show positive granular structures or completely negative staining for anti-p-Tau, indicated by thin white arrows. The co-existence of p-Tau labeled by PHF-1 and AT8 with p-mTor (S2448) in was further studied using immunofluorescent microscopy. A similar pattern of co-existence between p-Tau (PHF-1 and AT8) and p-mTor (S2448) (Fig.1 C1, C2, C3, D1, D2, D3, E1, E2, E3, F1, F2, F3, G1,G2 G3, H1, H2, H3) was found compared to the immunostainings visualized by DAB (A, B). More than 90% of the pretangles and more than 80% of the tangles co-existed with different extent of the p-mTor (S2448) aggregates (Table 3). A small portion of pretangle neurons and normal-looking neurons were positive only for p-Tau (PHF-1 and AT8) and for p-mTor (S2448) (not shown in picture), respectively. A relatively larger portion of tangle-bearing neurons only positive for p-Tau (PHF-1 and AT8) was found, and no tangle-bearing neurons were only positive for p-mTor (S2448). Dystrophic neurites positive for both PHF-tau (PHF-1 and AT8) and p-mTor (S2448) were also observed (data not shown). Antibodies against p-mTor (S2481) were also used, but not working properly in paraffin-embedded sections even after different approaches of antigen retrieving such as in 0.01 M citric sodium buffer pH 6.0 80ºC for 10 min by microwave or water bath, or > 100 ºC for 20 min by autoclave.

Phosphorylation of tau by mTor in vitro- To understand if mTor can directly phosphorylate tau and how the global tau phosphorylation profile is mediated by mTor, we carried out in vitro phosphorylation of tau (2N4R) by active mTor. Mass spectrometry resulted in >88% coverage of tau protein and uncovered two phosphorylation sites: S214 at
the flanking region and S356 at microtubule binding region (Fig.2A-C). These two sites were also confirmed by western blots (Fig.2D). Additionally, increased phosphorylation on T231 site that was not covered by mass spectrometry was identified by western blots (Fig.2D). Other antibodies such as anti-P-Tau S262 and PHF-1 were also used in western blot analysis, and no additional phosphorylation sites were recognized by these antibodies.

**Tau synthesis and phosphorylation in cells influenced by genetic interference of mTor activity** - To study how mTor is involved in tau synthesis and phosphorylation, we established stable cellular models with SH-SY5Y cells as background in which mTor activity is constitutively genetically modified. We centrifuged the cell lysates from these cell lines at 1000 × g to remove the nuclei, and the postnuclear samples were resolved by Western blotting. The SH-SY5Y cells that over-expressed wild type mTor (m-WT) or mTor mutated at rapamycin binding site (m-S) showed an increased level of p-S6K T389, the well-characterized downstream substrate of mTorC1, while no difference of p-S6K T389 was found in the SH-SY5Y cells that over-expressed mTor mutated at both rapamycin binding and kinase dead sites (m-SD) as compared to the SH-SY5Y cells carrying empty pcDNA3.0 vector (V1) (Fig.3A). The increased levels of p-S6K T389 in both m-WT and m-S cells indicated mTOR activity in these cell lines. Total S6K level was not changed among the four cell lines. In contrast, the SH-SY5Y cells in which mTor was knocked out (partial m-SR1; complete m-SR2) decreased level of p-S6K in a dose-dependent manner as compared to empty pLko.1 vector (V2). The level of total S6K was not changed among the different cell lines (Fig.3B).

We also analyzed the levels of tau in the postnuclear samples from these cell lines. Up-regulation of mTor activity by overexpression of m-WT increased levels of total (R134d), de-p-(Tau-1) and p-Tau (TG3 and PHF-1) at ~50 kDa as compared to V1 control, m-S and m-SD cells (Fig.3C). Down-regulation of mTor (m-SR1, and m-SR2) decreased the levels of all forms of tau (total, de-p-, and p-Tau) (Fig.3D). The increased level of de-p-Tau might represent the newly synthesized tau driven by up-regulated mTorC1 activity. The mechanism why m-S cells did not show increased total and phosphorylated levels of tau compared to V1 control cells remained to be further investigated.

Like reported by other groups (53,54), we also noticed multiple bands of tau in SH-SY5Y cell homogenates when loading 30μg/lane (data not shown). The molecular weights of these bands were judged by both the positions of the molecular markers used in every electrophoresis and existence of the positive bands (masses) in different preparations such as in differentiated SH-SY5Y cell lysates (SY5Y cells-diff), purified soluble AD-p-tau, and insoluble PHF-tau, homogenates prepared from murine neuroblastoma N2a cells, a case of AD medial cortex, and mouse brain cortical cortic. When the experimental conditions was optimized by titrating different concentrations of primary antibodies or loading different amount of proteins (not shown), only 50 kDa
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(55) and or 36 kDa bands remained when loading 40 µg/lane (data not shown): only 50 kDa band for R134d, Tau-1, TG3 (existing when in high protein amount, not shown), PHF-1 (existing only in high protein amount, not shown); only 36 kDa band for anti-p-Tau S262 and anti-p-Tau S214; both 50 kDa and 36 kDa bands with stronger intensity at 36 kDa was: anti-p-Tau S356; only 110 kDa was: anti-p-Tau T212. The faint 50 kDa band for TG3 and PHF-1 was observed when more than 80 µg/lane postnuclear supernatant from V1 control cells were loaded (not shown).

Two faces of S6K in tau synthesis- When the kinase dead form of S6K was stably overexpressed in SH-SY5Y cells (Fig.4A), a dramatic increase of total S6K and a dramatic decrease of p-S6K T389 were observed as compared to V3 empty control vectors (V3). Correspondingly, in a similar manner to mTor knockout shown in Figure 3D, all forms of tau (total, de-p- and p-Tau) were decreased. In an attempt to clarify the role of S6K in tau synthesis, we also tried to establish stable cell lines with S6K depletion. Although we could manage to achieve the clones but failed to passage the cell lines as the growth rate of both cell lines (S6K-SR1 or S6K-SR2) was very slow. To our surprise, the growth rate of S6K silence cells (S6K-SR1 or S6K-SR2) was slower than mTor silence m-SR1 and m-SR2 cells. From the limited amount of cell lysates we detected a decreased S6K and an increased total tau in S6K-SR1 cells as compared to V4 control vectors, and S6K-SR2 cells did not show significant change (Fig. 4B).

Rapamycin decreases tau synthesis-To further explore the role of mTor in tau synthesis, V1 control cells were cultured and treated with 100 µM or 300 µM zinc sulfate for 4h in the presence of 20 ng/ml rapamycin. Both 100 µM and 300 µM zinc could induce increased levels of total (Fig.5A), de-p-Tau (Tau-1) (Fig.5B), and p-Tau S214 (Fig.5C). However, a consistent decreased effect for all forms of tau was observed in V1 control cells treated with both 100 µM and 300 µM zinc in the presence of 20 ng/ml rapamycin, as compared to only 100 µM zinc treated controls, with exception for total tau in condition treated with 300 µM zinc in the presence of rapamycin.

Tau aggregation in cells influenced by genetic interference of mTor activity- To evaluate tau aggregation mediated by mTor, the soluble and insoluble forms of p-Tau were extracted with 1% Triton X-100 (Fig 6A and 6B). Tau in insoluble fraction represents aggregated tau. Regarding the different p-Tau sites in Western blots, PHF-1 mainly recognized p-Tau mass at ~50 kDA. Both anti-p-Tau S214 and anti-p-Tau S262 recognized p-Tau mass at ~36 kDa, while anti-p-Tau S356 recognized two p-Tau masses: 50 kDa and 36 kDa. P-Tau recognized by PHF-1 was predominantly observed in the soluble fraction and was increased in m-WT cells as compared to V1 control cells (Fig.6A, the histogram for PHF-1). In contrast, the increased level of p-Tau at S214 was predominantly located in the insoluble fraction of m-WT cells as compared to the V1 control cells (Fig.6A, the histogram for p-Tau S214). Levels of p-Tau S356 at ~50 kDa
showed a substantial increase in both soluble and insoluble fractions of m-WT cells, whereas levels of p-Tau S356 at ~36 kDa were not changed (Fig.6A, the histograms for p-Tau S356). Levels of p-Tau S262 were reduced in soluble fraction but not changed in insoluble fraction in m-WT cells compared to V1 control cells (Fig.6A, the histograms for p-Tau S262).

A large variation of p-Tau level was observed in soluble and insoluble fractions between V2, m-SR1, and m-SR2 cells (Fig.6B). In soluble fraction, PHF-1 was not changed in m-SR1, but increased in m-SR2, as compared to V2. In insoluble fraction PHF-1 signal was not changed between V2, m-SR1, and m-SR2 (Fig.6B, the histogram for PHF-1). A decrease of p-Tau S214 was found in insoluble fractions of m-SR1 cells, while an increase of p-Tau S356 at ~50 kDa found in both soluble and insoluble fractions of m-SR2 cells (Fig.6B, the histograms for both p-Tau S214 and p-Tau S356). Levels of p-Tau S262 were decreased in m-SR1 in the soluble fraction, and not changed in the insoluble fraction of both m-SR1 and m-SR2 (Fig.6B, the histogram for p-Tau S262).

Microtubule binding capacity of tau in cells influenced by genetic interference of mTor activity. As both mTor and S6K can phosphorylate the flanking and repeat regions of tau, the effect of mTor on the microtubule binding capacity of tau was analyzed. We found that m-WT cells showed a significant increase for total tau and p-Tau S356 in microtubule dependent fraction was also found in m-WT cells (Fig. 7B, the histograms for T Tau (R134d) and p-Tau S356) as compared to V1 control cells. Genetic inactivation of mTor activity in m-SR1 and S6K-KD cells reduced total tau in both microtubule independent and dependent fractions, as well as p-Tau S214 in microtubule independent fraction. m-SR1 but not S6K-KD reduced p-Tau S214 in microtubule dependent fraction. No change was found for p-Tau S356 in m-SR1 and S6K-KD cells compared to control cells.

Immunoreactivities of PKA, Akt, GSK-3β, Cdk5 and PP2A in cells influenced by genetic interference of mTor activity. The immunoreactivities of antibodies to these enzymes were measured in the postnuclear supernatants (Fig.8A). While V1 control cells were negative for p-Akt S473, the well-characterized downstream substrate of mTorC2, the m-WT, m-S, and m-SD cells showed a dramatic increase for p-Akt S473 (Fig.8B). A step-wise decrease of p-Akt S473 was observed from m-WT, m-S to m-SD. mTor partial deletion (m-SR1) showed a reduction of p-GSK-3β S9, while the mTor complete deletion (m-SR2) showed almost negative level of p-Akt S473 compared to V2 control. The S6K-KD cells showed a dramatic increase of p-Akt S473 compared to V3 control.

The level of p-GSK-3β S9 was increased in m-WT cells and not changed in both m-S and m-SD cells, as compared to V1 control cells (Fig.8D). The mTor partial deletion (m-SR1) showed a reduction of p-GSK-3β S9, while the mTor complete deletion (m-SR2) showed no change for p-GSK-3β S9.
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DISCUSSION

Previously we have extensively studied the role of mTor signaling and tau hyperphosphorylation in SH-SY5Y cells, murine N2a neuroblastoma cells, rat primary neurons, metabolically active rat brain slices that were treated with physiological or pathological dosage of zinc (21,31,44,51,56,57). In the present study the role of mTor in biochemical changes (translation, phosphorylation, and aggregation) of tau was studied in AD brains, by in vitro phosphorylation plus mass spectrometry, and in SH-SY5Y cells with different genetic modifications of mTor activity.

A key sensor of cellular stress, mTor, maintains protein homeostasis (23,58). Its phosphorylation at S2448 by Akt activates mTor, while phosphorylation at T2446 site by AMPK inactivates mTor (23,58). We previously found an ~3-fold increase by dot blots in p-mTor (S2481) in the homogenates of AD brains but no change in p-mTor (S2448) (20), a site that signals upstream AKT activity. However, a relatively more focal change for p-mTor (S2448) was found in special groups of neurons in the present study (Fig.1). The active forms of both S6K and Akt were reported to be aberrantly accumulated in tangle bearing neurons (21,32). Taken together, it suggests that both mTorC1 and mTorC2 are up-regulated in AD brains.

The increase of total tau in AD brains (59,60) is not likely caused by up-regulated transcription of tau gene, but by an up-regulated translation of tau mRNA since tau mRNA copy is not changed in sporadic AD brains (61,62), and tau mRNA has 5’top like
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structure that is preferentially regulated by mTorC1-S6K pathway (36,42). Inhibition of mTor with rapamycin induced decreased level of total tau both in vitro and in vivo (36,43,51). In the present study, we found that constitutive overexpression of mTor increased total tau, meanwhile constitutive deletion of mTor or constitutive overexpressions of the inactive form of mTor and S6K decreased total tau (Fig. 3 & Fig.4). Taking into consideration of the previous data reported in our lab that mTor signaling is up-regulated in AD brains (20,21), it is suggested that mTorC1-mediated tau translation contributes to the significant amount of normal tau remained in the homogenates of AD brains (59,60).

We reported that a ~10-fold increase in AD brains is selectively found for p-Tau epitopes T217, S202, T231, and T231/S235, these sites are located at flanking region (46). Pseudo-phosphorylating tau at the flanking and repeat regions (p-T212, p-T231, and p-S262) not only results in the loss of its normal function but also the gain of a toxic activity that causes disruption of the microtubule network and cell death (10). Tau in SH-SY5Y cells is hyperphosphorylated at some of the same sites as AD soluble P-tau purified from AD brains such as S262, T231/S235, S396/404 and S214 (55,63), it does not bind to taxol-stabilized microtubules but fully inhibits tau-promoted microtubule assembly in vitro, although about three times more SY5Y tau was required to achieve the same inhibition as AD P-tau. Interestingly, results from the in vitro phosphorylation assay and cellular models in the present study showed that mTor phosphorylates tau at S214, T231, and S356 (Fig.2-4), a similar group of epitopes shared by other pro-survival signalings such as Akt and S6K (36,64,65) that exclusively mediate the phosphorylation sites of tau at flanking and repeat regions, resulting in inhibition of microtubule binding (66,67). These signals are critical for converting tau into a toxic molecule.

Hyperphosphorylation contributes to the aberrant formation of insoluble tau aggregates (10,68), but the mechanisms that bridge the hyperphosphorylation and aggregation of tau are poorly understood. Epitopes such as S214 at ~36 kDa or S356 at ~50 kDa of tau induced by mTor were principally extracted in Triton-X-100 insoluble pellet, suggesting that mTor mediates the process for tau to be converted from soluble hyperphosphorylated form to aggregated form.

Efforts aiming to elucidate the mechanisms of abnormal tau hyperphosphorylation, a major step of tau deposition in neurons of AD brains, have led to identification of several tau protein kinases PKA, Akt, GSK-3β, S6K, and Cdk5, and tau protein phosphatase PP2A (21,27,28,31,35,55). Results from the present study confirmed that the phosphorylated level of GSK-3β is regulated by mTorC2. It is known that GSK-3β is constitutively phosphorylated at Y216 site when it is expressed (29,32), and GSK-3β S9 is sensitive to the phosphorylation regulation by Akt via PDK1 and PDK2 (mTorC2) in response to the fluctuation of upstream signals (22,23).

Data from the present study indicated that genetic up-regulation of mTor increases
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the phosphorylated levels of Akt and GSK-3β, the levels of both total and phosphorylated levels of PP2A, and the protein levels of both PKAα and PKAβ, but the level of the PI3K-mTorC1 down-stream target Cdk5 was decreased (Fig.8). It has also been shown that mTor phosphorylates and suppresses PP2A activity, and inhibition of both mTorC1 and mTorC2 with LY294002 and selective inhibition of mTorC1 with rapamycin decrease the phosphorylation of PP2A catalytic subunit (69). The impacts of mTor on the immunoreactivities of these enzymes suggest that the end phosphorylation status of tau in neurons with up-regulated mTor activity observed in AD brains is a synergistic action of multiple up- and or down- stream signaling targets of mTor including itself, with exception of GSK-3β and Cdk5 since they were suppressed by up-regulated mTor. The discrepancies regarding the effects of mTor on the activities of PKA, Akt, and PP2A, and the various aspects of tau homeostasis between m-WT, m-S and m-SD cells existed. It is speculated that mutation of mTor at rapamycin resistant site changes its interaction mechanism with tau and the up- stream signaling that regulate tau phosphorylation.

In summary, we have established a simple and yet highly reproducible cellular system of tau hyperphosphorylation and aggregation, recapitulating some key features of AD. We have employed this system to gain important insights in the role of mTor in tau hyperphosphorylation at the flanking and microtubule binding regions. Taking into consideration that mTor is a major intracellular hub integrating divergent intracellular and extracellular signals, and it is activated by these signals in neurons in aging process, it suggests a central role for mTor in the onset and progression of tau pathology in sporadic AD. Our cell-based cellular models not only provide a valuable tool for studying the pathogenesis of tau abnormal translation, hyperphosphorylation and aggregation, but it also offer a potential system for identifying therapeutic strategies against neurodegenerative tauopathies, such as small molecules inhibiting translation, hyperphosphorylation and fibrillation of tau or promoting microtubule stability.

References
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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Immunoreactivity of p-mTor (S2448) in neurons with different degrees of PHF-Tau involvement. Double immunostainings for p-mTor (S2448) and PHF-Tau (p-Tau S422 and PHF-1) in CA1 pyramidal neurons of AD hippocampus. The brown color shows anti-p-mTor (S2448) immunoreactivity and grey color anti-PHF-Tau (p-Tau S422 in A and PHF-1 in B). Neurons with both brown and grey color structures indicated by fat white arrow are classic tangles, and neurons with single brown punctuate structures or with both brown and grey punctuate structures indicated by thin white arrow are pretangles.

In the pre-tangle neurons, some of the granular stainings of p-mTor (S2448) are partially overlapped with dotted PHF-tau labeled by PHF-1 (C1, C2, C3) or fine filamentous structures labeled by AT8 (F1, F2, F3). In classic tangle-bearing neurons, the granular structures positive for p-mTor (S2448) distributes along with the tangled filamentous structures positive for PHF-1 (D1, D2, D3) or fine filamentous structures positive for AT8 (G1, G2, G3). A lot more of neurons bearing pretangles and tangles distinguished by the immunoreactivities of p-mTor (S2448) and PHF-tau (PHF-1 and AT8) were shown in E1,E2,E3 and H1,H2 H3.

Bar scale in panel B, D1, E1, G1, and H1 indicates the size of the structures.

FIGURE 2. mTor directly phosphorylates tau. Schematic diagram of the phosphorylation sites on the longest isoform of tau (2N4R) phosphorylated in AD brain (http://cnr.iop.kcl.ac.uk/ hang-erlab/tautable) (A). Sites phosphorylated by mTor identified by mass spectrometry: High energy collision induced dissociation (HCD) spectrum (and respective annotation) of the observed doubly charged precursor ion 829.8987 m/z matched to the phosphopeptide IGsLDNITHVPGGGNK. Observed fragment masses are shown in black whereas theoretical fragment masses are shown in red. Mass meas-
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Mass measurement errors are well under 0.01 m/z, in accordance with the high resolution employed (7500@400m/z). Ions are denoted b ions (if extending from the N-terminal) or y ions (from the C-terminal), and some ions have incurred also in loss of the phospho group (H3PO4), which is typical of phospho peptide HCD fragmentation spectra. The observed fragment ions are consistent with a phosphorylation located at Serine 3 (i.e. Serine 356 in the protein sequence). In this respect, of particular importance is the presence of the y9 ion (866.4479), which proves that Threonine 8 is unmodified, leaving the side chain of Serine 3 as the only possible phosphorylation site (B); High energy collision induced dissociation (HCD) spectrum (and respective annotation) of the observed doubly charged precursor ion 573.7858 m/z matched to the phosphopeptide TPSPTPPTR. Observed fragment masses are shown in black whereas theoretical fragment masses are shown in red. Mass measurement errors are well under 0.01 m/z, in accordance with the high resolution employed (7500@400m/z). Ions are denoted b ions (if extending from the N-terminal) or y ions (from the C-terminal), and some ions have incurred also in loss of the phospho group (H3PO4), which is typical of phospho peptide HCD fragmentation spectra. The observed fragment ions are consistent with a phosphorylation located at Serine 3 (i.e. Serine 214 in the protein sequence). In this respect, of particular importance is the presence of the ions y3, y4, y5, y6, y7 and b2, which prove that all Threonines in the peptide are unmodified, leaving the side chain of Serine 3 as the only possible phosphorylation site (C).

FIGURE 3. Tau synthesis and phosphorylation is influenced by genetic modification of mTor activity. Cell lines in panel A are SH-SY5Y cells with selection empty control pcDNA3.0 (V1), flag wild type mTor (m-WT), flag mTor mutated at S2035T rapamycin resistant site (m-S), and mTor kinase dead mutated at both rapamycin resistant and activity sites (S2035T/D2357E, m-SD). Cell lines in panel B are SH-SY5Y cells with partial or complete silence of mTor by mTor shRNA1 (m-SR1) or mTor shRNA2 (m-SR2). The selection empty control vector (V2) for this group of cells is pLko.1. Panels C and D show the impact on total (T), phosphorylated (p), and de-p tau with genetic modification of mTor activity. Anti-GADPH indicates the loading controls. Blots are representative from at least 3 independent experiments.

FIGURE 4. Tau synthesis is influenced by genetic modification of S6K activity. Panel A shows the characterization of control SH-SY5Y cells (V3) with both selection pcDNA3.0 and EECMV empty plasmids, and S6K kinase dead SH-SY5Y cells (S6K-KD). All forms of tau including total (T), phosphorylated (p), and de-p tau are decreased. Panel B shows the impact of S6K deletion on total tau. S6K shRNA SH-SY5Y cells (S6K-SR1 and S6K-SR2) and the control SH-SY5Y cells (V4) with both selection pcDNA3.0 and pSilencer 1.0-U6 empty plasmids were used. Anti-GADPH or anti-actin indicates the loading controls. Blots are representative from at least 3 independent experiments. The corresponding results from the independent experiments were presented as mean ± SD.* p<0.05 compared to control.

FIGURE 5. Pharmacological inactivation of mTor activity decreases tau synthesis. SH-SH-SY5Y cells with selection pcDNA3.0 empty vector (V1) were grown and treated with 100 μM or 300 μM zinc sulfate in the presence of 20 ng/ml rapamycin for 4 h in serum deprivation condition. Levels of Total (T) (R134d), de-phosphorylated (de-p-Tau) (Tau-1) and phosphorylated (p) Tau (p-Tau S214) were analyzed by Western blots. Blots are representative from at least 3 independent experiments. The corresponding results from the independent experiments were presented as mean ± SD.* p<0.05 and ** p<0.01, compared to control; * p<0.05 and **p<0.05, rapamycin + zinc compared with only zinc.
FIGURE 6. Tau aggregation is influenced by mTor. Triton X-100 soluble and insoluble samples were fractioned from cell homogenates at 100,000 x g, 4°C for 1h, and analyzed by western blots with anti-tau antibodies: total (T) and phosphorylated (p) tau. Two groups of SH-SY5Y cell lines are: 1) cells with selection pcDNA3.0 empty control vector (V1) and cells with flag wild type mTor (m-WT); 2) cells with selection pLko.1 empty control vector (V1), cells with partial deletion of mTor (m-SR1) and complete depletion of mTor (m-SR2). Blots are representative from at least 3 independent experiments. The corresponding results from the independent experiments were presented as mean ± SD.* p<0.05 and ** p<0.01 compared to control.

FIGURE 7. Microtubule binding capacity of tau is influenced by mTor. Panel A shows levels of total (T) and phosphorylated (p) tau in soluble supernatant free of microtubules, and panel B shows T Tau and p-Tau in insoluble microtubule fractions. The three groups of SH-SY5Y cell lines from left to right are: 1) cells with selection pcDNA3.0 empty vector (V1), cells with flag wild type mTor (m-WT), cells with flag mTor mutated at S2035T rapamycin resistant site (m-S), and cells with flag mTor kinase dead mutated at both rapamycin resistant and activity sites (S2035T/D2357E, m-SD); 2) cells with selection pLko.1 empty control vector (V2), cells with partial deletion of mTor (m-SR1); 3) control cells (V3) that contain both selection pcDNA3.0 and EECMV empty plasmids, and cells with S6K kinase dead (S6K-KD). Blots are representative from at least 3 independent experiments. The corresponding results from the independent experiments were presented as mean ± SD.* p<0.05, ** p<0.01, and *** p<0.001 compared to control.

FIGURE 8. Immunoreactivities of tau kinases and tau phosphatase PP2A are influenced by mTor. The three groups of SH-SY5Y cell lines from left to right are: 1) cells with selection pcDNA3.0 empty control vector (V1), cells with flag wild type mTor (m-WT), cells with flag mTor mutated at S2035T rapamycin resistant site (m-S), and cells with flag mTor kinase dead mutated at both rapamycin resistant and activity sites (S2035T/D2357E, m-SD); 2) cells with selection pLko.1 empty control vector (V2), cells with partial deletion of mTor (m-SR1) and complete depletion of mTor (m-SR2); 3) control cells (V3) that contain both selection pcDNA3.0 and EECMV empty plasmids, and cells with S6K kinase dead (S6K-KD). Blots are representative from at least 3 independent experiments. The corresponding results from the independent experiments were presented as mean ± SD.* p<0.05 and ** p<0.01, compared to control.
Table 1. Antibodies used in this study

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Regulation of tau by mTor

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T-total, r-rabbit, m-mouse, p-phosphorylated, de-p- dephosphorylated, IHC-immunohistochemistry, IF-immunofluorescence, WB-western blot.

Table 2. Case details

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<td>83</td>
<td>1</td>
<td>08:50</td>
<td>1120</td>
<td>33</td>
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</table>

AD-Alzheimer disease, Control-Non-demented control, f-female, m-male, Br.Staging-Neurofibrillary staging criteria by Braak & Braak, PMD-post-mortem delay, Br.wgt-Brain weight (g).

Table 3. Numbers of pretangles and tangles (labeled by PHF1 or AT8) co-stained with p-mTor (S2448)

<table>
<thead>
<tr>
<th>Pretangles</th>
<th>Classic tangles and ghost tangles at early stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Only positive for p-mTor (S2448)</td>
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<tr>
<td>PHF-1 fields) (120)</td>
<td>7</td>
</tr>
<tr>
<td>AT8 (120 fields)</td>
<td>4</td>
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</tbody>
</table>

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Regulation of tau by mTor

Fig. 1, Tang et al, 2013
Regulation of tau by mTor

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Regulation of tau by mTor

Fig. 2, Tang et al., 2013
Regulation of tau by mTor

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Fig.3, Tang et al, 2013

Fig.4, Tang et al, 2013
Regulation of tau by mTor

Fig. 6, Tang et al., 2013

Fig. 7, Tang et al., 2013
Regulation of tau by mTor

Fig. 7, Tang et al., 2013

Fig. 8, Tang et al., 2013
Regulation of tau by mTor

Fig. 8, Tang et al., 2013
mTor mediates tau dyshomeostasis: implication for Alzheimer disease
Zhi Tang, Erika Bereczki, Haiyan Zhang, Shan Wang, Chunxia Li, Xining Ji, Rui M. Branca, Janne Lehtiö, Zhizhong Guan, Peter Filipcik, Shaohua Xu, Bengt Winblad and Jin-Jing Pei

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