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Human secreted tau increases amyloid-beta production☆

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ABSTRACT

The interaction of amyloid-beta (Aβ) and tau in the pathogenesis of Alzheimer's disease is a subject of intense inquiry, with the bulk of evidence indicating that changes in tau are downstream of Aβ. It has been shown however, that human tau overexpression in amyloid precursor protein transgenic mice increases Aβ plaque deposition. Here, we confirm that human tau increases Aβ levels. To determine if the observed changes in Aβ levels were because of intracellular or extracellular secreted tau (eTau for extracellular tau), we affinity purified secreted tau from Alzheimer's disease patient–derived cortical neuron conditioned media and analyzed it by liquid chromatography-mass spectrometry. We found the extracellular species to be composed predominantly of a series of N-terminal fragments of tau, with no evidence of C-terminal tau fragments. We characterized a subset of high affinity tau antibodies, each capable of engaging and neutralizing eTau. We found that neutralizing eTau reduces Aβ levels in vitro in primary human cortical neurons where exogenously adding eTau increases Aβ levels. In vivo, neutralizing human tau in 2 human tau transgenic models also reduced Aβ levels. We show that the human tau insert sequence is sufficient to cause the observed increase in Aβ levels. Our data furthermore suggest that neuronal hyperactivity may be the mechanism by which this regulation occurs. We show that neuronal hyperactivity regulates both eTau secretion and Aβ production. Electrophysiological analysis shows for the first time that secreted eTau causes neuronal hyperactivity. Its induction of hyperactivity may be the mechanism by which eTau regulates Aβ production. Together with previous findings, these data posit a novel connection between tau and Aβ, suggesting a dynamic mechanism of positive feed forward regulation. Aβ drives the disease pathway through tau, with eTau further increasing Aβ levels, perpetuating a destructive cycle.

1. Introduction

Alzheimer's disease (AD) is characterized pathologically by amyloid-beta (Aβ) plaques and tau dominated neurofibrillary tangles (NFTs). Understandably, the field has focused on elucidating the mechanisms regulating these hallmark AD proteins and investigating their interplay in disease. Aβ is formed by the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretase. The accumulation of Aβ peptide in extracellular plaques is traditionally seen as the primary event in AD, with subsequent NFT formation and neuronal and synaptic loss following as a direct result; collectively known as the amyloid cascade hypothesis (Hardy and Higgins, 1992; Hardy and Selkoe, 2002).

Work performed with frontotemporal dementia mutant tau (P301L) transgenic mice injected with aggregated Aβ (Gotz et al., 2001) as well as experiments using APP and tau bigenic mice (Lewis et al., 2001) indicates an upstream role for Aβ oligomerization in initiating tau pathology. In vitro and in vivo models show Aβ induced neurotoxicity and Aβ induced axonal transport deficits are ameliorated by dose-dependent reductions in tau (Rapoport et al., 2002; Vossel et al., 2010). In hippocampal slice preparations, Aβ-induced impairment of long-term potentiation is inhibited in slices generated from $tau^{-/-}$ mice (Shipton et al., 2011). In the J20 APP transgenic mouse, reducing endogenous mouse tau decreased or eliminated learning and memory deficits, exploratory loco motor hyperactivity, interictal spiking, spontaneous seizures, and early mortality (Roberson et al., 2007, 2011). In the APP23 transgenic mice, reducing tau dose dependently improved memory in the

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T-maze and reduced seizures and premature mortality (Ittner et al., 2010). The exact downstream mechanism involved in tau mediating protection from Aβ toxicity has yet to be shown. Elimination of mouse tau in these APP transgenic animals mitigates disease phenotypes but does not alter Aβ levels or plaque burden (Ittner et al., 2010; Roberson et al., 2007).

In contrast to these findings, work done with double transgenic mice expressing both human mutant tau and APP showed greater Aβ deposition and NFT-like formation with increased neuronal loss in double transgenic animals when compared with single transgenic controls (Ribe et al., 2005). Furthermore, Braak and Braak (1995) have shown with their analysis of brain slices taken from patient autopsy samples still in the presymptomatic stages of AD, Braak-stage I-III, that intraneuronal tau alterations precede aggregated Aβ deposition (Braak and Del, 2004; Braak et al., 2013; Schonheit et al., 2004). They present evidence that tau tangles temporally develop either before or independent of Aβ plaques. These divergent findings indicate that the causal link between aberrant APP processing and tau alterations remains controversial.

Recent AD research places tau in the extracellular space under physiological conditions (Yamada et al., 2011) and shows endogenous tau to be actively secreted from human (Chai et al., 2012) and rat neurons (Pooler et al., 2013). In concert with these observations, others have found that tau pathology spreads in AD. Early in disease, tau tangles are detectable in the entorhinal cortex and as the disease progresses tau pathology spreads into the hippocampus and cortex (Braak and Braak, 1995).

The spread of tau tangle pathology occurs concomitantly with clinical disease progression, moving from memory loss to dementia. In in vivo models in which human tau is specifically overexpressed in the entorhinal cortex, tau is secreted and spreads along synaptic circuitry, resulting in tau pathology progression from the entorhinal cortex, through the hippocampus, and into the cortex (de Calignon et al., 2012; Dujardin et al., 2014; Liu et al., 2012). Furthermore, comparative analysis of AD and healthy patient cerebral spinal fluid (CSF) showed a clear increase of amino-terminal (N-terminal) tau fragments in AD patient CSF, with no evidence of full-length or carboxyl-terminal (C-terminal) tau (Meredith et al., 2013). This is in contrast to data suggesting that tau in the interstitial fluid (ISF) of P301L mice is of full length (Yamada et al., 2011).

Presynaptic release of secreted tau from cortical neurons has been shown to be mediated by neuronal activity. Yamada et al. (2014) used in vivo microdialysis in mouse brain to show that increasing neuronal and synaptic activity rapidly increases steady-state levels of transgenic extracellular tau. Additionally, in vitro treatment of rat neurons with the sodium channel blocker tetrodotoxin (TTX) prevents AMPA-mediated tau release (Pooler et al., 2013).

In fact both tau and Aβ have been shown to be affected by neuronal hyperactivity. Cirrito et al. (2005), (2008); DeVos et al. (2013); Kamenetz et al. (2003); and Verges et al. (2011) have demonstrated in vitro and in vivo that soluble Aβ levels in brain are directly influenced by neuronal, and more specifically, synaptic activity. They postulated a negative feedback role for Aβ in healthy neurons, functioning to keep neuronal hyperactivity in check by selectively depressing excitatory synaptic transmission. Taken together, these findings indicate a direct correlation between reductions in tau protein with reductions in neuronal hyperactivity, both of which correspond to a decrease in soluble Aβ levels.

Here, we have identified the major forms of human tau secreted from AD patient–derived cortical neurons to be a pool of secreted N-terminal tau fragments which we call eTau, for extracellular tau. We show that eTau, and more specifically the human tau insert region, increases soluble Aβ levels while simultaneously lowering the neuroprotective product of α-secretase APP cleavage, soluble amyloid precursor protein-α (sAPPα), suggesting that eTau modulates Aβ production. We show this eTau/Aβ relationship in both primary human in vitro neuronal cultures and in vivo tau transgenic models. Additionally, we observed that eTau induces neuronal hyperactivity when applied to cortical cultures and found that reducing or neutralizing eTau prevents aberrant neuronal hyperactivity. We suggest that eTau induced hyperactivity is negatively impacting neurons, and that eTau may elevate Aβ production in AD brain.

2. Methods

2.1. Primary human cortical neuron cultures

Human cortical neuron cultures were prepared as described (Wright et al., 2012) and cultured for 3 weeks in Neurobasal medium with B27 and Glutamax (Invitrogen) before the start of 20 days treatments. Human fetal cerebral cortical tissue was obtained by Advanced Bioscience Resources (Alameda, CA, USA) and complied with federal guidelines for fetal research and with the Uniformed Anatomical Gift Act.

2.2. Induced pluripotent stem cells patient-derived cortical neurons

Induced pluripotent stem cells (iPSC) were generated using the Yamanaka method (Takahashi et al., 2007) as previously described (Dimos et al., 2008) from skin biopsies taken from a familial presenilin-1 (A260V) AD patient aged 50 years and a sporadic AD patient aged 60 years. Briefly, iPSC were differentiated to neurons via dual SMAD (small body size mothers against decapentaplegic) inhibition (Chambers et al., 2009) followed by cortical neuron

Table 1
Patient information induced pluripotent stem cell derived cortical neurons (iPSC-CN)

Patient status	Mutation	Number of iPSC-CN lines tested/patient	iPSC line name	ApoE genotype	Age at biopsy	Used for eTau purification
fAD	PSEN1 A260V	3	fAD1.1 fAD1.2 fAD1.3	E3/E3	50	Y
fAD	PSEN1 L286V	1	fAD1.1	E3/E3	38	N
fAD	PSEN1 L418F	2	fAD1.1 fAD1.2	E3/E4	39	N
fAD	PSEN2 N141I	3	fAD2.1 fAD2.2 fAD2.3	E3/E3	81	N
sAD	Unknown	1	sAD1.1	E4/E4	60	Y

Key: apoE, apolipoprotein E; eTau, extracellular tau; fAD, familial AD; iPSC-CN, iPSC derived cortical neurons; iPSC, induced pluripotent stem cells; N, no; sAD, sporadic AD; Y, yes.

differentiation as described (Burkhardt et al., 2013). iPSC-derived cortical neurons (iPSC-CN) were grown between 70 and 108 days with media changes every 3 days unless otherwise noted. A list of AD patient samples used to generate individual iPSC clones, as well as their relevant mutations, is provided in Table 1.

2.3. Antibodies

2.3.1. iPierian tau antibodies

Tau sizes are represented based on 2N4R tau sequence length unless otherwise noted.

IPN001 and IPN002 (iPierian) are mouse monoclonal antibodies generated by standard immunization with in vitro aggregated 2N4R tau, followed by clonal selection and antibody purification from hybridoma. Both antibodies were selected for their high binding affinity to both secreted eTau as well as full length tau (recombinant Tau383, 0N4R form) and for their ability to neutralize secreted eTau's electrophysiological activity. They both bind to the N-terminus of tau with epitopes spanning amino acids 15–24.

2.3.2. Antibodies

MC1 (Peter Davies) is a conformational monoclonal antibody specific for a conformation change in tau found in AD brain, and paired helical fragment 1 (PHF1) (Peter Davies) detects the pSer396/404 tau epitope present on both normal adult brain tau and PHF tau (Jicha et al., 1997). Commercial mouse monoclonal antibodies against tau used are HT7 (aa 159–163, ThermoScientific), BT2 (aa 194–198, ThermoScientific), and T46 (C-terminal tau near amino acids 404–441, Invitrogen). Rabbit polyclonal antibodies used were against tau (aa 243–441, Dako) and against APP (C-terminal amino acids, Sigma). Mouse monoclonal control antibodies used were to β-actin (Abcam), α-tubulin (Cell Signaling), mouse IgG (R&D Systems), and mouse IgG whole molecule (Jackson).

2.4. Secreted tau characterization, purification, and liquid chromatography-mass spectrometry analysis

2.4.1. Secreted tau immunoprecipitation

iPSC-CN derived from a familial presenilin-1 (A260V) AD patient aged 50 years were differentiated for 70 days at which point media conditioned for 3 days was collected for tau immunoprecipitation. Conditioned media was centrifuged at 15,000 rpm (4 °C) and supernatants precleared on a mouse IgG (Jackson) Sepharose 4B resin (GE Healthcare) before immunoprecipitation on either an N-terminal tau antibody, IPN002, a mid-region tau antibody, HT7, or a C-terminal tau antibody, T46, each previously coupled to Sepharose 4B resins. After immunoprecipitation, all 3 bead sets were washed 3 times with mammalian protein extraction buffer (M-PER, ThermoScientific) and immunoprecipitated protein extracted from pelleted beads with 2X Laemmli reducing buffer (Sigma) and separated on NuPage Bis-Tris protein gels (Invitrogen). Western blots were probed for tau with IPN001 (1 μg/mL), analysis by Odyssey SA software version 1.1.7 (LiCor). To ensure the tau specificity of all 3 immunoprecipitations, iPSC-CN growth media was incubated with the 3 antibody conjugated resins in parallel with the conditioned media, and the resultant nonspecific eluate ran as Media control on the IPN001 Western blot.

2.4.2. Secreted tau affinity isolation

iPSC-CN generated from skin biopsies taken from a familial presenilin-1 (A260V) AD patient aged 50 years and a sporadic AD patient aged 60 years were cultured for 108 days in total, with media changes every 3 days. Starting at day 70 of iPSC-CN culture, conditioned media was collected every 3 days and pooled for secreted tau affinity isolation. Pooled conditioned media was centrifuged at 15,000 rpm (4 °C) and supernatants precleared on a mouse IgG Sepharose 4B resin before tau purification on N-terminal IPN002 anti-tau antibody Sepharose 4B coupled resin. Protein was eluted with 50 mM sodium citrate, pH 2.3, 150 mM NaCl, and neutralized with 1 M tris-buffered saline (TBS), pH 8.3. The eluate was concentrated, and buffer exchanged into phosphate-buffered saline (PBS) and here referenced as eTau. A mock protein control was generated from a second batch of pooled conditioned media, also precleared over the mouse IgG resin before mock affinity purification using mouse IgG resin.

2.4.3. Liquid chromatography-mass spectrometry analysis

Side-by-side lanes of IPN002 affinity purified eTau were separated on reducing and/or denaturing 10% NuPage gel (Invitrogen), and analyzed by Coomassie stain (LiCor) or transferred to nitrocellulose for Western blot analysis with iBlot (Life Technologies) and probed for tau with IPN001 (1 μg/mL). Four Coomassie stained and/or anti-tau confirmed bands were excised from the gel and submitted for LC/MS analysis (MS Bioworks). Protein was proteolytically digested out of the gel slices with trypsin, chymotrypsin, or elastase, and gel digests were analyzed by nano LC/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Orbitrap Velos Pro. Data were searched using Mascot. This analysis was verified using intact protein LC/MS analysis to ensure we identified the ends of the tau fragments. Liquid chromatography was performed on a C8 column (Agilent), and mass spec analysis was performed on a Q-ToF Premiere (Waters).

2.5. eTau secretion Brefeldin treatment

Familial AD PSEN1 (A260V) iPSC-CN were cultured for 70–108 days then washed once with growth media before addition of fresh growth media with and without 10 μg/mL Brefeldin A (Sigma); media was conditioned for 4 hours at 37 °C/5%CO_2 and collected for analysis by IPN001 (1 μg/mL) Western blot. Lysates from treated wells were also harvested with 50 μL M-PER and β-actin levels in the lysates were measured by Western blot for normalization of eTau data.

2.6. Primary human cortical neuron treatments

Primary human cortical neurons were treated with either 100 nM BACE1 inhibitor (BACEi) (Axon Medchem), tetrodotoxin (Tocris), 1 μM Accell siRNA pools against human *MAPT*, *BACE1*, *ADAM9*, *ADAM10*, *ADAM17*, or nontargeting control (Thermo Scientific/Dharmacon, catalog E-012488, E-003747, E-004504, E-004503, and E-003453, respectively), 50 nM or 500 nM purified secreted tau (eTau) or mock control (iPierian), *Escherichia coli* generated full-length rTau383(0N4R) (rPeptide), synthetic human eTau4 peptide (aa 2–68, 0N3R designation), scrambled human eTau4 peptide control, or the equivalent mouse tau sequence (aa 2–57) (Neobiolab), or with 1, 10, or 30 μg/mL protein A purified tau antibodies PHF1 and MC1 (Peter Davies), IPN002 or anti-mouse IgG. Treatments were diluted in basal media (Neurobasal media with Glutamax and B27, Invitrogen) and incubated on neurons for a total of 20 days; conditioned media was harvested every 5 days, and treatments were added again, for a total of 20 days of exposure. Conditioned media was stored at −80 °C until day 20 end of assay for full time course enzyme-linked immunosorbent assay (ELISA) analysis, and cells were lysed in 50 μL M-PER (ThermoScientific) or 50 μL Buffer RLT (Qiagen) for protein or messenger RNA (mRNA) analysis.

2.7. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity in in vitro conditioned media from iPSC-CN and human primary cortical cultures was

determined using the Lactate Dehydrogenase Activity Assay Kit (Sigma). Five day conditioned media of 25 μL or 50 μL was assayed with an equal volume LDH reagent and incubated for 30 minutes at room temperature. Plates were read on plate reader at 490 nm.

2.8. Human sAPPα and $A\beta_{40}$ and $A\beta_{42}$ ELISAs

Human sAPPα and $A\beta_{40}$ and $A\beta_{42}$ protein levels were measured in primary human cortical neuron conditioned media collected every 5 days over a 20-day treatment period using specific ELISA assays (Invitrogen #KHB0051, MilliporeEMD #EZHS40, and MilliporeEMD #EZHS42 respectively).

2.9. Mouse $A\beta_{40}$ and $A\beta_{42}$ ELISAs

Mouse Aβ protein levels were measured in mouse cortical brain homogenate or ISF using mouse $A\beta_{40}$ and/or $A\beta_{42}$ ELISAs (Immuno-Biological Laboratories #JP27720 and #JP27721). The mouse $A\beta_{42}$ ELISA lacked the sensitivity to reliably detect $A\beta_{42}$ in brain tissue.

2.10. Gene expression

Cultured neurons were lysed in Qiagen Buffer RLT and RNA isolated using an RNeasy kit (Qiagen). Complementary DNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). Quantitative real time polymerase chain reaction was performed using TaqMan Gene Expression Assays and Taqman Expression Master Mix (Life Technologies) on an Applied Biosystems HT 7900. Total *MAPT*, *BACE1*, *ADAM9*, *ADAM10*, *ADAM17*, *APP*, *PSEN1*, and *PSEN2* mRNA expression levels were normalized to *RPLPO* (housekeeping gene) mRNA expression levels and analyzed using the ΔΔCT method for relative expression. Invitrogen Taqman Gene Expression Assays used Cat#4331182 individual ID # *MAPT* Hs00902194_m1 (for all isoforms *MAPT*); *BACE1* Hs01121195_m1; *ADAM9* Hs00177638_m1; *ADAM10* Hs00153853_m1; *ADAM17* Hs01041915_m1; *APP* Hs01041915_m1; *PSEN1* Hs00997789_m1; *PSEN2* Hs01577197_m1; and *RPLPO* (Cat#4333761T). A probe recognizing only the 2N4R/1N4R/2N3R/1N3R isoforms of *MAPT* Hs00213484_m1 was used with *MAPT* Hs00902194_m1 to distinguish fetal 0N3R *MAPT* from other *MAPT* isoforms in both primary human cultures and iPSC-CN cultures.

2.11. Western blots

Cultured primary human neurons were lysed in M-PER Buffer. Cell lysates, primary cortical neuron conditioned media and iPSC-CN conditioned media, were diluted in 2X Laemmli reducing buffer, boiled, separated on NuPage gels, and transferred to nitrocellulose using iBlot. Western blots were blocked (LiCor) and probed with antibodies, IPN001 or IPN002 (1 μg/mL), HT7 (1 μg/mL), PHF1 (1 μg/mL), Dako tau (1:3000), β-actin (1:5000), α-tubulin (1:10,000), APP (1:1000 Sigma), and anti-mouse 680 or anti-rabbit 800 secondary antibodies (1:10,000, LiCor), analysis by Odyssey SA software (LiCor).

2.12. Animals

Two transgenic human tau mouse models were used in these studies. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines and were approved by the Institutional Animal Care and Use Committee. Schematic depicting overall study plans provided in Supplementary Fig. 3.

The 8-week study used homozygous JNPL3 mice (Taconic, Tg(Prnp-MAPT*P301 L)JNPL3Hlmc) as described by Lewis et al. (2000). The in-life study was conducted by Brains On-Line. In brief, the JNPL3 line overexpresses human 0N4R tau containing the most common frontotemporal dementia (FTDP-17) mutation P301L on a C57BL/6, DBA/2, SW mixed background, driven by the prion promoter. Homozygous JNPL3 mice express transgenic tau at levels about 2-fold over endogenous tau and demonstrate motor and behavioral deficits as well as age dependent development of NFT (Lewis et al., 2000).

The 26-week study used tau-4R/2N-P301L (van Leuven) mice as described by Terwel et al. (2005). The in-life study was conducted by reMYND. In brief, human 2N4R tau containing the FTDP-17 mutation P301L was overexpressed by the mouse *thy1* promoter on a pure Friend Virus B-type susceptibility genetic background. Mice display an age dependent hyperphosphorylation of tau, tau aggregation, and tangle formation with concomitant development of motor defects.

2.12.1. The 8-week study (JNPL3 P301L mice)

Female JNPL3 mice aged 3.5 months old were treated weekly with a single dose of 60 mg/kg irrelevant IgG1 or IPN002 antibody for 8 weeks by an intraperitoneal injection volume of 10 mL/kg. At the end of the study, mice were anesthetized using isoflurane (2%, 800 mL/min O_2) and stereotactically implanted with a microdialysis probe in the ventral hippocampus, a polyacrylonitrile membrane with 3 mm exposed surface and 1 megadalton cutoff (BrainLink, the Netherlands). Bupivacain and/or epinephrine was used for local analgesia and fynadine or carprophen for perioperative and/or postoperative analgesia. Push-pull microdialysis sampling was performed 24 hours after implantation surgery. Animal probes were connected with FEP tubing to a microperfusion pump (Harvard PHD 2000 Syringe pump) and perfused with artificial CSF containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$, and 0.15% BSA at a flow rate of 0.75 μL/min. Microdialysis samples were collected over 60 minutes; basal samples were collected after stabilization. Dialysis ISF samples were centrifuged at 15,000 rpm for 15 minutes at 4 °C. The animals were sacrificed immediately following microdialysis sampling and terminal brain, plasma, and CSF were collected (Brains On-Line).

2.12.2. The 26-week study (tau-4R/2N-P301L van Leuven mice)

Female mice aged 3–4 months old were treated weekly by an intraperitoneal injection (10 mL/kg) of 20 mg/kg irrelevant IgG1 or IPN002 antibody for up to 26 weeks (reMYND). At the study end, mice were sacrificed and serum, CSF, and brain were collected. CSF was collected via an incision in the neck muscle and drawn through a puncture with a 26-gauge needle from the cisterna magna then centrifuged at 10,000*g*/10 minutes at 4 °C. Brain regions were dissected from the left hemisphere (cerebellum, cortex, hippocampus, and hindbrain) for each mouse and homogenized in TBS (Sigma) (1:10 wt/vol) then centrifuged at 10,000*g*/10 minutes at 4 °C. Brain fraction homogenates were diluted to 1 mg/mL in TBS and total protein concentration determined by BCA Assay (Pierce); to generate a soluble fraction a portion of each homogenate was further spun 1 hour at 100,000*g* (4 °C) and soluble supernatants collected. Friend Virus B-type susceptibility wild-type cortex was provided by reMYND.

2.13. Tau ELISAs

2.13.1. Free-tau ELISA

We designed a homogeneous alphascreen (Perkin Elmer) assay to measure tau not bound by IPN002 in mouse ISF and CSF and in primary human cortical culture conditioned media and lysates. The tau antibodies used are HT7 (aa 159–163) and the N-terminal tau antibody IPN001 (aa 15–24) which competes with IPN002 binding

and therefore will not interact with tau already bound by IPN002. IPN001 is biotinylated, and HT7 is conjugated to AlphaLISA acceptor beads (Perkin Elmer). Samples are incubated overnight with both antibodies. Streptavidin-coated acceptor beads (Perkin Elmer) are added for 30 minutes and then the plate is read on Envision plate reader (Perkin Elmer) at excitation 630 nm, emission 615 nm. Tau levels were interpolated from an 8 point tau protein (rTau383, 0N4R) standard curve and analyzed using linear curve fit in Excel.

2.13.2. Total-tau ELISA

The total-tau ELISA uses the tau antibodies HT7 (aa 159–163) to capture and biotinylated BT2 (aa 194–198) for detection. HT7 diluted in PBS is coated on high binding ELISA plates (Costar-Corning) at 1 μg/mL overnight at 4 °C and plates blocked with 1% Casein (VectorLabs)/PBS for 2 hours at room temperature. Samples are diluted in 0.1%Casein/PBS and incubated overnight at 4 °C. Tau levels detected with 1 hour incubation with biotinylated tau antibody BT2, followed by addition of Streptavidin-HRP (Southern Biotech) and then 3,3′,5,5′-tetramethylbenzidine substrate (TMB) (ThermoScientific), assay was stopped with 1 M sulfuric acid (Sigma), and the absorbance read on a Beckman plate reader at 450 nm. Tau levels were interpolated from an 8 point tau protein (rTau383, 0N4R) standard curve diluted in 0.1%Caesin/PBS and analyzed using 4-Parameter Sigmoidal Curve Fit (GraphPad Prism).

2.14. Tau binding assay

To measure the k_D of various tau antibodies, high binding ELISA plates were coated overnight at 4 °C with 100 ng of recombinant Tau383(0N4R) or purified eTau (iPierian) diluted in PBS; wells were then blocked with 1% casein/PBS for 2 hours at room temperature. Tau antibodies (IPN001, IPN002, MC1, and PHF1) were diluted to 10 μg/mL in PBS/0.1% casein and 12-point titrations generated, added to the plate, and incubated 2 hours at room temperature. After washing with 0.05%Tween-20 (Sigma)/PBS, detection antibody goat anti-mouse HRP (Southern Biotech) diluted 1:2000 in PBS/0.1% casein was added and incubated at room temperature for 1 hour. Wells were washed as mentioned previously, and TMB substrate (ThermoScientific) added, assay was stopped with 1 M sulfuric acid, and absorbance read at 450 nm; k_D was calculated based on nonlinear regression analysis (GraphPad Prism).

2.15. Free IPN002 levels in CSF and ISF

To measure the amount of free IPN002 in mouse CSF or ISF, high binding ELISA plates were coated overnight at 4 °C with 100 ng of recombinant Tau383(0N4R) diluted in PBS; wells were then blocked with 1% casein/PBS for 2 hours at room temperature. CSF or ISF was diluted 50- or 200-fold in PBS/0.1% casein. Coated plates were washed twice with PBS, then diluted CSF or ISF samples were added to the plate in duplicate and incubated for 2 hours at room temperature. After washing with 0.05%Tween-20 (Sigma)/PBS, detection antibody goat anti-mouse HRP (Southern Biotech) diluted 1:2000 in PBS/0.1% casein was added and incubated at room temperature for 1 hour. Wells were washed as mentioned previously, and TMB substrate (ThermoScientific) added, assay was stopped with 1 M sulfuric acid, and absorbance read at 450 nm; IPN002 amounts calculated based on dilution factor and linear regression analysis equation of the IPN002 standard titration (GraphPad Prism).

2.16. Electrophysiology

Whole–cell patch-clamp recording was performed on primary human cortical cultures and familial AD PSEN1 (A260V) iPSC-CN cultured on a monolayer of normal human astrocytes (Lonza) using a micropipette (2–5 MOhm) filled with solution containing (mM): K-methyl-sulfate (140), NaCl (10), $CaCl_2$ (1), Mg-ATP (3); Na-GTP (0.4), EGTA (0.2), HEPES (10), phosphocreatine (10) with adjusted pH = 7.3, and mOsm = 305. Neurons were perfused (1–2 mL/min) with artificial cerebral spinal fluid containing (mM): NaCl (140), KCl (2.5), $MgCl_2$ (2) $CaCl_2$ (2), Hepes (10), D-Glucose (10), sucrose (20), adjusted pH = 7.3–4 mOsm = 310. Recordings were made using pClamp-10.3 data acquisition software (Molecular Devices) and MultiClamp 700B amplifier (Axon Instrument, Foster City, CA, USA). Puff application of eTau, full-length rTau383 (0N4R), and buffer or mock protein controls was performed using MiniSquirt micro-perfusion system (AutoMate, Berkeley, CA, USA). Bath application of eTau, IPN002 antibody, and buffer controls was performed for other recordings. Off-line data analysis used Clampfit 10.2 analysis software (Molecular Devices). Recordings were conducted at 34 °C–37 °C.

2.17. Statistical analysis

Statistics were determined using GraphPad Prism software. Data are expressed as mean ± standard deviation on an n = 3 (unless otherwise noted in figure legends); experiments were repeated a minimum of 4 times. Analysis of tau or Aβ levels, comparing the effects of one treatment versus control, at a single time point, were done by 2-tailed unpaired *t* test with Welch correction. Analysis of tau, Aβ, and sAPPα levels involving repeated measures between multiple treatment groups at different time points was done using 1-way analysis of variance with Tukey multiple comparison correction, unless otherwise noted. When significant (<0.05), *p*-values are noted.

3. Results

3.1. Human MAPT knockdown reduces Aβ (40 and 42) and increases sAPPα levels in vitro in human primary cortical neurons

To better understand the relationship between Aβ and tau, we asked whether tau can alter levels of soluble, endogenous Aβ produced from human neurons. Gene expression and Western blot analysis of primary human neuronal cultures derived from fetal tissue show that the cultures express neuronal APP695 and only the embryonic (0N3R) isoform of tau, as well as *BACE1*, the 3 α-secretase proteins (*ADAM9*, *ADAM10*, and *ADAM17*) and both *PSEN1* and *PSEN2* (Supplementary Fig. 1A–C).

To demonstrate that our primary human neuronal cultures express and regulate Aβ appropriately, we show that levels of $Aβ_{40}$ and $Aβ_{42}$ are lowered by a BACEi, a direct Aβ processing inhibitor (Supplementary Fig. 1D), and that siRNAs targeting both β- and α-secretase are capable of modulating Aβ and sAPPα levels, respectively (Supplementary Fig. 1E and F).

To investigate the potential regulation of $Aβ_{40}$ and $Aβ_{42}$ by human tau, we reduced *MAPT* gene expression and tau protein levels in our primary human cortical neuron cultures over 70% using Accell *MAPT* siRNA pools (Fig. 1A). Under these conditions non-targeting siRNA had no effect on Aβ levels; *MAPT* knockdown however resulted in a reduction of both $Aβ_{40}$ and $Aβ_{42}$ levels >50% (Fig. 1B).

We then asked if tau affects α- and/or β-secretase, 2 proteases opposed in Aβ generation that compete for APP substrate. Colombo et al. (2012) show that α- and β-secretase cleavage of their common substrate APP is partially coupled in primary mouse neurons. We confirmed these results in primary human neurons, showing that BACEi results in increased production of sAPPα (soluble APP cleaved at the α-secretase site) in the conditioned media, Fig. 1C left, as well

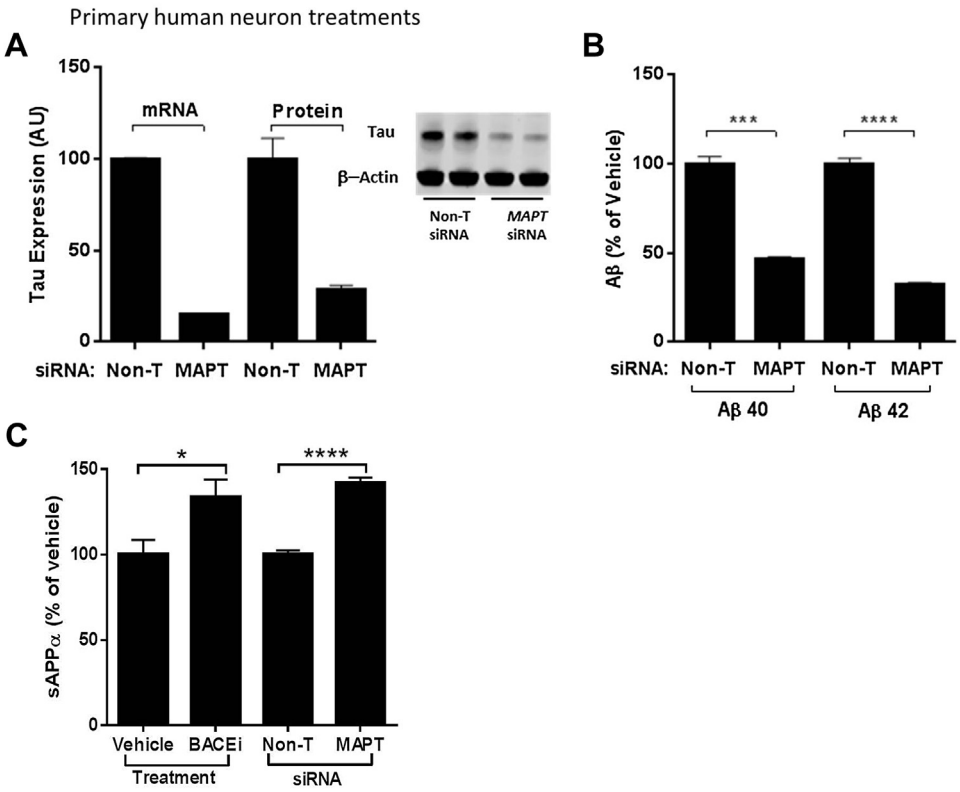

Fig. 1. Human tau increases Aβ and decreases sAPPα levels in vitro in primary human cortical cultures. Primary human neurons treated with *MAPT*, nontargeting (NT) siRNA or BACEi for to 20 days. Conditioned media (CM) collected every 5 days, cells retreated, cell lysates collected at day 20. Each experiment repeated 6 times, n = 3 for each condition; graphs are STD on mean. (A) siRNA transfection decreases *MAPT* mRNA levels by 85%. Tau protein levels decreased 72% (Western blot quantification shown normalized to β-Actin); both compared with NT control. Day 20 data shown. (B) *MAPT* siRNA treatment decreases $A\beta_{40}$ levels by 54% (6546 ± 262 pg/mL–3028 ± 215 pg/mL) and $A\beta_{42}$ levels by 68% (1356 ± 43 pg/mL–432 ± 64 pg/mL). Bars represent mean Aβ levels shown as percent of NT control; error bars are standard deviation; $^{***}p = 0.0001$ and $^{****}p < 0.0001$, by 2-tailed unpaired *t* test Welch correction. (C) BACEi treatment increases sAPPα levels in CM by 35% (42 ± 1 ng/mL–59 ± 1.3 ng/mL); *MAPT* siRNA treatment increases sAPPα levels in CM by 42% (42 ± 1 ng/mL–59 ± 1.2 ng/mL). Bars represent sAPPα mean levels as percent of control treatment value; error bars are standard deviation; vehicle versus BACEi, $^{*}p = 0.0129$; nontargeting siRNA versus *MAPT* siRNA, $^{****}p < 0.0001$, by 2-tailed unpaired *t* test Welch correction. Abbreviations: Aβ, amyloid-beta; BACEi, BACE1 inhibitor; mRNA, messenger RNA; sAPPα, soluble amyloid precursor protein-α; siRNA, small interfering RNA; STD, standard deviation.

as increased production of APP CTF-α in the lysate, Supplementary Fig. 1C. Furthermore, treatment of primary human neuron cultures with siRNAs directed against *BACE1* and *ADAM9/10/17* resulted in opposing effects on cellular Aβ and sAPPα levels (ELISA data Supplementary Fig. 1E and F).

In our primary human neuron cultures, we reduced *MAPT* gene expression and tau protein (Fig. 1A) levels over 70% using Accell *MAPT* siRNA pools. Under these conditions nontargeting siRNA had no effect on sAPPα levels; *MAPT* knockdown however resulted in a 42% increase in sAPPα (Fig. 1C right).

3.2. Secreted tau (eTau) identified from AD patient–derived iPSC cortical neuron conditioned media is composed of N-terminal tau fragments

In light of these findings and recent data showing tau to be secreted from human induced pluripotent stem cell (iPSC)-derived cortical neurons (Chai et al., 2012), we characterized tau release from our iPSC-CN derived from AD patients. To do so, we generated iPSC from a skin biopsy taken from a familial presenilin-1 (A260V) AD patient aged 50 years then differentiated and cultured iPSC-CN for up to 108 days. To characterize eTau secretion from these iPSC-CN, we collected conditioned media beginning at day 70 over time for 96 hours. As shown in the tau Western blot in Fig. 2A, tau is present in the conditioned media of iPSC-CN and quickly accumulates over time.

To ensure that the tau found in the conditioned media was not the result of cell lysis, we measured the LDH activity in the conditioned media of several of our iPSC-CN lines listed in Table 1, and found all to be free of cell lysis (Supplementary Fig. 2A). These data and an absence of other high abundance cytoplasmic proteins such as β-actin and α-tubulin (Supplementary Fig. 2B) in the conditioned media together confirm the lack of contamination by intracellular proteins in iPSC-CN conditioned media. The tau release we observed from our iPSC-CN is rapid, detectable within 2–5 minutes, and plateaus over 24–96 hours (Fig. 2A). iPSC-CN tau secretion is not modulated by Brefeldin A treatment, indicating that secretion is occurring independently of the canonical secretory process (Fig. 2B).

Because iPSC-CNs derived from AD patient samples secrete tau in the absence of neuronal lysis, we characterized the secreted tau species identified in the media. To do so, we attempted to immunoprecipitate tau from the media (conditioned 3 days) of 70-day-old iPSC-CNs derived from a familial presenilin-1 (A260V) AD patient with either an N-terminal tau antibody (IPN002), a mid-range tau antibody (HT7), or a C-terminal tau antibody (T46). Each of these antibodies efficiently isolates cytoplasmic full length tau (data not shown) but only IPN002 and HT7, not T46, were capable of immunoprecipitating secreted tau (Fig. 2C). The C-terminal tau antibody's failure to isolate secreted tau is shown by IPN001 Western blot analysis of the 3 immunoprecipitated products, indicating that tau is released from AD iPSC-CN predominately as N-terminal fragments ranging from 20 to 28 kDa (Fig. 2D) with

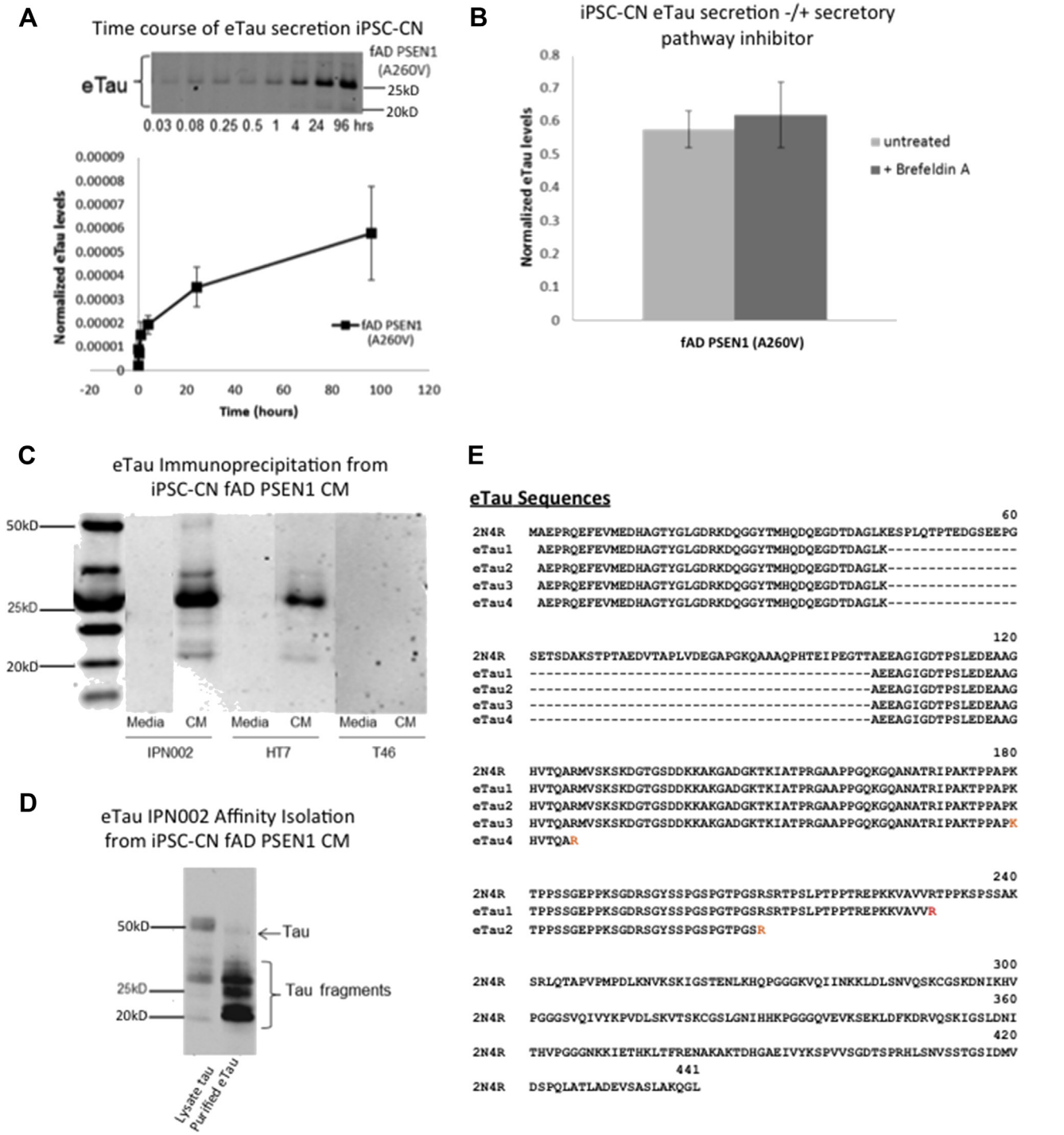

Fig. 2. Tau is secreted as N-terminal fragments from AD patient–derived iPSC cortical neurons (iPSC-CN). (A) IPN001 Western blot of tau fragments released over 96 hours into the CM of fAD PSEN1 (A260V) iPSC-CN cultures. At time 0, media removed, cells washed with media, fresh growth media added, and CM harvested at described time points, lysates harvested at final time point. Graph of IPN001 Western blot quantification normalized to total β-actin levels in lysate shows tau levels in the CM increasing with time. (B) fAD PSEN1 (A260V) iPSC-CN cultures were washed as described previously and treated ±10 μg/mL Brefeldin A for 4 hours, CM and lysates harvested; IPN001 Western blot of CM normalized to total β-actin levels in lysate show no difference in tau secretion with Brefeldin A. (C) IPN001 Western blot analysis of tau fragments immunoprecipitated from CM of iPSC-CN cultures using tau region specific antibodies; nonconditioned media (media) immunoprecipitated as control for each antibody. (D) IPN001 Western blot of 4 main tau fragments (eTau) affinity isolated and purified from AD patient derived iPSC-CN CM with IPN002. (E) Sequence from LC/MS verified eTau 1, 2, 3, and 4 are aligned with 2N4R tau sequence. Abbreviations: AD, Alzheimer's disease; CM, conditioned media; iPSC, induced pluripotent stem cells; LC/MS, liquid chromatography-mass spectrometry.

no evidence of C-terminal reactive fragments. These data are also consistent with reports showing that tau is secreted into AD patient CSF as N-terminal fragments (Meredith et al., 2013). We have conducted similar characterizations of eTau secreted from cortical neuron from healthy control subjects and found a similar molecular weight range of eTau fragments as those found for AD patients (data not shown).

To verify the 20–28 kDa bands as tau and to identify their sequence, we affinity isolated secreted tau from our iPSC-CN conditioned media with the tau antibody IPN002 and conducted

Table 2
Antibody reactivity to full length or secreted eTau measured using a direct binding assay

Antibody	Antigen	Binding to rTau383 (0N4R) k_D [M]	Binding to secreted tau k_D [M]	Epitope (amino acids)
MC1	AD tau (conformational)	1.35×10^{-9}	2.44×10^{-10}	7–9 and 313–322
IPN001	Aggregated tau	1.4×10^{-10}	1.51×10^{-10}	15–24
IPN002	Aggregated tau	1.61×10^{-10}	1.31×10^{-10}	15–24
PHF1	AD tau	1.05×10^{-9}	Does not bind	PS396/S404

Key: AD, Alzheimer's disease.

tryptic, chymotrypic, and elastase digestion followed by LC/MS analysis on the 4 major IPN002-affinity isolated, anti-tau reactive bands shown in Fig. 2D. We also used intact protein LC/MS to ensure we identified the end of the largest tau fragment. This analysis confirmed that secreted tau is a series of N-terminal tau fragments, all lacking the C-terminal amino acids of tau, including the microtubule binding repeats as shown in the sequence alignment in Fig. 2E. We named the 4 major fragments eTau 1, 2, 3, and 4 and refer to the pool of secreted tau as eTau (for extracellular tau).

Our iPSC-CNs only express the 0N3R embryonic isoform of tau (Supplementary Fig. 1B), and LC/MS analysis confirmed the n1 and n2 inserts to be missing in the sequenced fragments, Fig. 2E. All forms of eTau start at the second amino acid, alanine, with the N-terminal methionine removed and the alanine acetylated. This is a covalent modification traditionally indicative of a cytosolic protein, providing further evidence that eTau is released independent of the canonical endoplasmic reticulum and/or golgi secretory process. The 4 major forms of eTau we identified range in size from 67 amino acids (aa 2–44,103–126, eTau4) to 171 amino acids (aa 2–44, 103–230, eTau1), Fig. 2E. Phosphorylation of eTau was rarely detected but when found the residues phosphorylated were consistent with phosphorylation of cytoplasmic tau at residues threonine181 (p181), serine202 (p202), and threonine231 (p231).

We further characterized eTau by its reactivity with several tau antibodies, specific affinities are shown in Table 2. Both 0N4R recombinant full length tau-383 and eTau bind with high affinity to the N-terminal tau antibodies MC1 (a conformational specific tau antibody) (Jicha et al., 1997) and IPN002 (generated against aggregated tau, iPierian). Not surprisingly, eTau does not bind to the C-terminal anti-phospho-tau antibody PHF1 (Jicha et al., 1997) in either the tau binding assay (Table 2) or by Western blot (Supplementary Fig. 2C), indicating that eTau lacks this phosphoepitope, as expected based on LC/MS analysis.

3.3. eTau regulates Aβ (40 and 42) levels in vitro in primary human neurons

We found that this pool of N-terminal eTau fragments is also secreted from human primary cortical neurons at concentrations up to 3–4 nM, when allowed to condition for 5 days (data not shown). Total tau ELISA of the conditioned media of human primary neurons confirmed this tau secretion and demonstrated that *MAPT* knockdown in these primary human neurons significantly reduces secreted eTau levels in the conditioned media over time when compared with nontargeting siRNA control (Fig. 3A).

To determine if eTau plays a role in regulating $A\beta_{40}$ and $A\beta_{42}$, we treated primary human neurons for 20 days (media and treatments replaced every 5 days) with affinity purified eTau from AD patient derived iPSC-CN or mock protein control and saw a dose and time-dependent (as early as 10 days at 500 nM) increase in levels of $A\beta_{40}$ and $A\beta_{42}$ (Fig. 3B). The changes in Aβ levels are small but reproducible over multiple experiments and are statistically significant.

Because the conditioned media from primary human neurons, when allowed to condition on cells for 5 days, already contains secreted eTau at concentration up to 3–4 nM, a large excess of purified secreted eTau (500 nM) appears to be required to generate these additional small changes. Less eTau was needed (<50 nM) to see similar increases in $A\beta_{40}$ and $A\beta_{42}$ when protein was applied to primary human cortical cultures in which *MAPT* was reduced by >75% with siRNA transfection (data not shown). Similar experiments conducted with up to 1 μM of full-length rTau383 (0N4R) did not affect Aβ levels (data not shown), suggesting an eTau specific regulation of Aβ levels.

3.4. eTau regulates sAPPα levels in vitro in primary human neurons

Our data indicate that eTau may be affecting how Aβ is produced. To understand how eTau is affecting Aβ production at the mRNA level we measured the gene expression of *APP*, *BACE1*, *ADAM9*, *ADAM10*, and *ADAM17* in human primary neurons but did not detect any significant gene expression changes after treatment with either *MAPT* siRNA, purified secreted eTau, or tau binding antibodies (gene expression data not shown).

The addition of eTau to primary human neurons for 20 days caused a small but significant, dose-dependent decrease in sAPPα levels (Fig. 3C). The simultaneous increase in Aβ with the decrease in sAPPα indicates that eTau is affecting the production of Aβ in neurons.

3.5. eTau binding antibodies reduce Aβ (40 and 42) and increase sAPPα levels in vitro in primary human neurons

We tested each of several antibodies for binding affinity to tau and eTau. As expected, all tau antibodies tested bound with good to excellent Kd's and as expected IPN001, IPN002 bound to eTau, whereas PHF1 did not bind eTau, Table 2. Unexpectedly, we found that MC1 bound with high affinity to not only tau but also eTau, Table 2. This was unexpected because MC1 is reported to bind to a conformational epitope comprised both aa 7–9 and aa 313–322, but aa 313–322 are not present on eTau. This suggests that MC1 can also bind to eTau as well as to the conformation epitope as described previously. Peter Davies has also found that MC1 binds to the N-termini of tau, in the absence of the C-terminal epitope (personal communication). To confirm therefore that eTau regulates Aβ, we treated our human primary cortical cultures with various tau antibodies. We used the eTau binding antibodies IPN002 and MC1, as well as PHF1, shown not to bind to eTau. We found that when added to cortical neurons, MC1 and IPN002 both time dependently reduced $A\beta_{40}$ and $A\beta_{42}$ levels, whereas the IgG control and PHF1 had no effect (Fig. 4A). Additionally, eTau binding antibodies (MC1 and IPN002) increased levels of sAPPα over time, whereas PHF1 did not (Fig. 4B). APP Western blots of the cell lysates of IPN002 treated primary human cortical cultures confirm these ELISA results, showing a slight increase in APP CTF-α when compared with IgG control (Supplementary Fig. 1C).

To demonstrate that our IPN002 antibody is acting extracellularly, specifically binding, and neutralizing secreted and not intracellular tau, we used our IPN002-free-tau ELISA assay to analyze the conditioned media and lysate of primary human cortical neurons treated with IPN002. This is an IPN002 competitive tau assay, which only detects tau not already bound by IPN002. Analyzing the conditioned media of primary human neurons treated with either

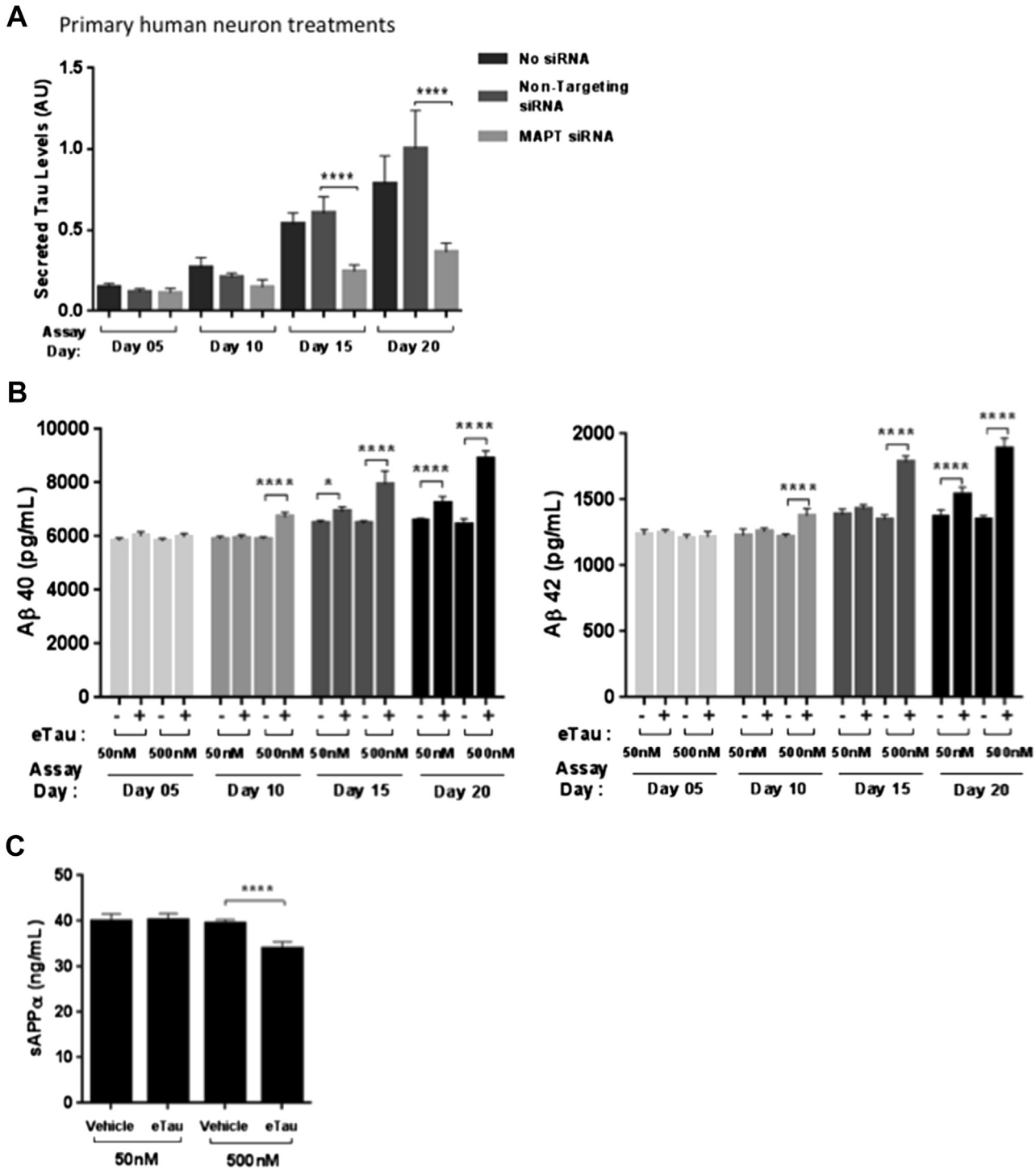

Fig. 3. eTau increases Aβ and decreases sAPPα levels in human primary cortical neurons in vitro. Experiments done on primary human cortical neurons, treatments present for 20 days in total; conditioned media harvested and cells retreated every 5 days with siRNAs or purified eTau protein. Total tau (A), $A\beta_{40}$ and $A\beta_{42}$ (B), and sAPPα (C) ELISAs ran on day 5, 10, 15, or 20 CM. Each experiment repeated 6 times, n = 3 for each condition; graphs are STD on mean. (A) Human total tau ELISA shows *MAPT* siRNA reduces secreted tau levels by greater than 50% by day 15 compared with control siRNA (0.6 ± 0.1 AU–0.25 ± 0.04AU); $^{****}p < 0.0001$ by 1-way ANOVA with Tukey multiple comparison correction. (B) Affinity purified human eTau treatment gradually increases both $A\beta_{40}$ and $A\beta_{42}$ levels above mock control (38% and 40%, respectively, at 500 nM) by day 20 in CM. Effects are dose and time dependent; $A\beta_{40}$ $^{****}p < 0.0001$, $^{*}p = 0.0113$; $A\beta_{42}$ $^{****}p < 0.0001$, by 2-way ANOVA with Tukey multiple comparison correction. (C) Affinity purified human eTau lowers sAPPα levels; day 20 data shown. Treatment of human primary neurons with 500 nM eTau for 20 days slightly but significantly decreases sAPPα levels by 14% (39.55 ± 0.77 ng/mL down–33.94 ± 1.5 ng/mL). eTau effects are dose dependent and time dependent (data not shown); 500 nM vehicle versus 500 nM secreted tau $^{****}p < 0.0001$, by 1-way ANOVA with Tukey multiple comparison correction. Abbreviations: Aβ, amyloid-beta; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; eTau, extracellular tau; sAPPα, soluble amyloid precursor protein-α; siRNA, small interfering RNA; STD, standard deviation.

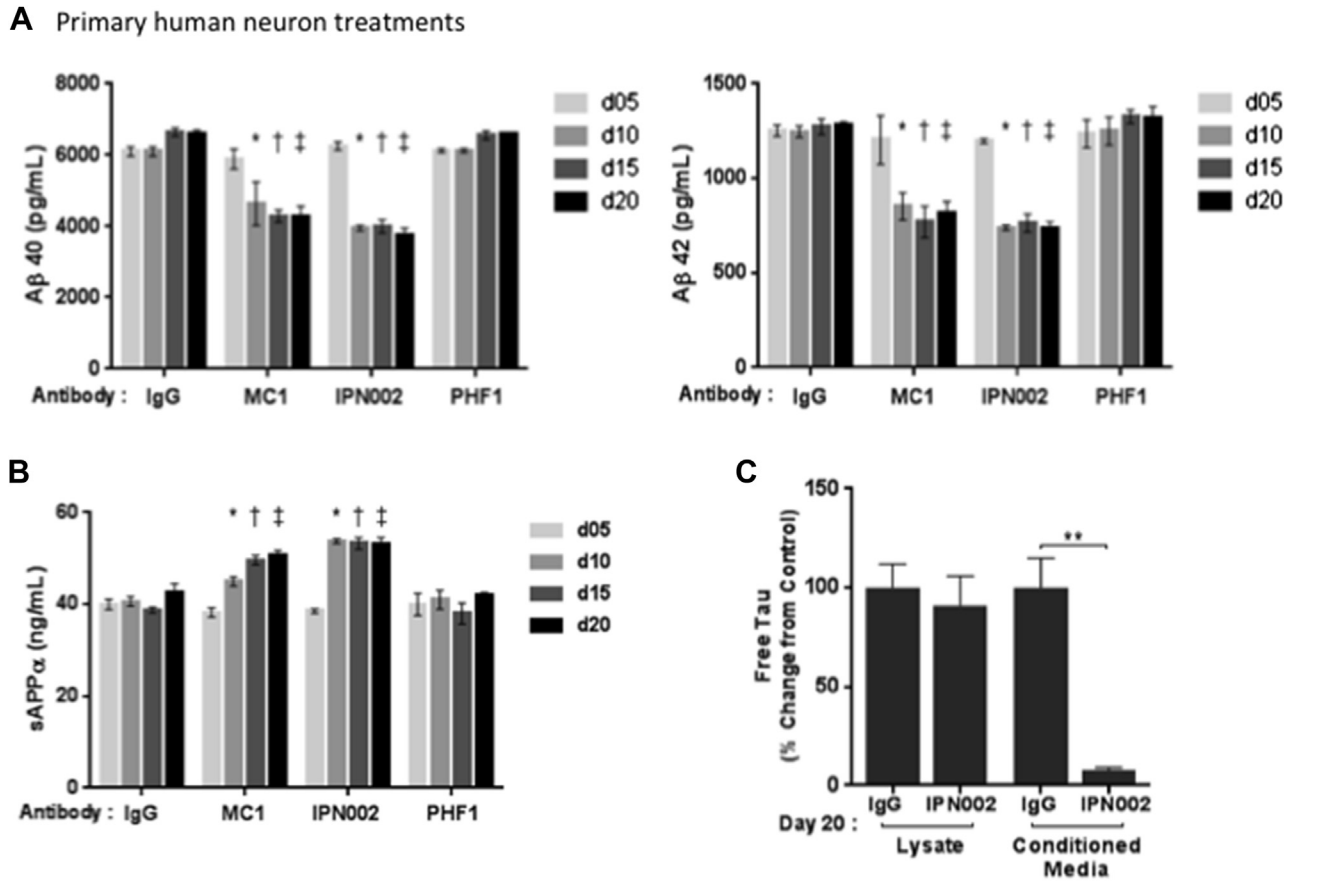

Fig. 4. eTau antibodies decrease $A\beta_{40}$ and $A\beta_{42}$ and increase sAPPα levels in vitro in primary human cortical cultures. Experiments done on primary human cortical neurons, treatments present for 20 days in total; conditioned media harvested and cells retreated every 5 days with tau antibodies. $A\beta_{40}$ and $A\beta_{42}$ ELISA (A), sAPPα ELISA (B), and free-tau ELISA assay (C). Each experiment repeated 6 times, n = 3 for each condition. (A) Secreted tau binding antibody IPN002 reduces $A\beta_{40}$ and $A\beta_{42}$ levels up to 43% and MC1 up to 40%. Nonsecreted tau binding antibody PHF1 has no effect on Aβ levels. Antibody effects are time dependent; graphs are STD on mean; * d10, IgG versus tau antibody $p < 0.0001$; † d15, IgG versus tau antibody $p < 0.0001$; ‡ d20, IgG versus tau antibody $p < 0.0001$, by 1-way ANOVA with Tukey multiple comparison correction. (B) IPN002 increases sAPPα levels up to 37% and MC1 up to 28% by 10 days of treatment; PHF1 has no effect on sAPPα levels. d10, IgG versus tau antibody $^*p < 0.0001$; d15, IgG versus tau antibody $\dagger p < 0.0001$; d20, IgG versus tau antibody $\ddagger p < 0.0001$ by 1-way ANOVA with Tukey multiple comparison correction. (C) Free-tau ELISA assay of lysate and CM harvested after 20 days of treatment shows IPN002 engages 92% eTau secreted into the media (free-tau levels in IgG treated CM 1.909 ± 0.28 AU vs. IPN002 treated CM 0.143 ± 0.018 AU); lysate levels of tau are unchanged with IPN002; STD on mean human tau free of IPN002 shown as % change from control; ** IgG versus IPN002 $p = 0.0083$ by 2-tailed unpaired t test with Welchs correction. Abbreviations: Aβ, amyloid-beta; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; eTau, extracellular tau; sAPPα, soluble amyloid precursor protein-α; STD, standard deviation.

IgG control or the N-terminal tau antibody IPN002 every 5 days for 20 days, we found that the vast majority of eTau in the conditioned media of these cultures was bound to IPN002, measured as a significant decrease in IPN002-free tau (Fig. 4C, right). Analyzing the lysates of these cultures, we found no change in free tau levels with IPN002 treatment (Fig. 4C, left). This indicates that IPN002 is not entering the cell in sufficient quantities to bind and/or neutralize intracellular tau, and suggests that the antibody's observed effect on Aβ levels is through its interaction with secreted eTau.

These results suggest that eTau plays a role in modulating Aβ levels in vitro. In contrast to the β-secretase inhibitor which rapidly (1 day) and dramatically (75%–85%) lowers Aβ levels (Supplementary Fig. 1D), IPN002 and MC1 lower Aβ levels more gradually (10 days) and modestly (30%–40%) (Fig. 4A). The gradual effect of these eTau binding antibodies is indicative of a modulatory rather than a direct, inhibitory role.

3.6. Human synthetic eTau 4 increases Aβ (40 and 42) levels in vitro in primary human cortical neurons

In vivo studies in APP transgenic mice show that reductions of endogenous murine tau do not impact Aβ levels (Ittner et al., 2010; Roberson et al., 2007, 2011). Therefore, to further understand our findings in the context of these studies involving mouse tau, we asked if human and mouse tau affect Aβ production differently. To do so, we generated a synthetic version of human eTau 4, a 67 amino acid peptide corresponding to the shortest secreted N-terminal tau fragment identified by LC/MS from iPSC-CN media. We also synthesized a peptide corresponding to the equivalent amino acid sequence in mouse tau (Fig. 5A). Because both our AD patient derived iPSC-CN cultures and our primary human cortical cultures predominately express embryonic 0N3R tau (Supplementary Fig. 1B) we did not include the n1 and n2 inserts in these synthetic sequences. Notably, mouse tau is missing the human tau insert, amino acids 17–28, which encompasses most of the IPN002 epitope (aa 15–24).

We treated primary human neurons with either the human or mouse eTau 4 peptide or a scrambled control peptide and looked for differential effects on Aβ levels. By using synthetic versions of the sequences, we eliminated any potential posttranslational differences between the 2 species tau and treated the same primary human neuronal cultures for direct comparison. Under these conditions, human eTau 4 treatment significantly increased $A\beta_{40}$ and $A\beta_{42}$ levels (18% and 26% respectively compared with scrambled control), although treatment with mouse eTau 4 did not increase $A\beta_{40}$ and $A\beta_{42}$ levels (Fig. 5B). Again, the increases in Aβ levels with human eTau4 are small but reproducible and highly significant.

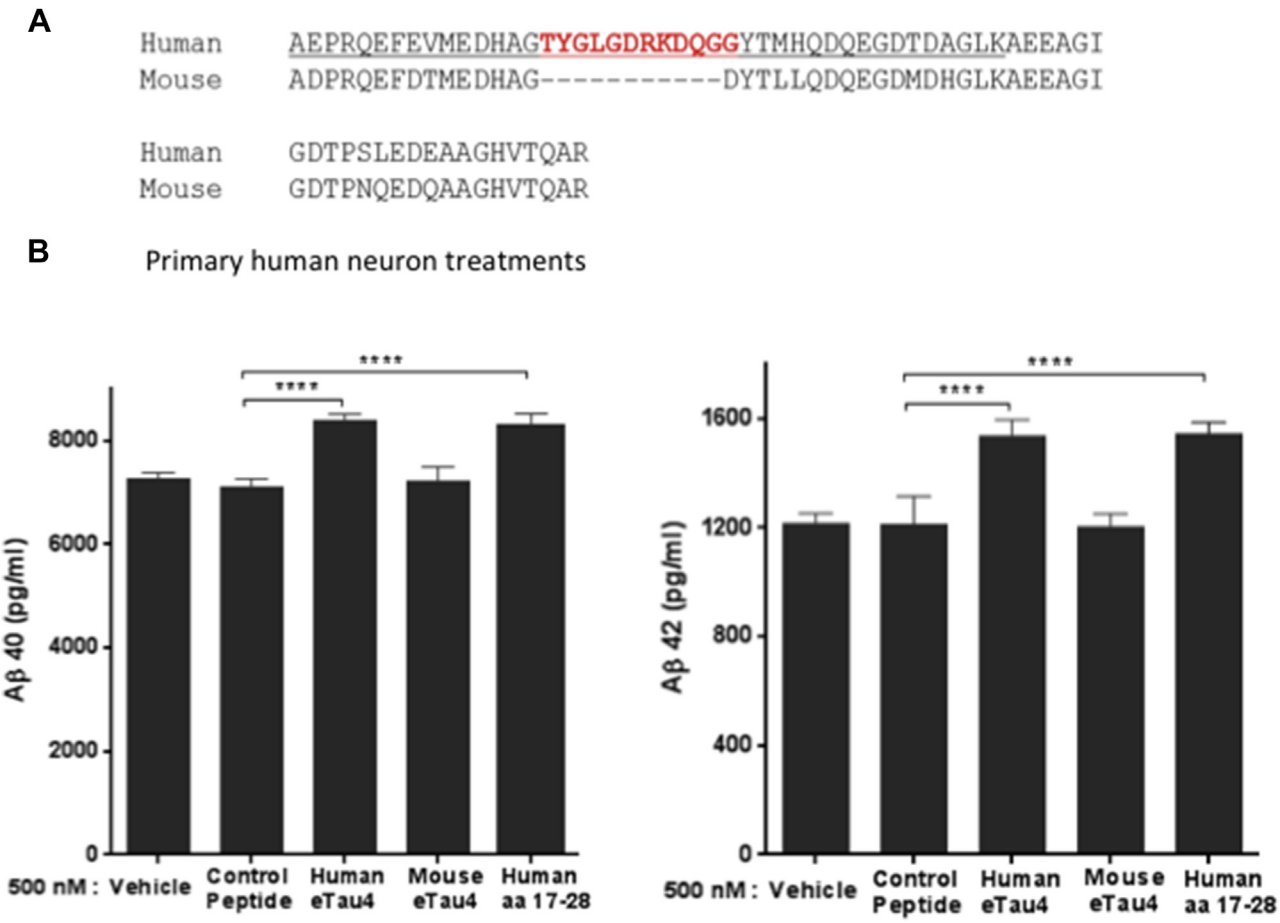

Fig. 5. Human tau insert increases Aβ levels in primary human cortical neurons. (A) Alignment of N-terminal human and mouse tau sequences comparing human eTau 4 sequence with 11 amino acid human tau insert (aa 17–28) to mouse eTau 4 sequence. (B) Synthetic human eTau 4 peptide and a peptide consisting only of the human tau insert (aa 17–28) both increase $A\beta_{40}$ and $A\beta_{42}$ levels significantly above scrambled control peptide by specific ELISAs. Human eTau 4 peptide treatment (500 nM) increases $A\beta_{40}$ and $A\beta_{42}$ levels above scrambled control; $A\beta_{40}$ 18% (scrambled peptide 7116.3 ± 147 pg/mL to human eTau 4 8402 ± 111 pg/mL) and $A\beta_{42}$ 26.4% (scrambled peptide 1216 ± 98 pg/mL to human eTau 4 1538 ± 57 pg/mL). Human tau insert peptide (500 nM) (aa 17–28) increases $A\beta_{40}$ 16% (scrambled peptide 7116.3 ± 147 pg/mL to 8286 ± 236 pg/mL for aa 17–28) and $A\beta_{42}$ 27% (scrambled peptide 1216 ± 98 pg/mL to 1546 ± 39 pg/mL for aa 17–28 peptide). Effects are dose and time dependent (data not shown); bars represent mean pg/mL $A\beta_{40}$ and $A\beta_{42}$ with STD; $A\beta_{40}$ $^{****}p < 0.0001$; $A\beta_{42}$ $^{****}p < 0.0001$ by 1-way ANOVA with Tukey multiple comparison correction. Experiments done on primary human cortical neurons, 500 nM peptides present for 20 days in total; conditioned media harvested and cells retreated every 5 days, each experiment repeated 4 times, n = 3 for each condition. Abbreviations: Aβ, amyloid-beta; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; eTau, extracellular tau; STD, standard deviation.

In fact, by treating the primary human neuronal cultures with an additional peptide encompassing only the human tau insert, we show that the 12 amino acids missing from mouse tau are capable of increasing $A\beta_{40}$ and $A\beta_{42}$ to the same level as the entire human eTau 4 peptide (Fig. 5B). These data confirm that human eTau increases Aβ production and suggest that the modulation of Aβ production is human eTau specific via an activity conferred by the human tau insert.

3.7. The eTau antibody, IPN002, reduces Aβ (40 and 42) levels in vivo

To test our hypothesis in vivo, we first used the transgenic P301L tauopathy model (JNPL3, Taconic) that releases mutant human tau into the ISF (Fig. 6A). We found that tau is present in the ISF of these mice at a truncated size of approximately 25 kDa, Supplementary Fig. 2D, similar to eTau found in the AD patient cortical neuron conditioned media. This is in contrast to the findings by Yamada et al. (2011) showing full-length tau in the ISF of P301L mice. We treated 3.5-month-old JNPL3 mice weekly with either IgG1 control or IPN002 for 8 weeks, collected ISF at 5.5 months of age, and measured the levels of IPN002 antibody, IPN002-free tau, and $A\beta_{40}$ and $A\beta_{42}$. To verify that IPN002 entered the central nervous system (CNS), we measured levels of IPN002 in the plasma and ISF of each animal at study termination and found IPN002 present in the ISF in all cases (Fig. 6B). Additionally, we found that IPN002 bound almost all the human mutant tau available in the ISF, measured by a 94% reduction in the free tau levels (Fig. 6C). $A\beta_{40}$ and $A\beta_{42}$ analysis of the animal's ISF at the end of the 8-week treatment showed IPN002 treatment reduced endogenous mouse $A\beta_{40}$ and $A\beta_{42}$ levels by approximately 25% (Fig. 6D).

We validated these initial findings in a second transgenic P301L tauopathy model, using the tau-4R/2N-P301L (van Leuven) mice, in which secreted human tau levels increase with age, (Fig. 6E). In this model, 3.5-month-old animals have <10 ng/mL human tau in their CSF, by 9.5 months their CSF tau levels increase to an average of 30 ng/mL (Fig. 6E). We treated these mice weekly, for up to 26 weeks, with 20 mg/kg IgG1 control or IPN002. To verify that IPN002 entered the CNS, we measured levels of IPN002 in the plasma and CSF of each transgenic animal at 9.5 months and found IPN002 present in the CSF in all dosed animals (Fig. 6F). CSF analysis by IPN002-free-tau ELISA assay confirmed that human mutant tau was fully engaged by IPN002, measured by a 98% reduction in free tau levels, Fig. 6E, IPN002 treated compared with IgG1 control-treated mice.

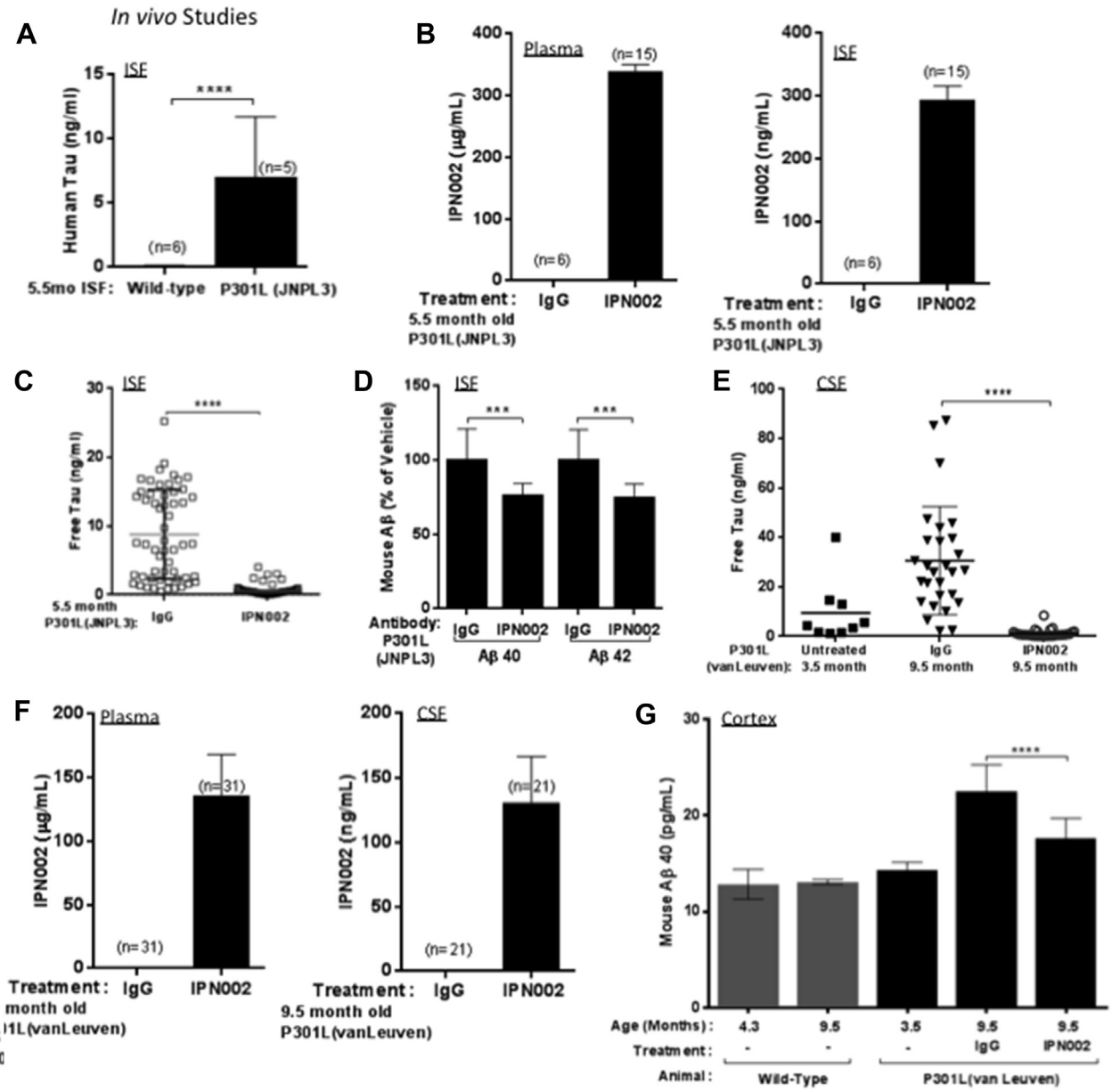

Fig. 6. IPN002 binds secreted tau and reduces Aβ levels in vivo. The 8-week study in P301L JNPL3 tau transgenic mice. (A) Human tau is present in ISF of P301L (JNPL3) tau transgenic mice (n = 5 animals). Bars represent mean ng/mL human tau by total tau ELISA; error bars STD; 2-tailed unpaired *t* test with Welch correction $^{****}p < 0.0001$. (B) IPN002 antibody levels measured in plasma and ISF of each P301L (JNPL3) mouse, in plasma IPN002 levels were 338.9 ± 10 µg/mL, in ISF IPN002 levels were 293.6 ± 21 ng/mL; n = 15 animals for IPN002, n = 6 animals for IgG; STD on mean levels of IPN002. (C) IPN002 decreases human free tau by 94% in ISF (IgG1 treated 8.78 ± 6.49 ng/mL vs. IPN002 treated 0.71339 ± 0.845 ng/mL); STD on mean levels human tau free of IPN002 in ISF by free-tau ELISA; 2-tailed unpaired *t* test with Welch correction $^{****}p < 0.0001$. (D) IPN002 treatment significantly decreases levels of mouse $A\beta_{40}$ by 24% (from 84 ± 17 pg/mL to 64 ± 9 pg/mL) and $A\beta_{42}$ by 25% (from 33 ± 6 pg/mL to 25 ± 4 pg/mL) in mouse ISF at the end of the 8-week study. Bars represent mean mouse Aβ levels as percent of control with STD; $A\beta_{40}$ $^{***}p = 0.0005$; Aβ 42 $^{***}p = 0.0003$ by 2-tailed unpaired *t* test with Welch correction. The 26-week study in P301L van Leuven transgenic mice. (E) In a 26-week study, human P301L tau levels significantly increase with age (3-fold) in CSF measured at 3.5 months (n = 10 animals) and at the 9.5 months end the study (n = 28). By 9.5 months end of study, IPN002 antibody treatment of transgenic animals (n = 32) engaged 97% of secreted human mutant tau in CSF, compared with IgG1 control treated mice (n = 32 animals), measured by free-tau ELISA, mean ng/mL tau free of IPN002 in CSF of each mouse (IgG1 treated 30.566 ± 21.8 ng/mL vs. IPN002 treated 1.150 ± 1.61 ng/mL), error bars are STD, 9.5 months IgG1 versus 9.5 months IPN002 $^{****}p < 0.0001$, statistics are 1-way ANOVA with Tukey correction. (F) Mean IPN002 antibody levels in the 9.5-month-old transgenic mice were 135.5 ± 32 µg/mL in plasma (n = 31) and 130.4 ± 35 ng/mL in CSF (n = 21); STD on mean levels of IPN002 in plasma and CSF. (G) $A\beta_{40}$ levels are increased by 57% with age in the soluble fraction of the cortex of transgenic mice, measured in 3.5 month old versus 9.5 month old P301L van Leuven mice (14.35 ± 0.78 pg/mL–22.52 ± 2.7 pg/mL). $A\beta_{40}$ levels are increased by 72% with disease progression in transgenic mice (13.11 ± 0.25 pg/mL–22.52 ± 2.7 pg/mL) comparing $A\beta_{40}$ levels in 9.5-month-old wild type C57/BL6 mice with 9.5 months P301L van Leuven mice. IPN002 antibody treatment significantly reduces $A\beta_{40}$ levels in the transgenic mice by 22% (22.52 ± 2.7 pg/mL–17.64 ± 2 pg/mL); $^{****}p < 0.0001$, 1-way ANOVA with Tukey correction. Mouse $A\beta_{40}$ levels do not change with age in wild type C57/BL6 mice approximately 4.3 months–9.5 months. Bars are mean pg/mL mouse $A\beta_{40}$; error bars are STD. Abbreviations: Aβ, amyloid-beta; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; CSF, cerebrospinal fluid; ISF, interstitial fluid; STD, standard deviation.

We measured $A\beta_{40}$ levels in both the total homogenate and the soluble fraction of the hindbrain (data not shown) and cortex; the results were consistent between fractions and brain regions. As shown in Fig. 6G, $A\beta_{40}$ levels in the soluble fraction of P301L mouse cortex increase from 14.4 pg/mL at 3.5 months to 22.5 pg/mL at 9.5 months (IgG1 control group). This substantial increase in levels of murine $A\beta_{40}$ in a tau mouse model may be because of either age, continued human tau expression, or a combination of both. To

distinguish between these possibilities, we measured $A\beta_{40}$ levels in the soluble fraction of the cortex of wild type C57/BL6 mice at 4.3 months and 9.5 months of age (Fig. 6G) and saw no change in Aβ with age. This suggests that the observed increase in mouse Aβ over time in this P301L tau mouse model is because of human tau expression.

To determine if elevated levels of $A\beta_{40}$ could be lowered with the human eTau binding antibody IPN002 we compared the $A\beta_{40}$ levels of IgG1 and IPN002 treated mice and found that IPN002 treatment significantly reduced levels of soluble mouse $A\beta_{40}$ by >25%, Fig. 6G. Together these data suggest that human tau released from neurons modulates Aβ production both in vitro and in vivo.

3.8. TTX treatment lowers Aβ and eTau levels in primary human cortical neurons in vitro

How does eTau modulate Aβ production? In AD, secreted tau is hypothesized to be released presynaptically and then taken up postsynaptically, inducing spread, tau pathology, neuronal dysfunction, and neuronal hyperactivity. Kamenetz et al. (2003) showed that picrotoxin induced hyperactivity in hippocampal slices dramatically elevated $A\beta_{40}$ and $A\beta_{42}$ although TTX treatment was able to lower Aβ levels; Cirrito et al. (2005), (2008) found a similar link between heightened neuronal and synaptic activity and increased Aβ production in living mouse ISF.

To determine whether our observed increase in Aβ could be the result of eTau induced neuronal hyperactivity, we treated our primary human cortical cultures with the sodium channel blocker TTX. As has been shown previously in vitro and in vivo, TTX is capable of significantly reducing soluble $A\beta_{40}$ and $A\beta_{42}$ in a dose and time-dependent manner (Fig. 7A), with no change to cell viability (data not shown). In these same cultures, eTau levels analyzed in the conditioned media of the TTX treated neurons were also reduced, by 60%, after 10 days of TTX treatment (Fig. 7B). eTau in the conditioned media of neurons is significantly reduced by day 10 of TTX treatment; however, Aβ levels are not affected until day 15, suggesting that suppressing neuronal activity reduces eTau levels and subsequently alters Aβ production.

3.9. eTau induces hyperactivity in human neurons

Increased neuronal activity potentiates Aβ levels in vitro and in vivo. This suggests that one mechanism by which eTau can modulate Aβ production is through increasing neuronal activity. To test this hypothesis, we performed whole–cell patch-clamp recordings to determine the effect eTau has on cortical neuron activity. Application of eTau increased the activity of both iPSCs-derived cortical neurons and primary human cortical neurons (Fig. 7C). Interestingly, application of full length tau (rTau383, 0N4R) at significantly higher concentrations (up to 3 μM) failed to produce any changes in neuronal activity similar to those caused by eTau treatment. This suggests that the eTau mediated neuronal activity effects may involve a specific interaction of the eTau protein with cortical neurons. This interaction appears to be specific to the N-terminal region of tau as it cannot be replicated with full length tau (rTau383, 0N4R). To test this hypothesis, we preincubate eTau with the neutralizing antibody IPN002 to block eTau mediated hyperactivity. At a 10:1 molar ratio of IPN002 to eTau, IPN002 inhibited eTau mediated hyperactivity (Fig. 7D). Together, these results suggest that eTau can modulate Aβ levels by promoting cortical neuron hyperactivity.

4. Discussion

In this study, we describe the identification of a novel secreted N-terminally truncated tau species (eTau), present both in vitro and in vivo, and show that these secreted tau fragments can regulate Aβ production. We initially purified eTau from the conditioned media of AD patient–derived cortical neuron cultures and found that secreted tau is composed predominantly of N-terminal fragments of tau. In vitro, we show that treating primary cortical cultures with eTau simultaneously increases Aβ levels while decreasing sAPPα levels. Globally, reducing tau with *MAPT* siRNA or treating neurons with neutralizing eTau antibodies lowers Aβ levels and increases sAPPα levels in parallel. Importantly, we validated these findings in vivo in 2 tau transgenic (P301L) mouse models. Levels of human tau are elevated in the ISF and CSF of the tau transgenic animals; treating these mice with an eTau-neutralizing antibody (IPN002) reduced endogenous murine Aβ in both their ISF and brain. Finally, in vitro we demonstrate that eTau treatment increases neuronal hyperactivity. We propose that this eTau driven neuronal hyperactivity increase, results not only in increased Aβ secretion but also in a further increase in eTau secretion, and that together eTau and Aβ create a feed forward disease mechanism that perpetuates the disease (Supplementary Fig. 4).

We examined the conditioned media of AD patient–derived cortical neurons for the presence of tau in the absence of neuronal lysis and found tau fragments. Analysis of the tau fragments by LC/MS for primary sequence and posttranslational modifications showed that this secreted tau is composed predominantly of 4 N-terminal fragments, all containing an N-terminal acetylated alanine and a low percentage (<5%) canonical tau phosphorylations (at p181, p202, and p231 using 2N4R designation). Immunoprecipitation and LC/MS analysis showed minimal evidence of full-length tau and no C-terminal tau fragments, suggesting that tau is processed to eTau in the cytoplasm before secretion. Future efforts could determine how and why tau is processed to these N-terminal fragments and may also help clarify the mechanism by which eTau is secreted from neurons.

The size of the identified eTau species varies from 171 amino acids for eTau1 (aa 2–44, 103–230) to 67 amino acids for eTau4 (aa 2–44,103–126). The molecular weight and immunoreactivity of the isolated eTau species is similar to that of the tau fragments found in the CSF of AD and control patients (Meredith et al., 2013; iPierian, data not shown). We furthermore show that tau in the ISF of P301L mice is also truncated. Our data, together with Meredith et al. (2013), suggest that the eTau secreted from patient-derived cortical neurons is physiologically relevant and is representative of the tau secreted into the CSF of AD patients in disease. This is in contrast to Yamada et al. (2011) which shows an immunoreactive band at 50 kd, suggestive of full-length tau, present in the ISF of P301L mice. The reason for these differences remains to be determined. Our results show for the first time that endogenously generated and secreted tau from AD patient neurons can been purified and characterized, making it now possible to study eTau's potential role in the accumulation of Aβ and tau pathology.

Using the purified eTau from conditioned media, we identified high affinity tau antibodies capable of recognizing all 4 eTau fragments. Some of these antibodies specifically neutralized eTau's effects; capable both of lowering Aβ production and of blocking eTau induced neuronal hyperexcitability. IPN002 is our strongest eTau neutralizing antibody.

To investigate the mechanism by which eTau may regulate Aβ levels we looked at the proteolysis of APP. Treating neurons with either purified endogenous eTau or synthetic eTau both increased Aβ levels, where treatment with eTau binding antibodies decreased Aβ levels. The mechanism by which α- and β-secretase compete for

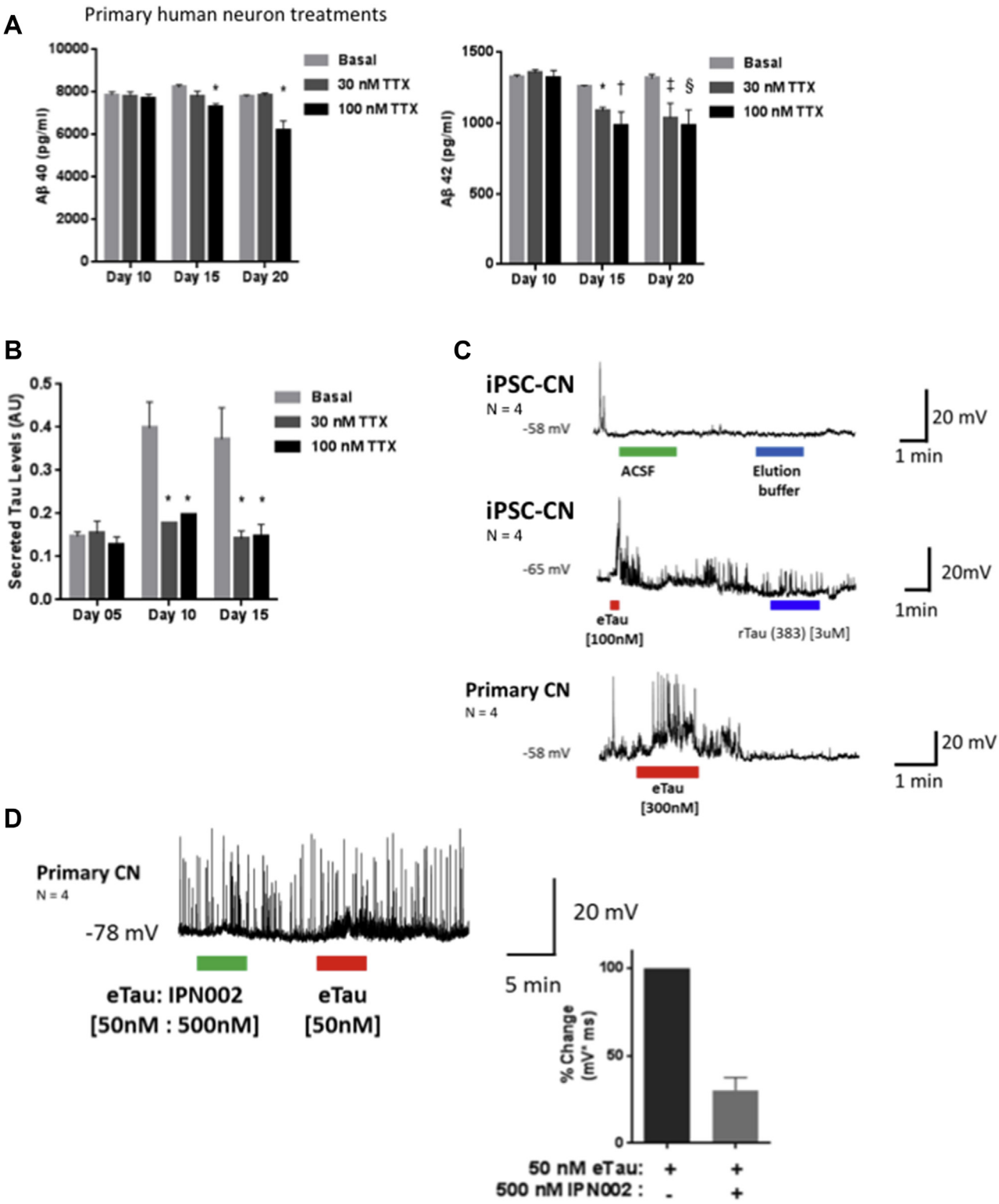

Fig. 7. Secreted tau and Aβ production are driven by neuronal excitability state. Human secreted tau induces neuronal hyperactivity. Primary human neuron cultures treated with TTX for Aβ and total tau ELISA endpoints; TTX present for 20 days in total; conditioned media harvested and cells retreated every 5 days. (A) In a dose and time-dependent manner, TTX treatment lowers $A\beta_{40}$ levels up to 20% (7778 ± 92 pg/mL–6203 ± 443 pg/mL) and $A\beta_{42}$ levels up to 25% (1316 ± 29 pg/mL–985 ± 108 pg/mL). Error bars are STD on mean pg/ml Aβ levels. One-way ANOVA with Tukey correction; $A\beta_{40}$, day 15 and 20, Basal versus 100 nM TTX $^*p < 0.0001$; $A\beta_{42}$, day 15, Basal versus 30 nM TTX $^*p = 0.0109$; day 15, Basal versus 100 nM TTX $\dagger p = 0.0001$; day 20, Basal versus 30 nM TTX $\ddagger p = 0.0001$; day 20, Basal versus 100 nM TTX $\S p < 0.0001$. (B) TTX treatment significantly lowers secreted eTau levels up to 60% by day 15 with 100 nM TTX (0.373 ± 0.07 AU–0.14823 ± 0.026 AU). Error bars are STD on mean tau ELISA units; 1-way ANOVA with Tukey correction day 10 and day 15, Basal versus 30 nM and 100 nM TTX $^*p < 0.0001$. Electrophysiology experiments done on both primary human cortical neuron cultures and iPSC derived cortical neuron cultures. (C) Current clamp recordings show that puff application of purified eTau, but not full length tau (3 μM rTau383), increases neuronal activity in both iPSC-CN (100 nM, upper panels) and in primary human cortical neuron cultures (300 nM, lower panel). (D) Bath application of purified eTau (50 nM) onto primary human cortical neuron cultures increases neuronal activity which is reduced by the addition of the anti-tau antibody, IPN002 (500 nM). Bar graph shows mean % reduction of eTau induced hyperactivity in primary human neurons when IPN002 is added to cultures, values are background normalized and averaged over 4 experiments. Abbreviations: Aβ, amyloid-beta; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; eTau, extracellular tau; iPSC, induced pluripotent stem cells; STD, standard deviation; TTX, tetrodotoxin.

APP is not fully understood; however, the amyloidogenic arm of APP processing is mitigated if α-secretase cleaves APP before β-secretase; α-secretase clips in the middle of the Aβ region releasing a soluble APP fragment, sAPPα leaving a short C-terminal cellular fragment. We found that eTau treatment of human cortical cultures results in a simultaneous increase in Aβ and a decrease in sAPPα, leading us to conclude that eTau affects the cleavage of APP. Our experiments showing the concurrent decrease in sAPPα levels with the increase in $Aβ_{40}$-$Aβ_{42}$ levels support the idea that α- or β-secretase cleavage of APP is coupled.

When we reduced tau protein levels with *MAPT* siRNA or neutralized eTau with MC1 and IPN002 tau antibodies, levels of sAPPα always moved in the opposite direction to $Aβ_{40}$-$Aβ_{42}$ levels. Analysis of the cell lysates of treated neurons by APP Western blot also indicate that neutralizing eTau favors the formation of the C-terminal cellular fragment cleavage fragment of APP over the β-secretase generated β-CTF.

Importantly, we were able to show that only the tau antibodies capable of binding eTau could affect Aβ production. The tau antibody PHF1, which does not bind to eTau, does not reduce Aβ levels, confirming that it is secreted eTau that is affecting APP proteolysis and Aβ levels and not full-length tau.

Our in vivo studies using human tau transgenic mice support the idea that eTau affects Aβ levels. The data from our 26-week tau-4R/2N-P301L van Leuven transgenic tau animal study show endogenous murine Aβ increases with age. This elevation was not observed in nontransgenic mice, suggesting that the observed increase in mouse Aβ in the older P301L mouse is driven by continued human tau expression. Dosing 2 strains of P301L tau transgenic mice, in 2 distinct studies with the eTau binding antibody, IPN002, significantly decreased endogenous murine Aβ levels.

Our studies, in vitro and in vivo, show that reducing or neutralizing eTau lowers Aβ production. This finding is in contrast to the in vivo hAPP mouse studies by Roberson et al. (2007) and Ittner et al. (2010). These hAPP studies demonstrate that dose-dependent reductions of mouse tau protect mice from hAPP driven neuronal dysfunction; however, eliminating mouse tau in these studies did not affect the levels or aggregation of transgenic Aβ. One possible explanation for this divergence in findings is that human tau but not mouse tau is capable of regulating Aβ levels. The findings in Ribe et al. (2005) that human tau overexpression increases the Aβ burden in a human APP and tau bigenic mouse model when compared with single transgenic control animals supports this claim.

We were able to confirm this putative activity difference in mouse and human tau by treating our primary human cortical cultures with human eTau 4 (aa 2–44,103–126) or the equivalent amino acid sequence from mouse tau. Synthetic human eTau 4, but not the equivalent mouse tau peptide, increased Aβ levels. Because these peptides were synthetically generated, the only difference between mouse and human eTau 4 treatments is the variation in their primary sequence. We were further able to show that a peptide composed only of the human tau insert region, the sequence absent from mouse tau, was able to increase Aβ production on its own. Thus, it appears that the human tau insert region at the N-terminus of tau regulates Aβ production and explains the human specificity of this observation.

Finding that N-terminally truncated tau regulates Aβ levels where full-length tau cannot suggest that a change in the secondary structure of the truncated fragments versus full-length tau confers an activity difference. Full-length tau protein in solution has been described alternatively as unordered (Schweers et al., 1994) and in other studies as having a "paperclip" conformation, where the amino- and carboxyl-terminal domains are folded back on each other (Jeganathan et al., 2006). In either case, our findings suggest that removing the C-terminus of the protein may change the secondary structure of tau, exposing the putative "active region" of eTau, the human tau insert region (aa 17–28), which being exposed can then affect Aβ levels.

In light of the observations that Aβ production is regulated by neuronal activity (Kamenetz et al., 2003) as well as observations implicating tau in the regulation of excitability and synchronization of neuronal networks (Holth et al., 2013), we asked if eTau could affect neuronal hyperactivity. We found that treatment of primary cortical neurons with eTau but not full-length tau increases neuronal hyperactivity and that neuronal hyperactivity caused further secretion of eTau. Additionally, we showed that reducing or neutralizing eTau lowers the production of $Aβ_{40}$ and $Aβ_{42}$ and prevents eTau-dependent neuronal hyperactivity. We therefore suggest that eTau induced neuronal hyperactivity may be the mechanism by which eTau is affecting Aβ production in the brain. Interestingly, it takes between 10 and 20 days for eTau to increase Aβ production, whereas eTau induces hyperactivity within seconds. This suggests that an adaptive process that is neuronal hyperactivity dependent may be responsible for this time lag in regulating Aβ production.

Finally, the amount of exogenous eTau needed to elicit neuronal hyperactivity as well as to affect Aβ and sAPPα levels is higher than the circulating levels of tau found in AD patient CSF or in the conditioned media of our primary cortical cultures and iPSC-cortical neurons. However, it has been proposed that activity-dependent secretion of tau may occur through synaptic transmission (Braak et al., 2013; Liu et al., 2012; Yamada et al., 2014). Therefore, eTau secretion through a synaptic mechanism would necessarily generate local eTau concentrations at the release site, and likely site of action, which are significantly higher than circulating levels of eTau.

The exact nature of the relationship between Aβ and tau, the 2 hallmark proteins involved in the pathology of AD has long been unclear. The amyloid hypothesis suggests that Aβ is the primary driver in AD. Patients with Down syndrome who carry an extra copy the APP gene or express hereditary APP mutations all suffer from earlier onset of dementia and a more severe disease pathology. Alternatively to the amyloid cascade idea, Braak and Schonheit work with human autopsy material has clearly found that Aβ deposits are seen later than intraneuronal tau changes in subregions of the AD brain (Braak and Del, 2004; Braak et al., 2013; Schonheit et al., 2004). Additionally, Ribe et al. (2005) using an APP/tau double transgenic mouse model have shown that human tau overexpression increases the Aβ burden over single transgenic animal controls, indicating that human tau is capable of modulating Aβ production or deposition.

Our studies, both in vitro and in vivo, indicate that tau plays a role in regulating Aβ production, an activity conferred by secreted N-terminal fragments of tau and not full-length tau. We propose that eTau is modulating Aβ levels through its induction of neuronal hyperactivity. In agreement with the observation that even partial reductions in soluble Aβ levels can have a profound impact on the development of Aβ pathology (McConlogue et al., 2007), our data identifying secreted eTau fragments and their effect on Aβ production are an important step forward to understanding the relationship of tau and Aβ in the progression of AD.

Our results taken together with previous findings align the 2 main players in AD, placing tau and Aβ in a disease-perpetuating loop as depicted in the diagram (Supplementary Fig. 4). The field has shown that tau mediates Aβ induced neuronal dysfunction, and we now show that secreted eTau modulates the process of APP cleavage, changing the putative balance between the neuroprotective sAPPα and amyloidogenic $Aβ_{40}$-$Aβ_{42}$ in a neuronal activity state dependent manner. In addition to strengthening the

connection between tau and Aβ, these findings underscore the potential utility in neutralizing eTau in the clinical setting of AD with therapeutics such as humanized versions of the IPN002 tau antibody. Neutralizing eTau in patients can potentially inhibit the spread of tau and tau pathology in the brain, reduce CNS Aβ levels and subsequent neuronal hyperactivity, and potentially slow the clinical progression into dementia. This strategy may offer the first of its kind dual therapy for this debilitating disease.

Disclosure statement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2014.09.007.

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