

Amyloid- β signals through tau to drive ectopic neuronal cell cycle re-entry in Alzheimer's disease

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Summary

Normally post-mitotic neurons that aberrantly re-enter the cell cycle without dividing account for a substantial fraction of the neurons that die in Alzheimer's disease (AD). We now report that this ectopic cell cycle re-entry (CCR) requires soluble amyloid- β ($A\beta$) and tau, the respective building blocks of the insoluble plaques and tangles that accumulate in AD brain. Exposure of cultured wild type (WT) neurons to $A\beta$ oligomers caused CCR and activation of the non-receptor tyrosine kinase, fyn, the cAMP-regulated protein kinase A and calcium-calmodulin kinase II, which respectively phosphorylated tau on Y18, S409 and S416. In tau knockout (KO) neurons, $A\beta$ oligomers activated all three kinases, but failed to induce CCR. Expression of WT, but not Y18F, S409A or S416A tau restored CCR in tau KO neurons. Tau-dependent CCR was also observed *in vivo* in an AD mouse model. CCR, a seminal step in AD pathogenesis, therefore requires signaling from $A\beta$ through tau independently of their incorporation into plaques and tangles.

Key words: Alzheimer's disease, Tau, Amyloid, Cell cycle, Phosphorylation

Introduction

Two types of insoluble, filamentous aggregates, amyloid plaques and neurofibrillary tangles, are the defining histopathological features of AD brain (Querfurth and LaFerla, 2010; Selkoe, 2001). Plaques are extracellular, and their principal components are the ~40 amino acid long $A\beta$ peptides produced by proteolysis of the amyloid precursor protein (APP). Tangles, in contrast, form inside diseased neurons and their major constituent is the axon-enriched microtubule-associated protein, tau. Although definitive diagnosis of AD typically requires postmortem, histological confirmation of plaques and tangles, there is a growing awareness that soluble forms of $A\beta$ and tau are major toxic agents in AD pathogenesis (Gandy et al., 2010; Kaye et al., 2003; Lambert et al., 1998; Lasagna-Reeves et al., 2010; Patterson et al., 2011).

One of the most significant, but poorly understood features of AD is that adverse effects of $A\beta$ typically depend on tau. Prominent examples include cytotoxicity (Nussbaum et al., 2012; Rapoport et al., 2002), disassembly of microtubules (Jin et al., 2011; King et al., 2006; Zempel et al., 2010), learning and memory deficits (Roberson et al., 2007), excitation/inhibition imbalance (Roberson et al., 2011), and impaired axonal transport (Vossel et al., 2010), dendritic function (Ittner et al., 2010; Zempel et al., 2010) and long term potentiation (Roberson et al., 2011; Shipton et al., 2011). Tau is therefore indispensable for many of the signature deleterious properties of $A\beta$, so deciphering the molecular mechanisms that underlie $A\beta$ -tau connections is bound to advance the development of sorely needed diagnostic and therapeutic tools for AD.

Most regions of normal adult human brain are nearly devoid of dividing neurons, but cortical neurons in AD brain commonly re-enter the cell cycle. Ironically, instead of dividing, these neurons apparently die up to a year after exiting G0 of the cell cycle (Greene et al., 2007; Herrup et al., 2004; Lee et al., 2009; Vincent et al., 1996). This ectopic cell cycle re-entry (CCR) evidently accounts for a significant fraction of the cortical neurons that are lost in AD (Arendt et al., 2010) and has also been observed in numerous mouse models of the disease (Li et al., 2011).

A key development in determining mechanisms that drive post-mitotic neurons back into the cell cycle was the finding that oligomeric $A\beta_{1-42}$ can induce genomic DNA replication in primary cultured neurons (Varvel et al., 2008). It thus became possible to use cell cultures as tools for systematically dissecting the pathway by which neurons ectopically re-enter the cell cycle in AD. Using this experimental approach, we tested the hypothesis that tau is also required for neuronal CCR triggered by $A\beta_{1-42}$ oligomers. We found that $A\beta$ -induced neuronal CCR does indeed depend on tau, along with multiple protein kinases that are activated by $A\beta_{1-42}$ oligomers and must then catalyze site-specific phosphorylation of tau. These results, coupled with our additional finding here of tau-dependent neuronal CCR *in vivo* in a mouse model of AD (Mucke et al., 2000), functionally link $A\beta$ and tau in a critical early step of AD pathogenesis before their respective integration into plaques and tangles.

Results

The principal method we used to monitor CCR in primary cultures of mouse cortical neurons was to expose the cells

for 24 hours simultaneously to $A\beta_{1-42}$ oligomers and bromodeoxyuridine (BrdU), after which they were fixed and stained for double immunofluorescence with antibodies to MAP2 and BrdU. The anti-MAP2 stained neurons, but not the occasional contaminating glial cells, and the anti-BrdU identified cells that had incorporated appreciable amounts of the thymidine analog into genomic DNA, and thus had entered or completed S phase (Varvel et al., 2008).

Neuronal CCR requires tau

Exposure of neurons from wild-type (WT) mice for 24 hours to oligomeric, but not monomeric $A\beta_{1-42}$, caused ~80% of the cells to become BrdU-positive (Fig. 1A). In contrast, comparable neurons from tau knockout (KO) mice (Dawson et al., 2001) rarely incorporated BrdU (Fig. 1A). BrdU uptake was quantified by 'in-cell westerns' (Chen et al., 2005), which measured total immunofluorescently labeled BrdU and MAP2 in individual cultures. This method indicated that $A\beta_{1-42}$ oligomers, but not monomers, caused a 4–5-fold increase in MAP2-normalized BrdU uptake in WT, but not tau KO cultures (Fig. 1B). Note that the method measured BrdU uptake in both neurons and glial cells, and thereby under-reported the enrichment of BrdU-positive neurons caused by $A\beta_{1-42}$ oligomers.

As shown in Fig. 2A, a shorter, 16 hour exposure to $A\beta_{1-42}$ oligomers produced a 10-fold increase in neurons in G1, from 1.4% to 14% of the neurons present, based on immunoreactivity with the nuclear G1 marker, cyclin D1 (Sherr, 1996) in cells marked by the neuron-specific nuclear protein, NeuN. Longer exposure to $A\beta_{1-42}$ oligomers, up to 72 hours, induced little, if any, neuronal apoptosis, however, as determined by the relatively

constant low level of cleaved caspase 3 immunoreactivity in cells that expressed MAP2 and tau at all time points examined (Fig. 2B).

Tau dependence for $A\beta$ -induced CCR was also observed *in vivo* (Fig. 3). In this case, quantitative double immunofluorescence was used to mark neurons with anti-MAP2 and cells that had re-entered the cell cycle with anti-cyclin D1. Nearly 60% of neurons in cortical layers 2, 3 and 6, the CA1 region of the hippocampus, and the entorhinal cortex were positive for cyclin D1 in 6-month-old hAPPJ20 mice, which overproduce human APP with Swedish and Indiana mutations, accumulate amyloid plaques, and express endogenous mouse tau (Mucke et al., 2000). In contrast, we never observed cyclin D1-positive neuronal nuclei in hAPPJ20 mice in a tau KO background, or in WT or parental tau KO mice. Note that the WT mice were of the C57BL/6 strain, from which all of the transgenic mouse strains used here were derived. The finding of cyclin D1-positive nuclei only in hAPPJ20 mice that expressed tau represents a cell biological parallel to earlier findings that hAPPJ20/tau KO hybrid mice do not develop the learning and memory deficits of the hAPPJ20 parental strain (Roberson et al., 2007).

Specific kinase activation and tau phosphorylation accompany CCR

AD tau is phosphorylated at many sites that are rarely phosphorylated in normal adult tau (Gong et al., 2005; Lee et al., 2001). To test whether tau phosphorylation at specific sites is necessary for CCR induced by $A\beta$ we focused on several protein kinases involved in tau phosphorylation, cell cycle regulation and AD pathogenesis (Fig. 4A,C,E,G). Pharmacologic inhibition of

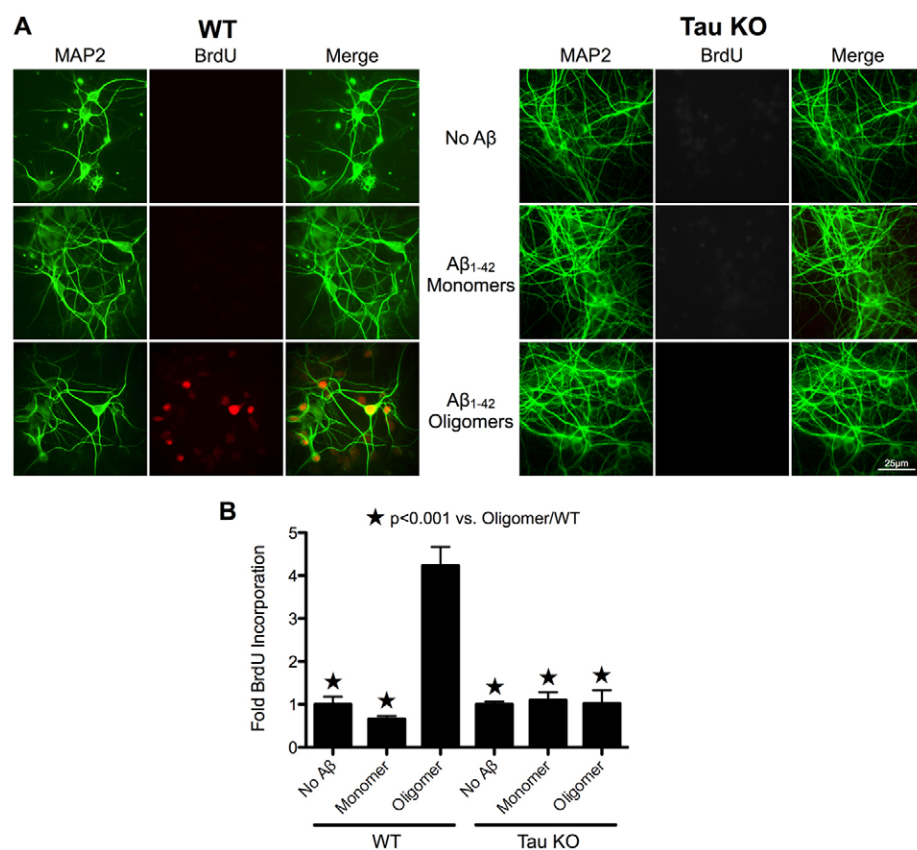


Fig. 1. Tau is required in cultured neurons for CCR induced by $A\beta$. (A) Primary neurons from WT and tau KO mice were incubated for 24 hours in the absence or presence of the indicated forms of $A\beta_{1-42}$ at 3 μ M total peptide in the presence of BrdU, and then were stained for immunofluorescence with antibodies to MAP2 and BrdU to identify neurons with newly synthesized nuclear DNA. Note the appearance of BrdU-positive neuronal nuclei only in WT neurons exposed to $A\beta_{1-42}$ oligomers (B) Quantification of BrdU incorporation by 'in-cell westerns' (Chen et al., 2005). Error bars represent s.e.m.

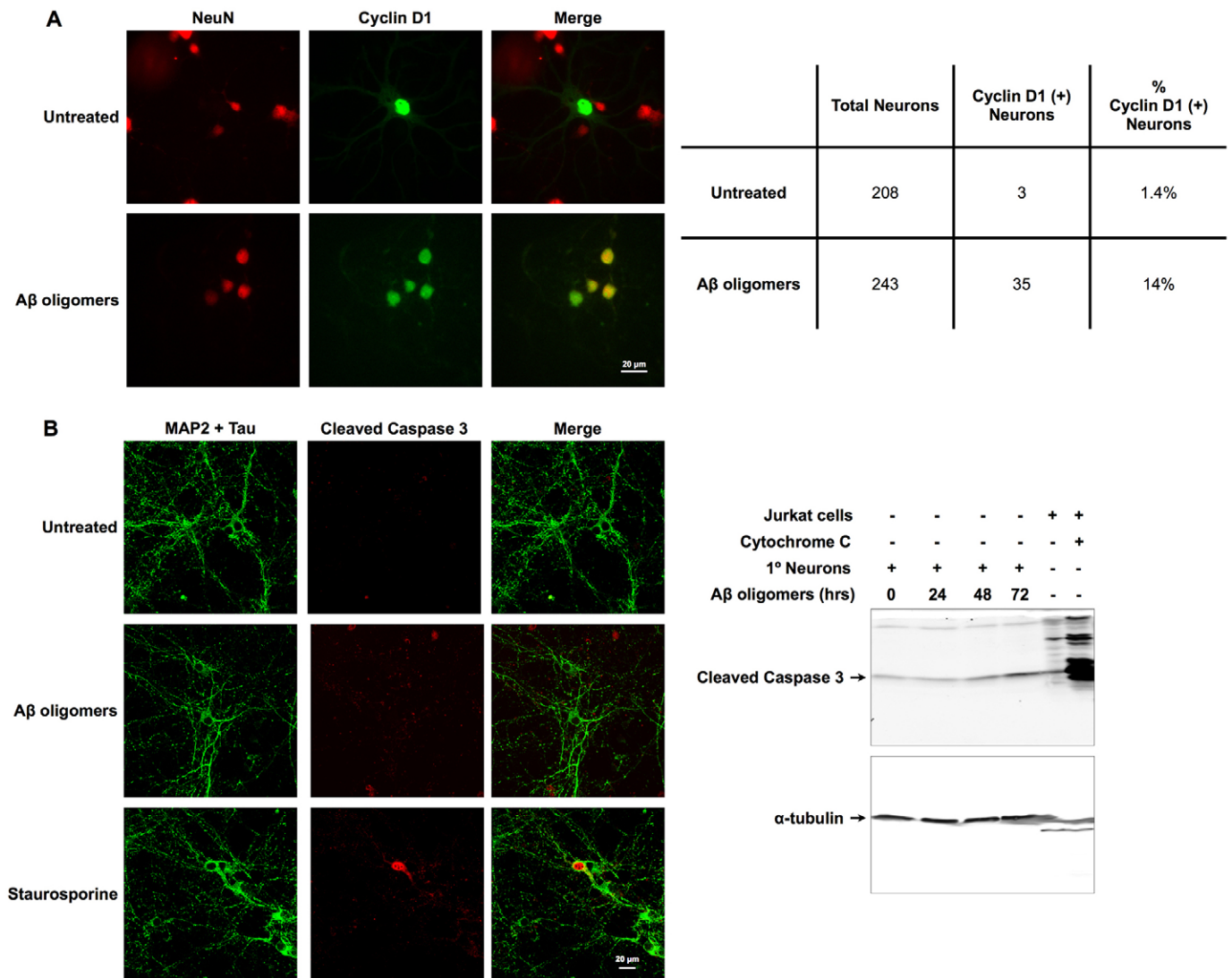


Fig. 2. A β_{1-42} oligomers induce expression of cyclin D1, but not cleaved caspase 3, in cultured neurons. (A) Primary neurons exposed for 16 hours to A β_{1-42} oligomers at 6 μ M total peptide were fixed, and stained for immunofluorescence with antibodies to the neuron-specific nuclear marker, NeuN, and the G1 marker, cyclin D1. Note the numerous G1-positive neurons in the oligomer-treated, but not the untreated, culture (left). Quantification indicated that A β_{1-42} oligomers induced a 10-fold rise (1.4% to 14%) in cyclin D1-positive neurons (right). (B) Primary neuron cultures exposed for 72 hours to A β_{1-42} oligomers at 6 μ M total peptide or to 300 nM staurosporine for 24 hours were fixed and stained for immunofluorescence with a mixture of mouse monoclonal anti-MAP2 (clone 2) and mouse monoclonal anti-tau (clone 5) to label neurons, and rabbit polyclonal anti-cleaved caspase 3 to reveal apoptotic nuclei. Note that staurosporine, but not A β_{1-42} oligomers, induced apoptosis (left). Western blotting of cultured neurons treated with A β_{1-42} oligomers for up to 72 hours confirmed the lack of cleaved caspase 3 induction, which was observed in Jurkat cells treated with cytochrome C (note: Jurkat extracts were obtained from Cell Signaling, Inc.; catalog no. 9663).

calcium-calmodulin kinase II (CaMKII) with KN-93, of the non-receptor tyrosine kinases, fyn and src, with PP2, or of the cyclic AMP-dependent protein kinase A (PKA) with KT5720 blocked BrdU uptake, and thus CCR, in WT neurons. CCR was also blocked by the mTOR inhibitor, rapamycin, as reported earlier (Bhaskar et al., 2009), but not by Ag1024, an inhibitor of IGF-1 and insulin receptor kinase, which act upstream of mTOR (Zoncu et al., 2011), nor by glycogen synthase kinase3 β (GSK3 β) inhibitor II, which potently blocks GSK3 β , a major tau kinase that can be activated by A β (Hernández et al., 2010).

Western blotting for total and phospho-activated CaMKII, fyn or PKA indicated that all three kinases were strongly activated by

A β_{1-42} oligomers and that inhibition of any individual kinase did not block activation of the others (Fig. 4B,D,F). CaMKII, fyn and PKA were therefore activated independently of each other. All three enzymes were also efficiently activated by A β_{1-42} oligomers in tau KO neurons (Fig. 4H), indicating that at least some neuronal responses to A β do not require tau.

Following their activation in WT neurons exposed to A β_{1-42} oligomers, fyn, PKA and CaMKII phosphorylated tau at Y18, S409 and S416, respectively (Fig. 5A). In contrast, A β_{1-42} oligomers did not cause increased phosphorylation of tau at S199, T205 or T231, which are GSK3 β substrates (Hanger et al., 2009), nor at the MARK/Par-1 sites, S262 or S356 (Drewes et al., 1997)

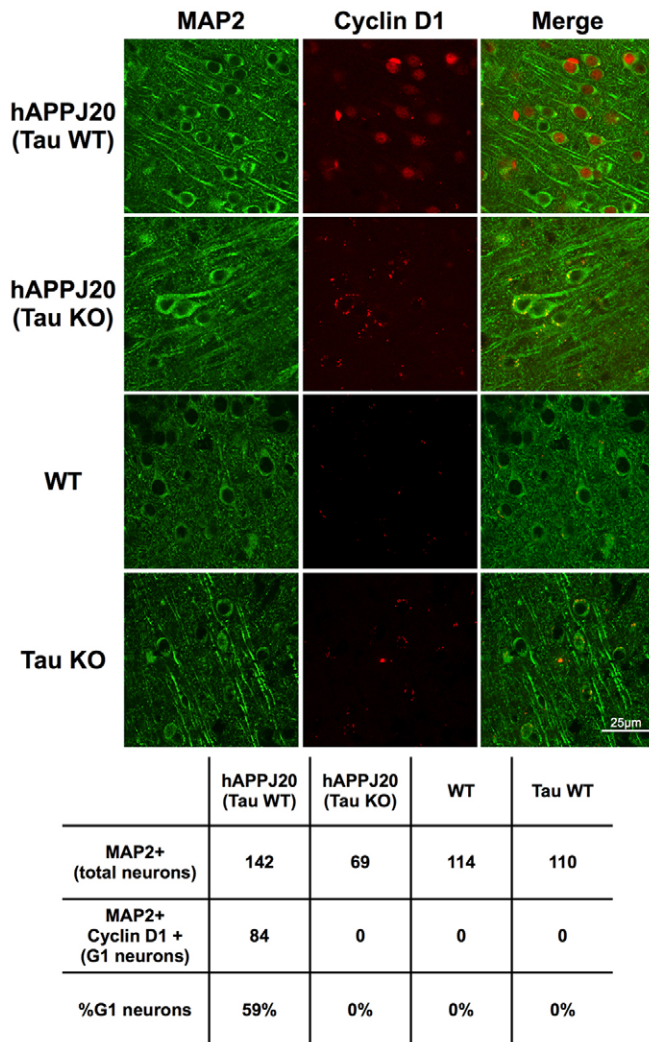


Fig. 3. Tau is required for neuronal CCR in an AD mouse model. Cortical forebrain sections from 6-month-old mice of the indicated genotypes were stained for immunofluorescence with antibodies to MAP2 and cyclin D1 to identify neurons in G1. The hAPPJ20 strain overexpresses human APP with Swedish and Indiana mutations, and accumulates amyloid plaques (Mucke et al., 2000). Note that abundant cyclin D1-positive neuronal nuclei were found in the brains of parental hAPPJ20 mice that expressed endogenous mouse tau, but not in the brains of hAPPJ20 mice that were crossed into a tau KO (Dawson et al., 2001) background. Although brain sections from all mouse strains examined did exhibit non-nuclear fluorescent puncta in the red (cyclin D1) channel, the puncta were bright in all other fluorescence channels and thus represented autofluorescent material. For quantification (lower panel), counts were derived from images like those shown here (upper panel) using brains from three different animals per strain, processed in two different experiments.

(Fig. 5B). Pharmacologic inhibition of fyn, PKA or CaMKII blocked phosphorylation respectively at Y18, S409 or S416, and with one exception, did not interfere with phosphorylation at any of the other sites. The exception was CaMKII, whose inhibition by KN-93 also blocked PKA-catalyzed tau phosphorylation at S409 (Fig. 5A). Because KN-93 did not inhibit PKA activation after exposure of neurons to Aβ₁₋₄₂ oligomers (Fig. 4F), S409 is inaccessible to PKA until CaMKII phosphorylates tau at one or more sites, possibly including S416. Note that all amino acid

numbers are based on the sequence of human 2N4R tau, the 441 residue isoform and the largest tau isoform in brain (NCBI Reference Sequence: NP_005901.2).

Site-specific tau phosphorylation is required for CCR

Finally, we tested whether tau phosphorylation at Y18, S409 and S416 were merely coincidental with CCR or essential for the process by using lentiviruses to re-introduce WT tau or tau that could not be phosphorylated at these sites into tau KO neurons (Fig. 6). Transduced tau KO neurons expressed tau at levels nearly identical to the level of endogenous tau in WT neurons. WT tau expression in tau KO neurons enabled more than 30% of the cells to incorporate BrdU. In contrast, expression of tau with Y18F, S409A or S416A mutations, which respectively prevented phosphorylation by fyn, PKA and CaMKII at those residues, did not restore CCR induced by Aβ₁₋₄₂ oligomers. We therefore conclude that a minimum of three residues on tau, Y18, S409 and S416, must be phosphorylated to enable neurons to re-enter the cell cycle.

Discussion

The collective results presented here define an Aβ-induced, tau-dependent, pathway to massive cell death in AD: aberrant re-entry of neurons into the cell cycle. The pathway includes three sequential stages (Fig. 7): (1) Aβ oligomer-induced activation of fyn, PKA and CaMKII; (2) tau phosphorylation by these kinases at Y18, S409 and at S416, respectively; and (3) exit of neurons from G0 into the cell cycle. Notably, this destructive pathway requires soluble forms of Aβ and tau prior to their incorporation into plaques and tangles.

Evidence for neuronal CCR has been known since the mid-1990s, when several phospho-epitopes characteristic of dividing cells were discovered in AD brain, but not in age-matched normal brain (Vincent et al., 1996). Subsequent studies have implicated numerous proteins involved in this process, examples of which include DNA polymerase-β and the p53 tumor suppressor (Copani et al., 2007); a group of chromatin modifying proteins that are activated by a complex of cyclin D1 and Cdk4 (Greene et al., 2007); and a C-terminal cytoplasmic domain of APP (Neve and McPhie, 2007). A possible involvement of tau in this process was revealed by studies of transgenic *Drosophila* expressing human tau, which triggered a neurodegeneration pathway that requires expression of cell cycle markers in affected neurons prior to apoptosis (Khurana et al., 2006). At least two important features of the fly pathway differ from what we describe here for mammalian neurons. First, whereas the fly pathway requires Sgg, a homolog of mammalian GSK3 (Khurana et al., 2006), we found that exposure of mouse neurons to GSK3β II inhibitor, which blocks the activity of both mammalian GSK3 isoforms (Watcharaset al., 2003), did not prevent Aβ-induced CCR (Fig. 4G), nor did treatment of neurons with Aβ oligomers affect phosphate levels at three tau residues (S199, S205 and S231) that can be phosphorylated by GSK3β (Fig. 5B). Therefore, despite the widely recognized importance of GSK3β-catalyzed phosphorylation of tau in AD (Hernández et al., 2010), GSK3β does not appear to be involved in neuronal CCR. Secondly, the present study establishes that the CCR-mediated pathway of neurodegeneration in AD results from a direct functional link between Aβ and tau, the principal building blocks of plaques and tangles.

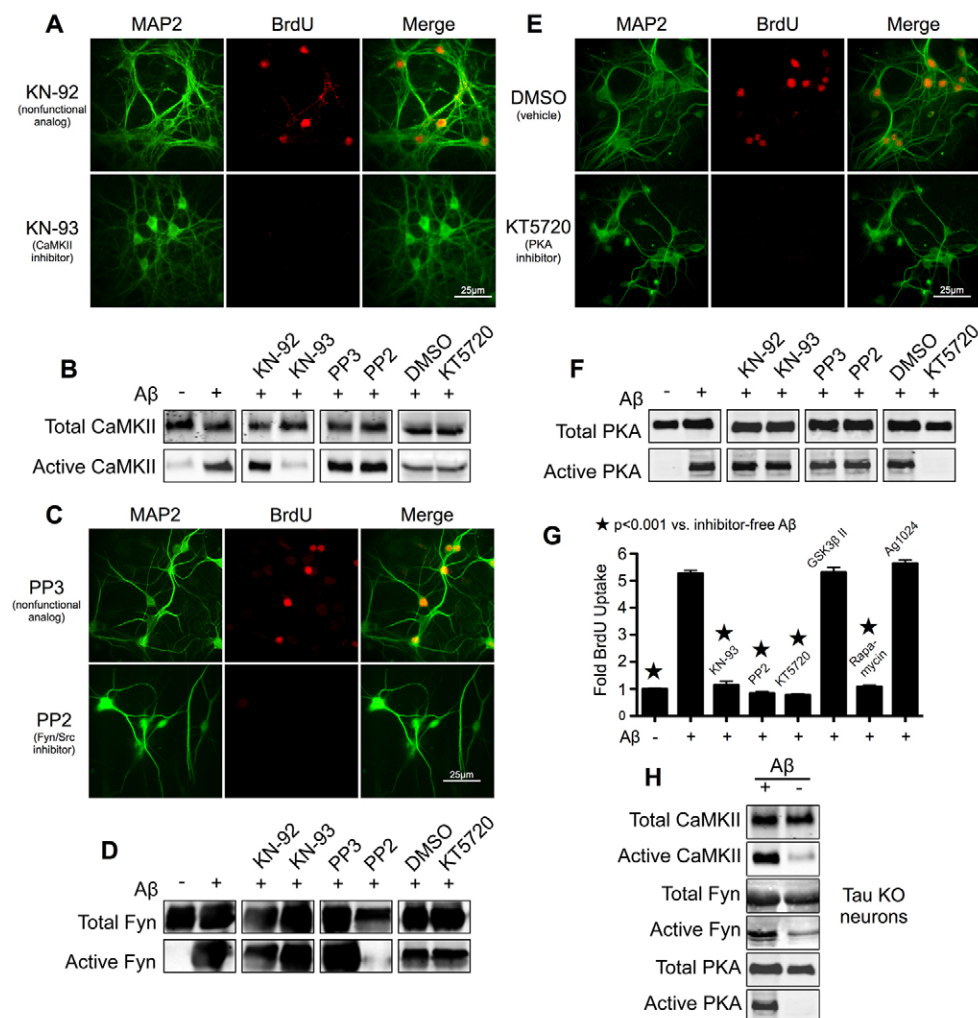


Fig. 4. CaMKII, fyn and PKA activities are required for CCR induced by A β . Primary WT neurons were incubated for 24 hours with A β _{1–42} oligomers at 3 μ M total peptide in the presence of the indicated protein kinase inhibitors or corresponding controls. (**A,C,E**) To monitor CCR, BrdU was present during exposure to A β , after which cells were stained for immunofluorescence with antibodies to MAP2 and BrdU to identify neurons with newly synthesized nuclear DNA. To analyze activation states of CaMKII, fyn and PKA, whole cell lysates from primary WT (**B,D,F**) and tau KO (**H**) neurons were assayed by western blotting with antibodies that discriminated total from activated forms of each kinase. (**G**) Quantification of BrdU uptake by 'in-cell westerns' (Chen et al., 2005). Error bars represent s.e.m. Note that CCR induced in WT neurons by A β _{1–42} oligomers required activation of CaMKII, fyn and PKA, which also occurred, but did not lead to CCR, in tau KO neurons.

While our results implicate three protein kinases, fyn, PKA and CaMKII, and three corresponding tau phosphorylation sites, Y18, S409 and S416, in CCR, the data do not exclude the possibility of additional phosphorylation substrates or kinases being essential for CCR. For example, fyn, PKA and CaMKII can phosphorylate tau at multiple sites that were not examined in the current study (see <http://cnr.iop.kcl.ac.uk/hangerlab/tautable>), and might have additional requisite substrates on proteins other than tau. Likewise, activation of kinases that were not examined here might be essential for CCR, and if so their required substrates could include tau, other proteins or both. The possibility that downregulation of tau phosphatases, such as PP2A (Sontag et al., 1999), contributed to the increased tau phosphorylation reported here also cannot be dismissed. Although resolving these issues will require a comprehensive analysis of the kinome for CCR, the current study defines a protein phosphorylation framework that underlies the process and depends on the most conspicuous proteins associated with AD, A β and tau.

Remarkably, the Y18, S409 and S416 tau phosphorylation sites identified in this study as being required for A β oligomer-induced CCR, have not been regarded as canonical AD phospho-tau sites, such as those recognized by the monoclonal antibodies, PHF-1 (pS396/pS404) (Otvos et al., 1994), AT8 (S199/pS202/

pS205) (Porzig et al., 2007), AT100 (pT212/pS214) (Zheng-Fischhöfer et al., 1998) or AT180 (pT231/pS235) (Goedert et al., 1994). Nevertheless, the evidence presented here establishes pY18, pS409 and pS416 as markers for a seminal step in AD pathogenesis, and emphasize the importance of direct interactions between tau and fyn (Ittner et al., 2010; Lee et al., 1998; Lee et al., 2004). Tau has a binding site for fyn (Lee et al., 1998), and despite being a predominantly axonal protein, it targets fyn to dendrites, where fyn phosphorylates, and thereby regulates the activity of the NMDA receptor (Ittner et al., 2010). As first described in 2004 (Lee et al., 2004), and as documented here in Fig. 4D, Fig. 5A and Fig. 6D, the Y18 residue on tau is also a substrate for fyn-catalyzed phosphorylation. Until the present study, the specific functional significance of tau phosphorylation at Y18 has been largely a matter of speculation, but we can now conclude that ectopic neuronal CCR requires that tau modification.

There are other mechanisms by which the tau-fyn connection may contribute to CCR. For example, it was recently demonstrated that A β accumulation *in vivo* can drive excess tau, along with its bound fyn, into dendrites, and that the overabundant dendritic fyn enhances NMDA receptor phosphorylation, thereby exaggerating calcium influx through the receptor and causing excitotoxicity (Ittner et al., 2010).

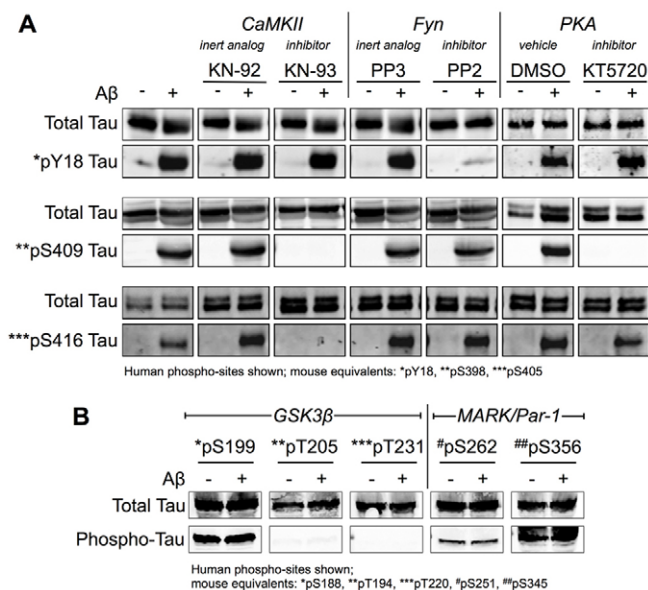


Fig. 5. Aβ₁₋₄₂ oligomers upregulate tau phosphorylation by fyn, PKA and CaMKII, but not GSK3β or MARK/Par-1. Primary WT neurons were incubated for 24 hours with Aβ₁₋₄₂ oligomers at 3 μM peptide in the presence (A) or absence (B) of the indicated protein kinase inhibitors or corresponding controls. Whole cell lysates were then assayed by western blotting with antibodies to total tau or the indicated phospho-tau epitopes. Note that Aβ₁₋₄₂ oligomers enhanced phosphorylation of Y18 by fyn, S409 by PKA and S416 by CaMKII, but did not alter tau phosphorylation at three sites catalyzed by GSK3β (S199, T205 or T231) or at two sites catalyzed by MARK/Par-1 (S262 and S356).

Complementing these results is another recent study showing that Aβ₁₋₄₂ oligomers cause dendritic mistargeting of tau in cultured neurons and corresponding local increases in free calcium (Zempel et al., 2010). It is thus tempting to speculate that the tau dependency of adverse neuronal responses to Aβ oligomers is coupled downstream to rises in cytoplasmic calcium. The exact responses apparently depend on which Aβ species provide the insult, the size distribution and specific conformational features of the oligomers, and the oligomer concentration and duration of exposure to Aβ. As documented here for instance, oligomerized Aβ₁₋₄₂, at low micromolar concentrations insufficient to induce extreme calcium influx and acute, lethal excitotoxicity, can induce neuronal CCR. In contrast, we also recently reported that low-*n* oligomers seeded by pyroglutamylated Aβ₃₋₄₂, but containing predominantly Aβ₁₋₄₂, are conformationally distinct from pure Aβ₁₋₄₂ oligomers, and are potently and acutely cytotoxic to cultured neurons under otherwise nearly identical conditions (Nussbaum et al., 2012). Unraveling structure-function relationships for the myriad forms of Aβ is therefore a matter of high priority.

Aberrant re-entry into the cell cycle adds to a growing list of pathological effects on neurons that are triggered by Aβ, depend on tau and drive the synaptic dysfunction and destruction that cause the behavioral symptoms of AD. For example, Aβ can induce tau-dependent microtubule depolymerization (Jin et al., 2011; King et al., 2006; Zempel et al., 2010), which compromises protein and organelle trafficking in axons (Vossel et al., 2010) and dendrites (Ittner et al., 2010; Zempel et al., 2010), leading to impairment of synaptic activity (Roberson et al., 2011; Shipton

et al., 2011), and by extension, learning and memory (Roberson et al., 2007). Moreover, neuronal cell death, the ultimate cause of synapse loss and cognitive impairment in AD, can be provoked acutely by Aβ (Nussbaum et al., 2012; Rapoport et al., 2002), or as a delayed consequence of ectopic neuronal CCR (Arendt et al., 2010), provided in both cases that the neurons express tau. The etiology of AD thus depends profoundly on toxic signaling from Aβ through tau, and a comprehensive understanding of AD pathogenesis must therefore await deeper knowledge of how Aβ and tau interact to undermine neuronal homeostasis.

Materials and Methods

Primary neuron cultures

Cultures were prepared from E16-17 WT (C57/BL6) and tau KO (Dawson et al., 2001) mouse embryos according to approved University of Virginia Institutional Animal Care and Use Committee (IACUC) guidelines. Unless otherwise specified, tissue culture reagents were obtained from Invitrogen/Gibco. Neurons preparation followed standard protocols (Kaech and Banker, 2006; Roy et al., 2008). Briefly, cortical tissue was dissected from brain and dissociated in Hank's balanced salt solution (HBSS), 0.25% trypsin, and DNAaseI (Worthington Biochemical Corp.), then incubated for 30 minutes at 37°C. Dissociated cells were washed in HBSS and triturated using a glass Pasteur pipette. Cells were plated at a density of 50,000 cells/cm² on poly-L-lysine coated six-well dishes, in Neurobasal Media supplemented with 5% fetal bovine serum, B27, 2 mM GlutaMAX-I, penicillin/streptomycin, 15 mM glucose, and maintained at 37°C and 5% CO₂ in a humidified incubator. Medium was replaced 3 hours post-plating with fresh medium that lacked serum, but was otherwise identical. Two days after plating cytosine arabinoside was added at 10 μM to the cultures, which then were grown for 8–10 days, with 50% medium changes every 3 days. For experiments in which DNA replication was monitored, BrdU (Sigma-Aldrich Co.) was added to medium at 10 μM at the same time as Aβ₁₋₄₂. The protein kinase inhibitors (KN-93, PP2 and KT5720, GSK3β II and Ag1024) and non-functional analogs (KN-92 and PP3) were purchased from Santa Cruz Biotechnology, and used at 5–10 μM. Rapamycin was purchased from Sigma and was used at 4 μM.

Aβ oligomer preparation

Lyophilized synthetic Aβ₁₋₄₂, a generous gift from Dr Charles Glabe of the University of California Irvine, was resuspended as previously described (Lambert et al., 1998). Samples were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich Co.) to 1 mM, which was evaporated overnight at room temperature. The dried powder was resuspended in DMSO at 5 mM and sonicated for 10 minutes in a water bath. The peptide was then diluted to 100 μM in Neurobasal media, and either incubated overnight at 4°C with rocking, to prepare oligomers, or immediately added to cells as monomers.

Immunofluorescence microscopy of cultured neurons

All steps were performed at room temperature. Neurons growing on No. 1 thickness glass coverslips were rinsed in PBS and fixed for 10 minutes in 4% paraformaldehyde (PFA). Next, they were then permeabilized and blocked for 30 minutes in 0.4% Triton X-100 plus 5% BSA in PBS, and rinsed in PBS. This was followed by a 20 minute incubation in 2 N HCl to increase BrdU antigenicity, and acid neutralization with three washes in 0.1 M sodium borate, pH 8.6. Fixed neurons were incubated for 1 hour with primary (rabbit polyclonal anti-MAP2 and mouse monoclonal anti-cyclin D1, or as indicated otherwise) and secondary antibodies diluted into PBS containing 1% BSA, and were washed five times with PBS after each antibody step. Finally, the coverslips were mounted onto glass slides using Fluoromount-G (Southern Biotech, Inc.) supplemented with 2.5% DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma-Aldrich Co.) to retard photobleaching. Samples were imaged on a Zeiss Axiovert 100 equipped with a CARV spinning disk confocal head, using a 40×/1.3 NA or 63×/1.4 NA oil-immersion, planapochromatic objective, a Hamamatsu 9100-13 Imagem cooled EMCCD, and Improvision Volocity software (PerkinElmer, Inc.) for controlling hardware and image analysis. TIFF images saved using Volocity were assembled into multi-figure, labeled montages using Keynote (Apple, Inc.), and the montages were exported as TIFFs.

In-cell western analysis

Primary neurons were cultured for 8 days as described earlier, but were plated into 96-well dishes at a density of 10,000 cells per well. BrdU was added at 10 μM to wells, which were left untreated, or were treated overnight with 3 μM monomeric or oligomeric Aβ₁₋₄₂, with or without the indicated drugs. All subsequent steps were performed at room temperature. Cells were fixed in 4% PFA for 15 minutes and then permeabilized with 0.1% Triton X-100/PBS (PBS/TX100) for 15 minutes. Fixed cells were incubated at room temperature in a humidified

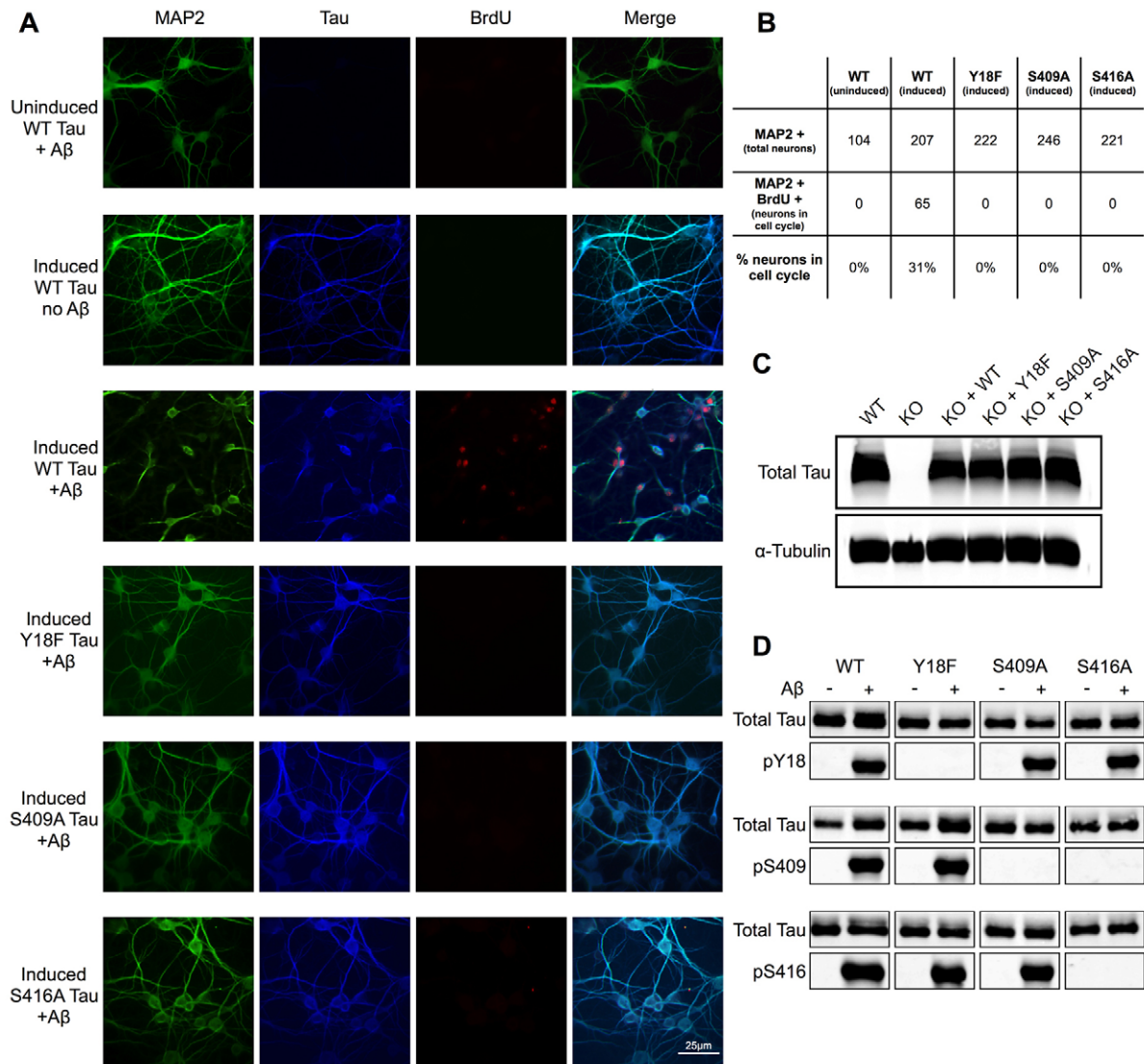


Fig. 6. Tau phosphorylation at Y18, S409 and S416 are required for CCR induced by A β . (A) Primary tau KO neurons with doxycycline-inducible, lentivirus-driven expression of WT or mutated tau were incubated for 24 hours with A β_{1-42} oligomers at 3 μ M total peptide in the presence of BrdU, and then were stained for immunofluorescence with antibodies to MAP2 and BrdU to identify neurons with newly synthesized nuclear DNA. (B) For quantification, counts were derived from two experiments using images like those shown in A. (C,D) Whole cell homogenates obtained from cultures like those shown in A were analyzed by western blotting with the indicated antibodies. Note that: (1) BrdU-positive nuclei appeared in neurons induced to express WT tau, but not tau mutated to prevent phosphorylation at residues 18, 409 or 416; (2) total tau levels in lentivirus-transduced neurons were nearly identical to the level of endogenous tau in WT neurons; and (3) the Y18F, S409A and S416A mutations prevented A β -induced tau phosphorylation only at the mutated sites.

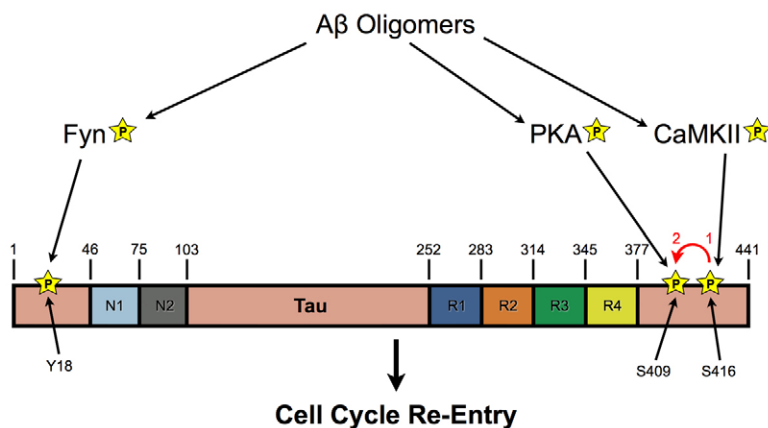


Fig. 7. A three-stage pathway for tau-dependent cell cycle re-entry induced by A β_{1-42} oligomers. The pathway includes three sequential steps: (1) A β_{1-42} oligomer-induced activation of fyn, PKA and CaMKII; (2) phosphorylation by these kinases of tau at Y18, S409 and at S416, respectively; and (3) exit of neurons from G0 into G1.

chamber for 30 minutes with Odyssey Blocking Buffer (LI-COR), then for 1 hour with primary (mouse anti-BrdU and rabbit anti-MAP2) and infrared-labeled IRDye secondary antibodies (LI-COR) diluted into PBS supplemented with Odyssey Blocking Buffer to 20% total volume. Following each antibody step, the cultures were washed 5 times in PBS/TX100. The dishes were allowed to dry for 2 hours in the dark, and then were scanned using the LI-COR Odyssey imaging station according to the manufacturer's instructions, with a 3.0 mm offset and a scan intensity of 8.0. The intensity ratio for BrdU to MAP2 was calculated and normalized to the level in untreated samples, and was expressed as mean \pm s.e.m. and statistically analyzed by an unpaired *t*-test (Prism, GraphPad Software, Inc.).

Immunofluorescence microscopy of mouse brain tissue

hAPPJ20 mice (Mucke et al., 2000) express human amyloid precursor protein (hAPP) with the Swedish and Indiana mutations, and were on a congenic C57/BL/6J background. Both males and females were used. Animals were housed in a pathogen-free barrier facility with a 12 hour light/12 hour dark cycle, and *ad libitum* access to food and water under a protocol approved by the IACUC of the University of Alabama at Birmingham. Brain sections were prepared as previously described from 6-month-old mice after transcardial perfusion with saline (Palop et al., 2011). Hemibrains were drop-fixed for 48 hours in 4% PFA, then cryoprotected in 30% sucrose. 30 μ m thick coronal sections were cut on a sliding microtome. All incubation steps for antibody staining were performed at room temperature using tissue sections suspended in appropriate solutions. Initially, sections were washed three times for 15 minutes each in PBS to remove cytoprotectant, and then were blocked for 1 hour with 10% normal goat serum, 1% non-fat dry milk, and 0.1% gelatin in PBS. Primary (rabbit polyclonal anti-MAP2, and mouse monoclonal anti cyclin D1) and secondary antibody incubations were for 4 hours each with gentle rocking using antibodies diluted into PBS containing 2% normal goat serum and 0.05% Tween 20 (PBS/Tween20). There were three PBS/Tween20 washes of 10 minutes each following each antibody step. Finally, the sections were rinsed once in PBS, transferred to a 1% gelatin solution, mounted on glass microscope slides, dried for 1 hour at room temperature, and then sealed with No. 1 thickness coverslips and Fluoromount-G with 2.5% DABCO. Sections were imaged on a Leica TCS SP5 X scanning laser confocal microscope (Leica Microsystems) equipped with a 63 \times /1.40 NA oil-immersion objective and a white light laser module, and controlled by Leica LAS AF software (Sun et al., 2009). TIFF images saved using the Leica software were assembled into multi-figure, labeled montages using Keynote (Apple, Inc.), and the montages were exported as TIFFs.

Immunoblotting

Samples were resolved by SDSPAGE using 10% acrylamide/bis-acrylamide gels, and transferred to 0.22 μ m nitrocellulose (Bio-Rad). Membranes were blocked with Odyssey blocking buffer, and were incubated with primary (rabbit polyclonal anti-MAP2 and mouse monoclonal anti-BrdU) and secondary IRDye antibodies in PBS for 1 hour at room temperature or overnight at 4°C. Three washes of 5 minutes each with PBS and 0.1% Tween 20 were performed after each antibody step. Finally, the membranes were dried between sheets of filter paper prior to imaging with the LI-COR Odyssey.

Lentivirus construction and infection

Full-length WT human 2N4R tau and the non-phosphorylated mutants, Y18F, S409A and S416A, were expressed in tau KO neurons using the TRIPZ Trans-Lentiviral ORF kit (Open Biosystems Products). Tau cDNA in the pTRIPZ plasmid was point mutagenized at the aforementioned sites using the QuikChange mutagenesis kit (Agilent Technologies, Inc.). Following the manufacturer's instructions, the tau expression plasmids and the packaging mix were transfected into HEK293T cells by calcium phosphate. After 48 hours of incubation the lentivirus-conditioned media were collected, and virus particles were pelleted by ultracentrifugation, resuspended in Neurobasal medium, titered and finally stored at -80°C. Cultures of tau KO neurons were infected with a viral MOI of 25 and incubated for 24 hours at 37°C. Infected cells were then selected with 3 μ g/ml puromycin, and tau expression was induced with 1 μ g/ml doxycycline (Sigma-Aldrich Co.). Twenty-four hours after selection and induction, the cells were treated with A β for subsequent analysis by immunofluorescence microscopy or immunoblotting.

Antibodies

For antibodies used in this study, please see supplementary material Table S1 (primary antibodies) and supplementary material Table S2 (secondary antibodies).

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Author contributions

M.E.S. performed all of the biochemical and most of the cell biological experiments, and along with G.S.B., participated in the overall design and analysis of the research. E.S. prepared most primary neuron cultures and provided guidance for immunofluorescence of brain sections. E.D.R. provided the brain sections and editorial advice. J.N.C. and R.L. performed immunofluorescence microscopy of brain sections. A.R. and A.N. contributed to the cultured neuron immunofluorescence. G.S.B. supervised the project and authored the manuscript.

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Table S1. Primary Antibodies used for this study

<i>Antigen</i>	<i>Host</i>	<i>Application</i>	<i>Dilution/ Concentration</i>	<i>Source/ Catalog #</i>
A (6E10)	Mouse (monoclonal)	WB	1 µg/ml	Covance/ SIG- 39300
BrdU (BU-33)	Mouse (monoclonal)	IF	3 µg/ml	Sigma/ B8434
BrdU	Rat (monoclonal)	IF	2 µg/ml	Abcam/ Ab6326
CaMKII (Active) pS286/T287	Rabbit	WB	1:2500	Millipore/ 06-881
CaMKII (Total)	Mouse (monoclonal)	WB	250 ng/ml	BD/ 611292
Cleaved Caspase- 3	Rabbit	IF	1:400	Cell Signaling 9661S
Cyclin D1	Rabbit	IHC, IF	1 µg/ml (IHC), 0.5 µg/ml (IF)	Abcam/ ab16663
Fyn-Activated (pY416)	Rabbit	WB	1:1000	Cell Signaling 2101
Fyn-Total	Mouse	WB	2.5 µg/ml	Invitrogen/ 13-7800
MAP2	Rabbit	IF	10µg/ml	Sigma/ M3696
MAP2 (clone 2)	Mouse (monoclonal)	IHC	undiluted conditioned medium	GS Bloom/ U Virginia
NeuN	Mouse (monoclonal)	IF	1 µg/ml	Millipore/ MAB377
PKA (Active) pS197	Rabbit	WB	1:1000	Abcam/ ab75991
PKA (Total)	Mouse	WB	200 ng/ml	Santa Cruz/ SC-365615
Tau (clone 5)	Mouse	WB, IF	0.25-2.5 µg/ml	LI Binder/ Northwestern U
Tau pS199	Rabbit	WB	1:1000	Invitrogen/ 44734ZG
Tau pS262	Rabbit	WB	1:1000	Invitrogen/ 44750ZG
Tau pS356	Rabbit	WB	1:1000	Invitrogen/ 44751ZG
Tau pS409	Rabbit	WB	1:1000	Invitrogen/ 44760ZG
Tau pS416	Rabbit	WB	1:1000	Antibodies- online/372727
Tau pT205	Rabbit	WB	1:1000	Invitrogen/ 44738ZG
Tau pT231	Rabbit	WB	1:1000	Invitrogen/ 44746ZG
Tau pY18	Rabbit	WB	1:500- 1:1000	G. Lee/ U of Iowa
Tau pY18	Mouse	IHC	1:500	G. Lee/ U of Iowa
Tubulin (DM1A)	Mouse	WB	100 ng/ml	Sigma/ T6199

IB: Immunoblot, IF: Immunofluorescence (cultured cells); IHC: Immunohistochemistry (brain)

Table S2. Secondary Antibodies used for this study (made in goats)

<i>Antigen</i>	<i>Label</i>	<i>Application</i>	<i>Dilution/ Concentration</i>	<i>Source</i>
Mouse-IgG	HRP	WB	200 ng/ml	KPL/ 474-1086
Mouse IgG	IRDye 800	WB	100 ng/ml- 200 ng/ml	LiCor/ 926-32210
Rabbit-IgG	IRDye 680	WB	100 ng/ml- 200 ng/ml	LiCor/ 926-68171
Mouse-IgG	Alexa Fluor 488	IHC	2 µg/ml	Invitrogen/ A-11001
Rabbit-IgG	Alexa Fluor 488	IF	8 µg/ml	Invitrogen/ A-11008
Mouse-IgG	Alexa Fluor 555	IF	8 µg/ml	Invitrogen/ A21424
Mouse-IgG	Alexa Fluor 594	IF	4 µg/ml	Invitrogen A-11005
Rabbit-IgG	Alexa Fluor 594	IF, IHC	4 µg/ml	Invitrogen/ A-11012
Rat-IgG	Alexa Fluor 647	IHC	2 µg/ml	Invitrogen/ A-21247

IB: Immunoblot, IF: Immunofluorescence (cultured cells); IHC: Immunohistochemistry (brain)