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Research paper

Tau antibody chimerization alters its charge and binding, thereby reducing its cellular uptake and efficacy

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ABSTRACT

Background: Bringing antibodies from pre-clinical studies to human trials requires humanization, but this process may alter properties that are crucial for efficacy. Since pathological tau protein is primarily intraneuronal in Alzheimer's disease, the most efficacious antibodies should work both intra- and extracellularly. Thus, changes which impact uptake or antibody binding will affect antibody efficacy.
Methods: Initially, we examined four tau mouse monoclonal antibodies with naturally differing charges. We quantified their neuronal uptake, and efficacy in preventing toxicity and pathological seeding induced by human-derived pathological tau. Later, we generated a human chimeric 4E6 (h4E6), an antibody with well documented efficacy in multiple tauopathy models. We compared the uptake and efficacy of unmodified and chimeric antibodies in neuronal and differentiated neuroblastoma cultures. Further, we analyzed tau binding using ELISA assays.
Findings: Neuronal uptake of tau antibodies and their efficacy strongly depends on antibody charge. Additionally, their ability to prevent tau toxicity and seeding of tau pathology does not necessarily go together. Particularly, chimerization of 4E6 increased its charge from 6.5 to 9.6, which blocked its uptake into human and mouse cells. Furthermore, h4E6 had altered binding characteristics despite intact binding sites, compared to the mouse antibody. Importantly, these changes in uptake and binding substantially decreased its efficacy in preventing tau toxicity, although under certain conditions it did prevent pathological seeding of tau.
Conclusions: These results indicate that efficacy of chimeric/humanized tau antibodies should be thoroughly characterized prior to clinical trials, which may require further engineering to maintain or improve their therapeutic potential.
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1. Introduction

Passive tau immunotherapy for the treatment of Alzheimer's disease (AD) has the potential to clear intracellular tau aggregates and prevent spreading of tau pathology in the brain. In animal models, success in reducing tau pathology has been achieved targeting multiple disease relevant epitopes as well as with pan-tau antibodies. Findings from us and other groups suggest two different mechanisms by which these antibodies exert their effects, namely intracellular clearance and extracellular blockage/clearance. Multiple groups have shown that antibodies can enter neurons via bulk- or receptor-mediated endocytosis, even when peripherally administered in animal models (for review see [1,2]).

Abbreviations: AD, Alzheimer's disease; FTDP, frontotemporal dementia with parkinsonism; PHF, paired helical filament; IEP, isoelectric point; IEF, isoelectric focusing; LDH, lactate dehydrogenase; HMD, hexamethylene diamine; PLP, periodate-lysine paraformaldehyde; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; BDNF, brain derived neurotrophic growth factor; cat4E6, cationized 4E6; h4E6, human chimeric 4E6.

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Research in context

Evidence before this study

As more antibodies move from pre-clinical studies to human trials, a thorough understanding of how antibody properties influence efficacy is crucial for a successful outcome. One such important antibody characteristic is charge, which can substantially impact their tissue uptake, specificity, Fc receptor binding and clearance. However, this important issue has not been well examined in brain cells, and not at all for antibodies targeting the tau protein. We and other groups have previously shown that tau antibodies can target pathological tau both extracellularly and intracellularly. Further, antibody uptake is required for efficacy when pathological tau is only found inside neurons. Thus, diminished antibody uptake into neurons is likely to limit its efficacy.

Added value of this study

We first examined four tau mouse monoclonal antibodies that have a varying overall charge and that bind to different sites of the tau protein. Only one of them was effective in preventing toxicity and seeding of both extracellular and intracellular tau. The other antibodies' lack of efficacy corresponded to much lower degree of neuronal uptake. We then generated a human chimeric version of the best antibody, which has a proven efficacy in various culture and animal tauopathy models. This substantially altered its charge, reduced its neuronal uptake and efficacy, as well as changed its tau binding profile even though the binding sites remained the same as in the mouse antibody.

Implications of all available evidence

It is essential to confirm the efficacy of humanized or human chimeric tau antibodies in appropriate models prior to clinical trials, in addition to the standard procedures of assessing their binding, target engagement and basic pharmacokinetic properties such as half-life and brain entry. Ideally, the efficacy assays should examine not only the ability of the antibodies to prevent seeding of tau pathology but also how well they block tau toxicity as these two properties do not necessarily go hand in hand.

Several groups have shown expression of Fc receptors in neurons, and that these are the major route of entry for some antibodies [3–7]. Blocking receptor-mediated uptake, either pharmacologically or through the use of Fc blocking antibodies, reduces tau antibody uptake [7–9]. Within neurons, these antibodies colocalize with tau within the endosomal/lysosomal system, analogous to α-synuclein antibodies [7–16].

However, not all tau antibodies enter neurons [17–20]. The spreading of tau pathology presents a second potential mechanism through which antibodies could alter disease course. Tau lesions appear in the hippocampus before showing up in or spreading to other regions [21,22]. This can be recapitulated in animals that express tau only in specific brain regions or where exogenous tau is injected intracranially, with tau pathology spreading over time to anatomically connected areas [23–30]. In both human patients and animal models, tau is detected in interstitial fluid [31,32] and tau expressing cells have been shown to secrete it in culture [33–38]. Extracellular pathological tau can then be taken up by other cells, and in turn seed further tau pathology [8,23–25,29,39–47]. Importantly, this process can be blocked by antibodies [8,9,18,20,46]. Although both mechanisms are valid means of reducing pathology in model systems, an antibody which is capable of entering neurons is likely to be more efficacious because most of pathological tau is intraneuronal.

Passive tau immunotherapy has advanced to clinical trials, and understanding which factors influence efficacy will allow the development of better antibodies. As stated above, there are two viable mechanisms of action for tau antibodies, intracellular clearance and extracellular blockage/clearance. We have been able to model these two mechanisms in culture using solubilized paired helical filaments (PHF) enriched from AD brain. PHF was added to neuronal cultures and monoclonal tau antibodies were added either immediately or 24 h later, after the PHF had been internalized by the cells. In both cases, one of our antibodies, 4E6, prevented PHF-induced toxicity and pathological seeding [8]. Our findings indicate that while antibody uptake into neurons is not required when the PHF is external, once the pathological tau has entered the cells, blocking antibody uptake reduces its ability to prevent tau-induced changes [8]. These results demonstrate that antibody uptake is important to achieve maximum efficacy.

Multiple factors influence the ability of antibodies to cross into the brain and enter neurons. Isoelectric point (IEP), the pH at which a molecule carries no charge, is one of these. How IEP affects antibody uptake is complex. Association with the cell surface, as well as uptake and transport across cell monolayers and the blood-brain-barrier, can be enhanced by raising the IEP, from acidic to basic, or within a basic range [48–50]. However, this change can also result in increased clearance and lower bioavailability [51]. Other reports indicate that lowering IEP from neutral to acidic, within an acidic range, or within a basic range may increase target tissue uptake, while increasing clearance and lowering non-specific uptake [52], or conversely increase serum half-life without reducing bioavailability [53].

Here, we examined the effects of antibody charge on uptake and efficacy of tau monoclonal antibodies, 1B9 (IgG1κ), 2C11 (IgG2aκ), or Tau-5 (IgG1), which recognize tau epitopes pThr212 and pSer214, pSer262, and amino acids 210–241, respectively (see Supplemental Table 1 and Supplemental Figs. 1 and 2 for source and characterization data). Which factors influence neuronal uptake of antibodies is still not well understood. We utilized both novel and commercially available tau monoclonal antibodies with differing charge, as well as directly modified the charge of 4E6, an antibody we have previously reported on [7,8,12,54]. Our results indicate that there is a narrow range of optimal charge for neuronal uptake, and deviation from this range in either the basic or acidic direction reduces uptake and thereby antibody efficacy in preventing tau toxicity and enhancing tau clearance. Most importantly for the development of antibody-based therapies, we found that human chimerization alters antibody charge and thereby profoundly reduces its neuronal uptake and efficacy. These findings have major implications for ongoing and future clinical trials on therapeutic tau antibodies as well as for similar trials targeting other intraneuronal protein aggregates.

2. Materials and methods

2.1. Animals

Pups from the homozygous JNPL3 mouse line (RRID: IMSR_TAC:2508) were collected at postnatal day zero for use in culture experiments. These mice express the 0N4R human tau isoform containing the P301L mutation found in human FTDP patients [55]. Mice were housed at NYU School of Medicine animal facilities and cared for by the veterinary staff in AAALAC-approved facilities. All the procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the university, and are in accordance with NIH Guidelines, which meet or exceed the ARRIVE guidelines.

2.2. Antibodies

Tau monoclonal antibodies 4E6, 1B9, and 2C11 were generated by our laboratory using the services of GenScript Inc. Wild type BALB/c mice were immunized with peptides corresponding to the following phospho-epitopes: cTDHGAEIVYK(pS)PVVSGDT(pS)PRHL (4E6-p396/404); cPGSRSR(pT)P(pS)LP (1B9-p212/214); IG(pS)TENLKHQPGc (2C11-p262).

Peptides were conjugated via cysteine residue to keyhole limpet hemocyanin. Hybridomas were prepared from mice showing sufficient response to immunization, and antibodies were purified with a low endotoxin version of a protein A affinity column. Antibodies were tested for binding to the target epitopes, and for staining on tissue and immunoblotting (See Supplemental Figs. 1 and 2 for 1B9 and 2C11, for 4E6 characterization see [7,8,12]). Tau-5 was purchased from Invitrogen (RRID:AB_61816).

Commercially available antibodies were used in immunoblotting and immunocytochemistry experiments. NeuN (RRID:AB_10807945), Pan-tau (RRID:AB_10013724) and pSer199 (RRID:AB_2533737) polyclonal primary antibodies were purchased from Millipore, Dako and ThermoFisher. HRP-conjugated mouse and rabbit, and Alexa488-conjugated mouse secondary antibodies were from ThermoFisher (RRID: AB_228313, AB_228341, AB_2534069). See Supplemental Table 1 for the source and product information for all antibodies used.

2.2.1. 4E6 cationization

Because direct comparison of uptake between antibodies is complicated by the difference in epitope recognized, we raised the IEP of 4E6 with 2 M hexamethylene diamine (HMD), pH 6.8 and 200 mg N-Ethyl-N′-(3-(dimethylamino) propyl)- carbodiimide. The reaction was carried out with rotation for 3 h at room temperature. The reaction was stopped with 1 M glycine and incubated at room temperature for a further 30 min. Following this step, the antibody was dialyzed overnight in PBS. Cationization was confirmed by running the original and modified 4E6 on an IEF gel.

2.3. Expression and purification of 4E6 human chimeric monoclonal antibody

The murine mAb 4E6 was sequenced by GenScript and the variable domains of the heavy and light chains of 4E6 were cloned into the pVRC8400 mammalian expression vector containing the human IgG_1 constant domains. Plasmids containing the heavy and light chains were transiently co-transfected into 293F cells in DMEM media containing 5% PenStrep following the manufacturer's protocol. After 5 days of expression, supernatant was clarified by centrifugation and 0.22-μm filtration. Chimeric 4E6 was then isolated by using a low endotoxin version of Protein A affinity chromatography and further purified with size-exclusion chromatography. We recently reported on the crystal structure of this human chimeric antibody [56].

2.4. Antibody sequencing

Unmodified and human chimeric 4E6 were digested both in-gel and in-solution. For in-gel samples, the heavy and light chains were separated by SDS–PAGE. Gel bands were cut, and proteins were reduced with DTT and alkylated with iodoacetamide, then digested with trypsin gold in ProteaseMAX™ surfactant at 50 °C for one hour. For in-solution samples, antibodies were dialyzed to Tris buffer, pH = 8. Samples were boiled at 100 °C for 5 min, then reduced with DTT and alkylated with iodoacetamide, then digested with trypsin gold at 37 °C for 30 min, 1 h, 2 h, and overnight. Half of the in-gel and in-solution samples were desalted using C8 StageTip columns and half of the samples were desalted using C18 StageTip columns. Eluates were collected, dried, and dissolved in 0.1% formic acid. Samples were analyzed using a Thermo-Fisher Q-Exactive mass spectrometer coupled directly to an EASY-nLC 1000 liquid chromatography system (Thermo-Fisher, RRID: SCR_014993). Buffer A (0.1% formic acid in water) and Buffer B (0.1% formic acid in 100% acetonitrile) were used as mobile phases for gradient separation. Samples were loaded onto an in-house packed C18-reversed phase column (PeproSil-Pur C18-AQ 3 μm resin, 20 cm long, 75 μm inner diameter). The column temperature was set to 50 °C. Peptides were separated with a linear gradient listed as following: 0-50 min 3% -40% B, 50–100 min 40–90% B, 100–110 min 90% B, with a flow rate of 300 nl/min. The mass spectrometer was operated in data dependent mode. Full MS scans were acquired in the Orbitrap mass analyzer over a range of 300–3000 *m/z* with resolution 70,000 (m/z 200). The target value was 3.00E+06, with a maximum fill time of 20 ms. Tandem mass spectra were acquired in the Orbitrap mass analyzer with a resolution of 17,500 at m/z 200. The width of the precursor isolation window was 1.6 Th. The target value was 3.00E+06, with a maximum fill time of 60 ms. The ten most intense peaks with charge state ≥2 were fragmented in the HCD collision cell with normalized collision energy of 27 eV and a dynamic exclusion duration of 6 s was enabled.

Data analysis was performed with MaxQuant software (Version 1.5.2.8, Max Planck Institute of Biochemistry, RRID: SCR_014485). The fragmentation spectra were used to search the UniProt mouse protein database containing the two antibody sequences allowing up to four missed tryptic cleavages. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine and protein N-terminal acetylation were used as variable modifications for database searching. Both peptide and protein identifications were filtered at 1% false discovery rate (FDR).

2.5. Primary neuronal cultures

Neuronal cultures were prepared from the cortex and hippocampus of day 0 JNPL3 pups as described [7,8]. All buffer and media components were purchased from Invitrogen. Briefly, tissue was washed in buffer before incubation with trypsin for 20 min at 37 °C. Tissue was then subjected to further washing before mechanical dissociation. Samples were lightly centrifuged to remove debris and added to wells containing plating media. After 24 h, plating media was replaced by neurobasal media. Cultures were then allowed to recover for 7 days prior to use in experiments.

2.6. Neuroblastoma cultures

Human neuroblastoma SH-SY5Y cells (RRID:CVCL_0019) were obtained from American Type Culture Collection (ATCC). Cells were plated in chamber glasses coated with Pluripro Protein Matrix (Cell Guidance Systems) and incubated in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, GlutaMAX (Invitrogen) and 10,000 units/ml pen/strep. Cells were allowed to recover for 2 days before double differentiation. First, cells were incubated in DMEM containing 1% FBS and 10 μM retinoic acid for 5 days. Then cells were washed with fresh DMEM and incubated with 50 ng/ml brain derived neurotrophic growth factor (BDNF).

2.7. Paired helical filament (PHF) isolation

Human AD brain was used as the source of the enriched PHF used in all experiments. Tau was isolated using procedures described previously, with some modifications [8,57]. Tissue was homogenized in buffer (pH 6.5; 0.75 M NaCl, 1 mM EGTA, 0.5 mM $MgSO_4$, and 100 mM 2-(*N*-morpholino) ethanesulfonic acid) and centrifuged at 11,000 x g for 20 min. The resulting supernatant was retained and further centrifuged at 100,000 × g for 60 min, and the pellet resuspended in extraction buffer (10 mM Tris; 10% sucrose; 0.85 M NaCl; and 1 mM EGTA, pH 7.4). This fraction was centrifuged an additional time at 15,000 × g for 20 min, with the supernatant retained and incubated with 1% sarkosyl. Following sarkosyl incubation, the samples were

centrifuged at 100,000 x g for 60 min, to yield the sarkosyl soluble supernatant and insoluble pellet. The pellet was then aliquoted into two fractions. The first was not subjected to any additional treatment and is herein referred to as the sarkosyl insoluble fraction. The second was resuspended in 50 mM Tris-HCl buffer, briefly heated to 37 °C and dialyzed in PBS overnight for use in cell cultures. This procedure removes potentially toxic detergent residue and promotes PHF solubility. In the experiments described below, the PHF is soluble at the doses used (1 and 10 μg/ml). Data from previous work suggests that PHF can be soluble at concentrations up to 100 μg/ml [57]. Additionally, we have previously reported that high speed centrifugation of this fraction does not produce a pellet, confirming that this PHF fraction does indeed remain soluble [8].

2.8. PHF and antibody experiments

After allowing cultures to recover, the cultures were utilized for several different experiments. First, cells were treated with antibody alone (4E6, 1B9, 2C11, Tau-5) for 7 days to assess uptake and whether antibodies induced toxicity or promoted tau clearance. Antibodies (4E6, 1B9, 2C11, Tau-5, cat4E6, h4E6) were then utilized in combination with PHF to determine their efficacy. For the PHF and antibody experiments, cells were treated as previously described [8]. Briefly, two different dosing paradigms were used, PHF + Ab and PHF → Ab. In the first, 10 μg/ml of the human-derived PHF and 1 μg/ml of antibody were added to the cultures together. In the second, 10 μg/ml of PHF was added, followed 24 h later by 1 μg/ml of antibody. For each set of conditions, control cells prepared from the same animal were collected prior to treatment (day 0). As a further control against any naturally occurring changes in protein levels which may occur, a group of cells was left untreated for 7 days and then compared to their own day 0 samples. At collection, cells were washed with PBS and collected in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethylsulfonyl flouride (PMSF), 1mM NaF, 1mM Na_3VO_4, 1 μg/ml complete protease inhibitor cocktail (Roche Applied Science).

Additional cells were grown on glass coverslips. Cultures were incubated with 1 μg/ml of either 4E6, 1B9, 2C11 or Tau-5 for 24 h prior to fixing and staining.

2.9. LDH assays

Media was also collected from each of the conditions and time points and retained to examine cell viability. Media was added to 96 well plates and processed using an LDH kit per manufacturer's instructions. Plates were developed using a BioTek Synergy 2 plate reader.

2.10. Immunoblotting

Western blotting was carried out as described [7]. Cells were lysed in 250 μl of RIPA buffer, assayed for total protein concentration, and normalized accordingly. Untreated control samples prepared from the same animal were run on the same gel for each of the experimental conditions to serve as an internal control. All samples were then added to O+ loading buffer (62.5 mM Tris-HCl (pH 6.8), 5% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 1mM EDTA, 1mM ethylene glycol bis (2-aminoethyl) tetraacetic acid (EGTA), 1mM PMSF, 1mM NaF, 1mM Na_3VO_4 and 1 μg/ml complete protease inhibitor cocktail (Roche Applied Science)) and boiled for 5 min. Cell lysate was run on a 12% polyacrylamide gel and then transferred to nitrocellulose membranes. For antibody characterization, blots were probed with 1B9 and 2C11 at a 1:1000 dilution in Superblock (Invitrogen) overnight at 4 °C. An HRP-conjugated mouse secondary was used at a 1:5000 dilution for 1 h at room temperature. Blots in PHF and mAb experiments were probed with rabbit antibodies against neuronal marker NeuN, total tau (Dako) and tau phosphorylated at Ser199 (Invitrogen) at a 1:1000 dilution in SuperBlock (Invitrogen) overnight at 4 °C. Each experiment was carried out using cells prepared from the same animal to limit variability. Membranes were incubated in HRP-conjugated rabbit secondary antibody (1:5000) for 1h at room temperature. In all experiments, chemiluminescent signal was visualized using a Fuji LAS-4000 and quantified with Multigauge (RRID:SCR_014299).

When probing for levels of intracellular 4E6, cat4E6, 1B9, 2C11 or Tau-5, blots were run and blocked as described above. Following this, they were incubated overnight in a 1:1000 dilution of HRP-conjugated mouse secondary antibody. Blots were then washed and developed without further addition of antibodies.

2.11. Dot blots

To compare the binding of mouse secondary antibodies to 4E6, 1B9, 2C11 and Tau-5, aliquots were spotted onto a nitrocellulose membrane at 200 mg/ml. The membrane was then blocked in 5% milk in TBS-T and incubated overnight in a 1:5000 dilution of HRP-conjugated mouse secondary antibody (ThermoFisher). Blots were then washed in TBS-T and developed using a Fuji LAS-4000. For the comparison between 4E6 and cat4E6, or 4E6 and h4E6, serial dilutions of the antibody were spotted onto the membrane (1 mg/ml, 0.25 mg/ml, 0.062 mg/ml). Membranes were then blocked and incubated with either HRP-conjugated mouse (ThermoFisher) or human (Abcam) secondary as appropriate. The same secondary antibodies were used for all immunoblot and ELISA experiments. Blots for 4E6 and h4E6 were incubated with different secondary antibodies, but washed and developed together to minimize any differences in handling.

2.12. Immunohistochemistry

Additional neuronal cultures were plated on glass coverslips for uptake experiments. Cells were fixed in periodate-lysine paraformaldehyde (PLP) and stained with a total tau antibody. Two fluorescently labeled secondary antibodies were used, rabbit polyclonal to detect the total tau, and monoclonal mouse to detect 4E6, 1B9, 2C11 and Tau-5. Coverslips were imaged using a Nikon Eclipse Ti confocal microscope at 40x magnification.

For antibody characterization experiments, tissue from an AD brain was stained with 1B9 and 2C11 using methods previously described [58]. Paraffin embedded sections were mounted on gelatin coated glass slides. To remove the paraffin and expose the epitopes, slides were immersed in xylene followed by a series of solutions with decreasing concentrations of ethanol. Slides were then boiled in a citric acid buffer (10 mM citric acid, 0.05% Tween 20) and incubated with 80% formic acid. Slides were then blocked and incubated with primary antibody (1:200) overnight at 4 °C. Following this, slides were washed and incubated with HRP-conjugated mouse secondary antibody (1:1000, ThermoFisher) for one hour at room temperature and an avidin-peroxidase solution for 30 min. Subsequently, slides were washed in a 0.2 M sodium acetate solution and then reacted with a solution of diaminobenzidine tetrahydrochloride (DAB) and nickel ammonium sulfate to visualize antibody staining. Samples were dehydrated using increasing concentrations of ethanol and xylene, then coverslipped using DEPEX mounting medium. Images were collected using Leica DM5000 B microscope.

2.13. Live cell imaging

Primary neuronal cultures were grown in glass bottomed plates and incubated with 1 μg/ml of CypHer 5 labeled unmodified or human chimeric 4E6 for 24 h. The CypHer 5 tag is acid sensitive and fluoresces in acidic compartments such as endosomes and lysosomes. Images were collected using an Axio Observer inverted confocal microscope at 20x magnification.

Neuroblastoma cells were prepared as described above and incubated with 20 μg/ml of CypHer 5 labeled unmodified or human chimeric 4E6 for 3 h. Images were then collected using an API DeltaVision Personal DV microscope.

2.14. Confocal image quantitation

Images from the 4E6, 1B9, 2C11, and Tau-5 uptake experiments were imported into Image J (RRID:SCR_003070) and the color channels split. Then using a colocalization finding macro [14,59,60], the percentage of pixels in each image that have a signal in both channels, total tau and antibody, was calculated.

For live imaging experiments, each picture was imported into Image J. The threshold was adjusted until the fluorescent signal was highlighted, and the percentage of pixel area occupied by the antibody signal was determined for each image. This method was used for both primary neurons and neuroblastoma cells.

2.15. Isoelectric focusing

Aliquots from each of the tau antibodies were added to IEF sample buffer and run on a precast 3–9 IEF gel (BioRad) as per manufacturer's instructions. Following running, the gel was stained overnight in a 20% Comassie blue solution (50% methanol, 50% H_2O) at room temperature. The gel was then destained in a solution of 50% methanol, 40% H_2O and 10% acetic acid until bands were visible.

2.16. ELISA assays

Two different ELISA assay procedures were used in these experiments. In the first, plates were coated overnight in a cold-room with 1 μg per well of the target (either tau isolated from human brain, or tau peptides). For human tau binding assays, protein from the sarkosyl soluble- and sarkosyl insoluble tau fractions as well as soluble PHF was utilized. In other experiments, peptides comprising the c-terminal region containing either phosphorylated Ser396/404, or its non-phosphorylated version were coated onto the plates. The plates were then blocked for 30 min using Superblock (ThermoFisher), followed by addition of serial dilutions of each antibody to the plate, and subsequent incubation for 2 h. Plates were then washed in TBS-T, and secondary antibody (1:5000), either mouse (ThermoFisher) or human (Abcam) was added to the plate for 1 h. Following further washing, plates were developed using TMB Peroxidase (ThermoFisher), stopped using 2M sulfuric acid, and read by a BioTek Synergy 2 plate reader.

For competitive ELISA assays, a single concentration of antibody was chosen, and incubated with increasing concentrations of solubilized PHF for 30 min before adding it to the assay plate. The rest of the procedure was identical to the non-competitive ELISA.

2.17. Statistical analysis

Statistical analysis was carried out in GraphPad 7 (SCR_002798). For all PHF and antibody experiments, treated samples were compared to untreated control cells prepared from the same animal. Two-way ANOVA analysis was used in LDH, NeuN and tau seeding experiments and post-hoc testing carried out using Dunnett's multiple comparisons test. A two-tailed *t*-test was used to determine significance when comparing uptake of normal and cationized, or normal and human chimeric 4E6.

Because of the multiple groups included, statistical analysis was only conducted between untreated and PHF-treated groups at day 7 (#) to verify PHF toxicity/tau seeding, and between day 7 groups treated with PHF alone or PHF and the individual antibodies (*), to verify antibody efficacy in preventing PHF toxicity/tau seeding. Various other group differences exist as can be appreciated in the figures but were not specifically analyzed.

3. Results

3.1. Prevention of PHF-induced toxicity depends on antibody and dosing method

To promote tau pathology in neurons, and more closely model the human condition, cells were incubated with 10 μg/ml of Alzheimer's brain derived enriched paired helical filament (PHF) tau and treated with 1 μg/ml of mouse tau monoclonal antibodies (mAbs) 4E6, 1B9, 2C11, or Tau-5 for 7 days. In all groups, samples were assessed by comparing them to control cells prepared from the same animal and collected prior to the experiment at day 0 (set at 100%). A further set of cells was incubated for 7 days without treatment (shown in white), and compared to their own day 0 controls, to ensure that differences observed were not the result of normal changes that occur in culture. The tau mAb 4E6 served as a positive control. Three different dosing paradigms were used in these studies, PHF alone, PHF and antibody added at the same time (PHF + Ab), and antibody added 24 h after PHF addition (PHF → Ab). Note that in this last condition, extracellular PHF was washed away prior to adding the antibody for the interaction between the two to be primarily intracellular. Toxicity was assessed using LDH assays and immunoblotting for neuronal marker NeuN.

3.2. LDH assay

Media from cultures treated with PHF and antibody was collected after 7 days in culture. (Fig. 1). A two-way ANOVA showed significant antibody, treatment group and interaction effects between the two parameters ($p < .0001$). Addition of PHF alone significantly increased LDH signal (120% above control, $p = .0017$) compared to the day 7 untreated cells. In the PHF + Ab paradigm, 4E6, 2C11 and Tau-5 significantly blocked PHF toxicity (20% above, 87% of, and 14% above control, $p = .006$, $p < 0.0001$, and $p = 0.005$). However, with the PHF → Ab dosing method, only 4E6 prevented PHF toxicity (25% above control, $p = .01$) and Tau-5 enhanced it with LDH levels 293% above that of internal controls and 173% above PHF alone ($p < .0001$ for both).

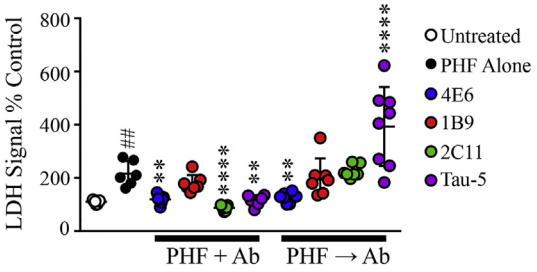

Fig. 1. Prevention of PHF induced toxicity, as measured by LDH levels, depends on antibody and dosing method. JNPL3 primary neurons were exposed to 10 μg/ml of human- derived PHF and 1 μg/ml tau monoclonal antibody in one of two dosing methods. In the first, PHF and antibody were added simultaneously. In the second, PHF was added, followed 24 h later by the antibody. Media was collected from day 0 control cells and those 7 days post treatment and analyzed using a commercially available LDH assay kit. An additional group of cells was left untreated for 7 days and compared to their own day 0 controls (shown in white). Significant antibody, treatment group and interaction effects were seen using a two-way ANOVA ($p < .0001$). Addition of PHF significantly increased LDH levels relative to day 7 untreated control cells (120% above control, $p = .0017$). In the PHF + Ab paradigm, 4E6, 2C11 and Tau-5 significantly blocked PHF toxicity (20% above, 87 of, and 14% above control, $p = .006$, $p < .0001$, and $p = .005$). Using the PHF → Ab dosing method, only 4E6 prevented PHF toxicity (25% above control, $p = .01$) and Tau-5 enhanced it with LDH levels 293% above that of internal controls and 173% above PHF alone ($p < .0001$ for both). All samples are included in the graph with mean ± SD. $^{\#\#}p \le .01$, $^{**}p \le .01$, $^{****}p \le .0001$, $^{\#}$difference compared to untreated cells, *difference compared to PHF alone treated cells.

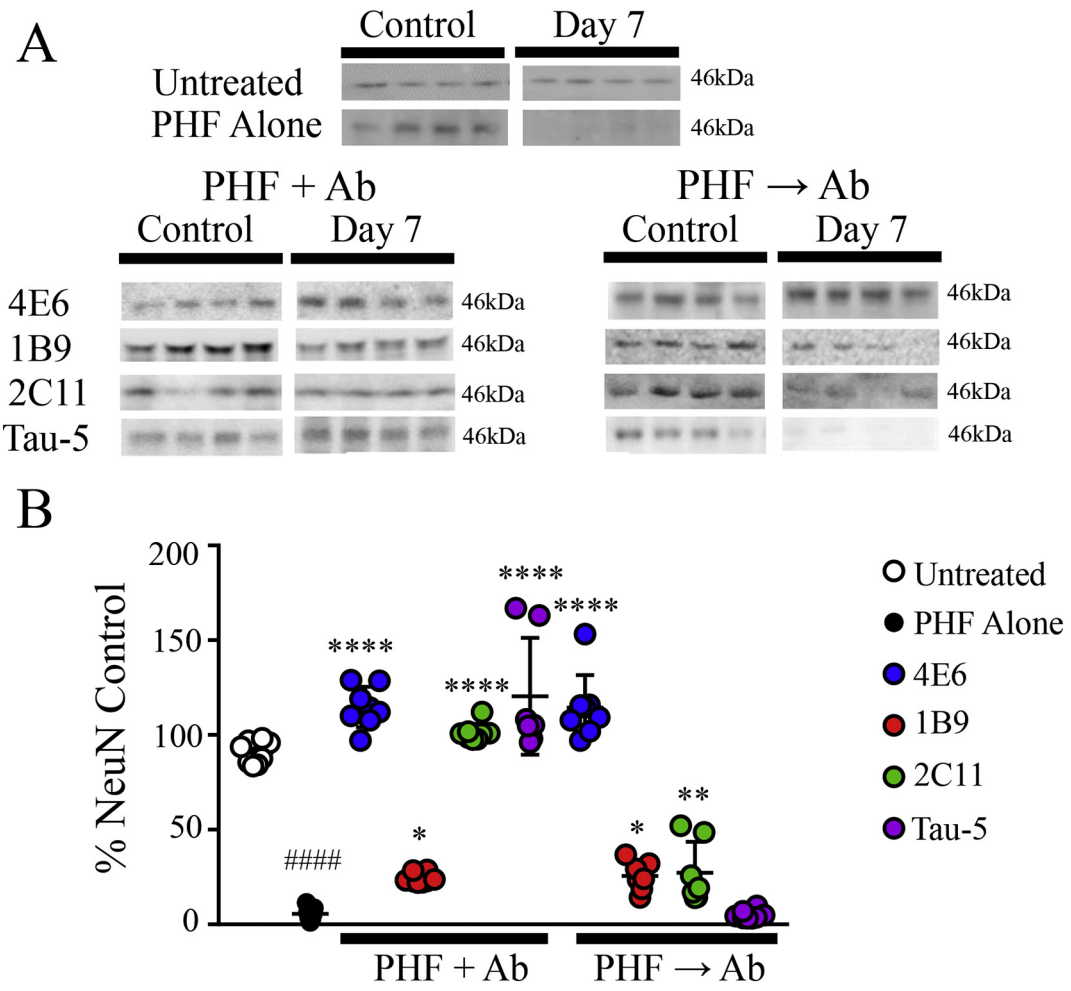

Fig. 2. Prevention of PHF induced toxicity, as measured by NeuN levels, depends on antibody and dosing method. Lysate from the JNPL3 neuronal cultures used in LDH experiments were collected at day 0 and 7 days after the final treatment. An additional group of cells was left untreated for 7 days, then compared to their own day 0 controls, to account for normal changes in NeuN (shown in white). All samples were normalized using control cells prepared from the same animal. A. Blots from day 0 controls, day 7 untreated, as well as PHF and tau mAb treated cells were incubated with a commercially available NeuN antibody (Cell Signaling) and developed to show levels in cells treated with PHF and 1B9, 2C11, and Tau-5. B. A two-way ANOVA revealed significant antibody, treatment group and interaction effects ($p < .0001$). Exposure to PHF induced substantial toxicity (a 95% decrease in NeuN, $p < .0001$). Under PHF + Ab conditions, all four antibodies tested prevented this toxicity (15% above, 24% of, 99% of and 20% above their internal day 1 control (set at 100%) for 4E6, 1B9, 2C11 and Tau-5, $p < .0001$, $p = .01$, $p < .0001$, and $p < .0001$). In the PHF → Ab paradigm, 4E6, 1B9 and 2C11 were able to prevent the PHF induced toxicity (114%, 25%, and 27% of control respectively, $p < .0001$, $p = .01$, and $p = .005$). (Control and day 7 samples were run on the same blot). All data points are included in the graph with mean ± SD. $^{\#\#\#\#}$, $^{****}p \leq .0001$, $^{\#}$difference compared to untreated cells, *difference compared to PHF alone treated cells.

3.3. NeuN immunoblotting

NeuN levels were measured in cell lysate collected at day 0 prior to treatment and at 7 days post-treatment (Fig. 2A), a time point at which PHF toxicity and tau antibody efficacy are clearly evident [8]. Western blots were prepared and probed with a commercially available NeuN antibody (see Supplementary Table 1 for product information). Two-way ANOVA revealed significant antibody, treatment group and interaction effects ($p < .0001$). Exposure to PHF induced substantial toxicity (a 95% decrease in NeuN, $p < .0001$) compared to day 7 untreated cells. We have previously reported that this loss of NeuN also corresponds to changes in the cultures that can be seen with a visual inspection [8]. Cell density decreased and debris was visible in the plate. Remaining neurons showed a reduced and retracted network of processes. This toxicity is also evident by a lack of color change of the media that is associated with neuronal growth. Under PHF + Ab conditions, each of the antibodies tested reduced or prevented this toxicity (15% above, 24% of, 99% of and 20% above their internal day 0 control (set at 100%) for 4E6, 1B9, 2C11 and Tau-5, respectively, $p < .0001$, $p = .01$, $p < .0001$, and $p < .0001$, Fig. 2B). Notably, although 1B9 prevented some of the PHF-induced toxicity, the efficacy was less than that seen with the other mAbs. In the PHF → Ab paradigm, 4E6, 1B9 and 2C11 prevented the PHF-induced toxicity (114%, 25%, and 27% of control values respectively, $p < .0001$, $p = .01$, and $p = .005$), whereas Tau-5 had no effect. Again, 1B9 was less effective than 4E6, and 2C11 showed reduced efficacy compared to that seen in the PHF + Ab paradigm.

Using the two different methods to measure toxicity, a pattern emerges. In both LDH and NeuN assays, 4E6 prevented PHF-induced toxicity in both dosing paradigms. Likewise, 2C11 and Tau-5 blocked toxicity under the PHF + Ab dosing method in both assays. However, their efficacy was impaired or lacking against intracellular tau (PHF → Ab). Using LDH, 2C11 showed no difference compared to PHF alone, and Tau-5 enhanced toxicity. In NeuN immunoblots from the same samples, 2C11 reduced toxicity, but to a much lesser extent than in the PHF + Ab condition. Tau-5's ability to prevent NeuN loss was completely abolished in the PHF → Ab paradigm. With 1B9, similar results are seen in both dosing conditions, namely limited protection against the PHF-induced loss of NeuN signal, and no efficacy by LDH. Overall, while the results of the two measurements (LDH and NeuN) show

similar patterns, differences in degree may be explained. LDH increases with disruption of cell membranes and neuronal death. However, loss of NeuN signal, in addition to neuronal loss, can occur in response to retraction of neuronal processes or stress.

Together, the results of toxicity experiments indicate a likely reason for the differing efficacies. In the PFH + Ab paradigm, both the pathological tau and antibody are extracellular. However, in the PHF → Ab paradigm the tau has been internalized by the neurons before the mAb is added. Both 2C11 and Tau-5 show full efficacy in the PHF + Ab, but reduced or no efficacy in the PHF → Ab condition. This suggests that 2C11 and Tau-5 cannot enter the neurons, and thus are not able to target intracellular PHF. With 1B9, the ability to prevent toxicity in either paradigm is low and may be epitope related.

3.4. Prevention of tau seeding depends on antibody and dosing method

In addition to measuring toxicity, we analyzed the efficacy of antibodies in preventing human-derived PHF tau seeding in neuronal

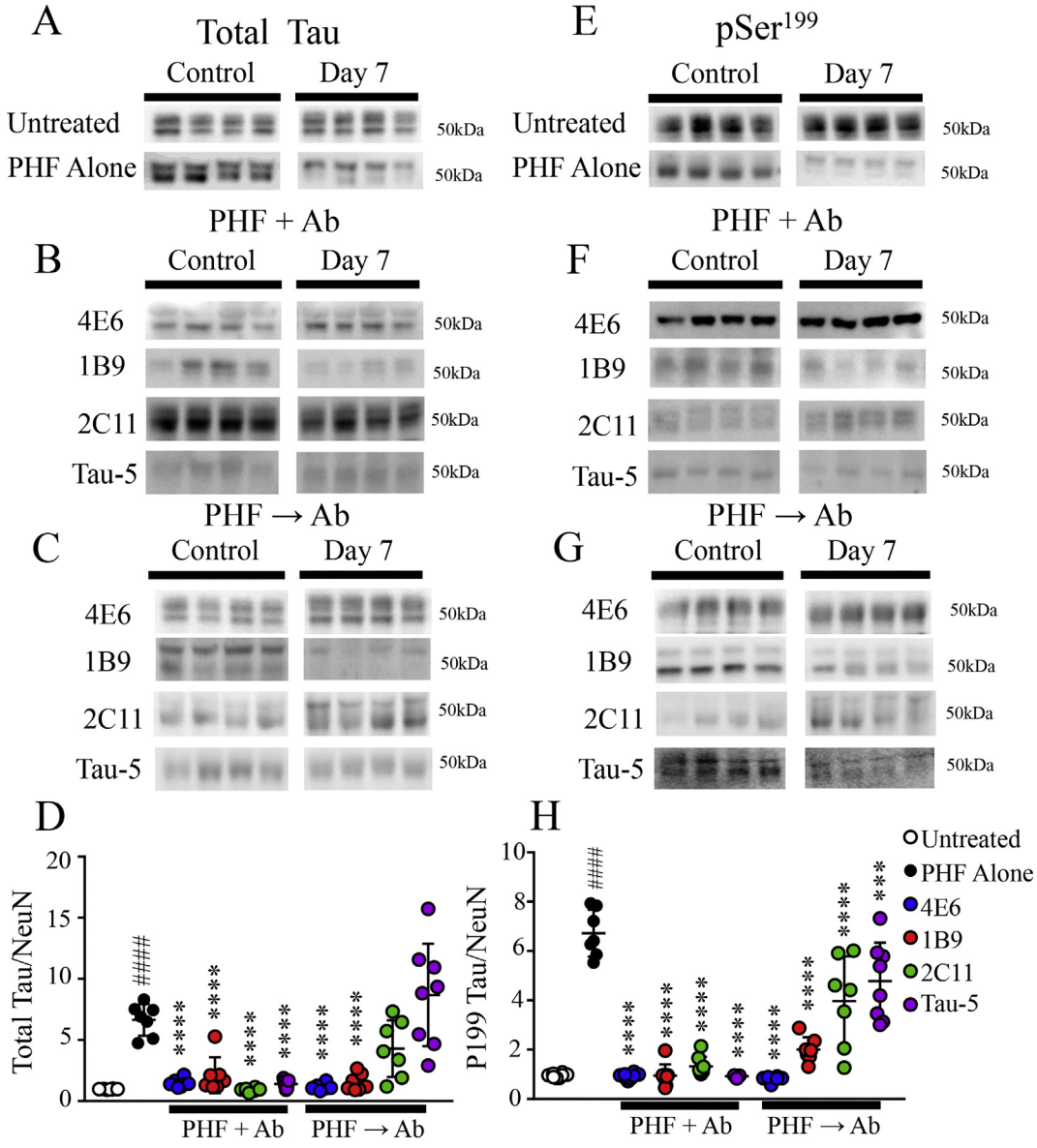

Fig. 3. Dosing method affects antibody efficacy in preventing increased intracellular tau. In addition to NeuN levels, samples from the same JNPL3 primary neuronal cultures were assayed for total and phospho-tau. To account for any loss of cells due to PHF toxicity, total and phospho tau levels were normalized using NeuN. A-C. Immunoblots were incubated with commercially available pan-tau (Dako). Control cells prepared from the same animal were also collected at day 0 prior to treatment. A second group of untreated cells were collected after 7 days and also compared to their day 0 samples to account for normal changes in tau levels (shown in white). Developed blots show total tau levels in cells treated with PHF and either 4E6, 1B9, 2C11 or Tau-5 in PHF + Ab and PHF → Ab paradigm. Data from 4E6 experiments is included to serve as a positive control. D. A two-way ANOVA showed significant antibody, treatment group and interaction effects ($p < .0001$ for all). PHF added to cultures led to an increase in tau levels in the remaining cells over 7 days (5.6 fold above control, $p < .0001$) compared to 7 day untreated samples (shown in white). Under the PHF + Ab condition, all four antibodies, 4E6, 1B9, 2C11 and Tau-5, significantly prevented increased tau levels compared to PHF alone (Tau/NeuN ratio 1.4 for 4E6, 2.0 for 1B9, 0.90 for 2C11, and 1.4 for Tau-5, $p < .0001$ for all, with untreated control at 1.0). Under the PHF → Ab paradigm only 4E6, and 1B9 had significantly lower tau levels compared to PHF alone (Tau/NeuN 1.1 and 1.5, $p < .0001$ for both). E-G. Immunoblots showing pSer199 tau levels in day 0 controls, untreated cells, and cells treated with PHF and either 4E6, 1B9, 2C11 or Tau-5 in PHF + Ab and PHF → Ab paradigm. H. A two-way ANOVA of pSer199 revealed significant antibody, treatment group and interaction effects ($p < .0001$). PHF alone increased intracellular phospho-tau (5.7 fold above control, $p < .0001$) compared to untreated cells. In the PHF + Ab condition, all four antibodies lowered pSer199 levels (pSer199/NeuN 0.93 for 4E6, 0.94 for 1B9, 1.3 for 2C11, and 0.91 for Tau-5, $p < .0001$ for all, with untreated control at 1.0). In the PHF → Ab paradigm, all of the antibodies also had significantly lower phospho-tau levels compared to PHF alone (pSer199/NeuN 0.8 4E6, 2.0 1B9, 3.9 2C11 and 4.7 Tau-5, $p < .0001$, $p < .0001$, $p < .0001$, and $p = .002$) (Control and day 7 samples were run on the same blot) All data points are included in the graph with mean ± SD. $^{***}p \le .001$, $^{\#\#\#\#}$, $^{****}p \le .0001$, $^{\#}$difference compared to untreated cells, *difference compared to PHF alone treated cells.

cultures. Preliminary experiments conducted in JNPL3 neurons in the absence of exogenous PHF showed that antibodies have varying efficacy in clearing endogenous tau, with Tau-5 producing a transient reduction in total tau, and 4E6 a more lasting one (Supplemental Fig. 3). Media containing PHF is removed after 24 h, but we have observed that tau levels continue to increase over the course of the experiment, suggesting seeding of tau pathology [8]. However, because of the significant toxicity associated with PHF exposure, changes in intracellular tau levels may be masked. In some cases, tau levels may seem stable or to decrease, and therefore must be normalized to show the effects more clearly. In these experiments we utilized NeuN levels to correct for any loss of cells/processes caused by PHF toxicity. Immunoblots were probed with commercially available rabbit polyclonal pan tau and pSer199 antibodies (See Supplemental Table 1 for product information). As with NeuN, all samples were compared to control cells prepared from the same animal, which were not exposed to either antibody or PHF. Additional cells were incubated for 7 days in culture and left untreated to account for any normal changes in tau levels.

Two-way ANOVA showed significant effects of antibody and treatment group on total tau levels, as well as their interaction ($p < .0001$ for all). PHF added to cultures led to an increase in tau levels in the remaining cells over 7 days (5.6 fold above control, $p < .0001$, Fig. 3A, D). Under the PHF + Ab condition, all four antibodies, 4E6, 1B9, 2C11 and Tau-5, significantly lowered intracellular tau levels compared to PHF alone after 7 days in culture (Tau/NeuN ratio 1.4 for 4E6, 2.0 for 1B9, 0.90 for 2C11, and 1.4 for Tau-5, $p < .0001$ for all, with untreated control at 1.0; Fig. 3B, D). Under the PHF → Ab paradigm only 4E6, and 1B9 significantly lowered tau levels compared to PHF alone (Tau/NeuN 1.1 and 1.5, $p < .0001$ for both, Fig. 3C, D).

Western blots probing for phospho-tau levels were also carried out and gave similar results as for total tau (Fig. 3E-G). A two-way ANOVA revealed significant antibody, treatment group and interaction effects ($p < .0001$). PHF alone increased the levels of intracellular phospho-tau (5.7 fold above control, $p < .0001$, Fig. 3E, H). In the PHF + Ab condition, all four antibodies lowered pSer199 levels (pSer199/NeuN 0.93 for 4E6, 0.94 for 1B9, 1.3 for 2C11, and 0.91 for Tau-5, $p < .0001$ for all, with untreated control at 1.0; Fig. 3F, H). In the PHF → Ab paradigm, all of the antibodies also significantly lowered phospho-tau levels compared to PHF alone (pSer199/NeuN 0.8 4E6, 2.0 1B9, 3.9 2C11 and 4.7 Tau-5, $p < .0001$, $p < .0001$, $p < .0001$, and $p = .0002$; Fig. 3G, H). Again, both 2C11 and Tau-5 show diminished efficacy in the PHF → Ab condition compared to the PHF + Ab paradigm. Together with the results of toxicity assays, these data further suggest that these two antibodies may have difficulties entering the neurons to block PHF toxicity and to neutralize its tau seeding.

3.5. Monoclonal antibodies vary in their tau binding

In previous experiments [8], antibody binding to soluble pathological PHF tau rather than sarkosyl insoluble tau, was a predictor of efficacy. To determine if this also applied to these antibodies, we characterized their binding to three different tau species, sarkosyl soluble tau, soluble PHF, and sarkosyl insoluble tau. These fractions were prepared from brain homogenate obtained from the same AD patient and produced during the PHF extraction process. ELISA plates were coated with each of the different fractions and then incubated with serial dilutions of each antibody. 1B9 had significantly higher binding to sarkosyl soluble tau compared to 4E6, 2C11 and Tau-5 at the 1/200

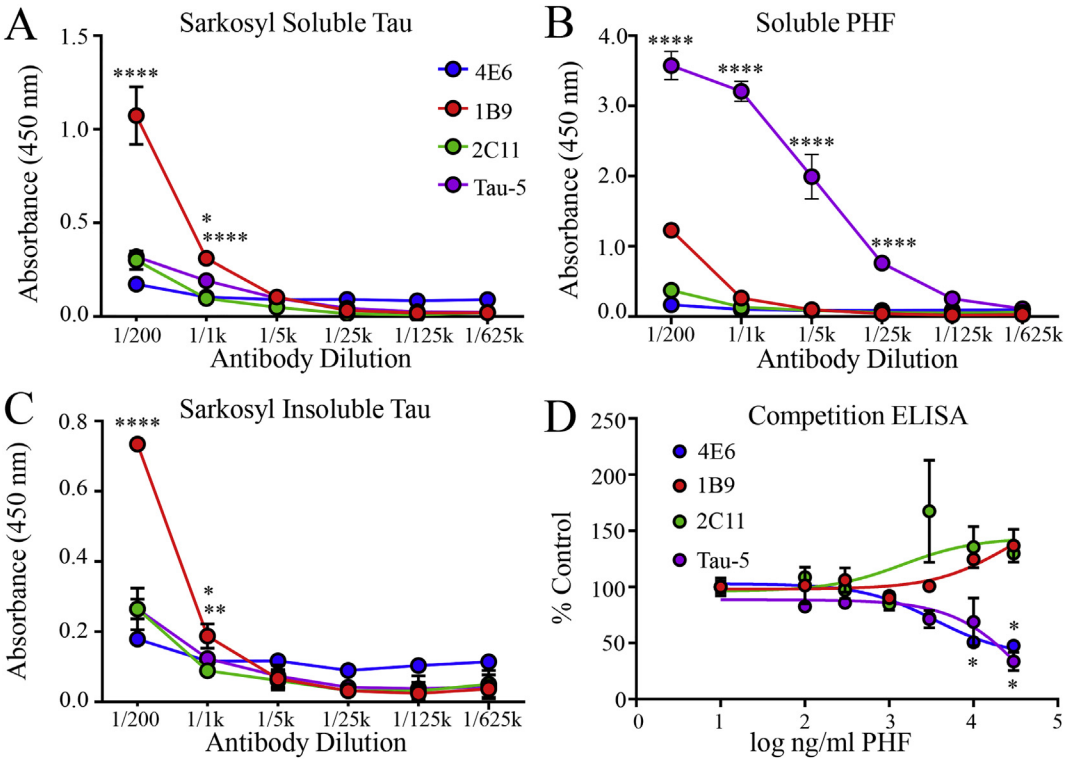

Fig. 4. Monoclonal antibodies vary in their tau binding. Assay plates were coated with one of three different tau fractions prepared from the same AD patient, sarkosyl soluble tau, soluble PHF tau and sarkosyl insoluble tau. A. When plates were coated with sarkosyl soluble tau 1B9 had significantly higher binding than all other antibodies at the 1/200 ($p < .0001$ for all) and 1/1000 dilutions ($p < .0001$ for 4E6 and 2C11 and $p = 0.012$ for Tau-5). B. Tau-5 had significantly higher binding to soluble PHF compared to 1B9 and 2C11 from 1/200–1/25 k ($p < .0001$ for all). C. On plates coated with sarkosyl insoluble tau, 1B9 again had significantly higher binding than all other antibodies at the 1/200 dilution ($p < .0001$ for all), and was significantly higher than 4E6 and 2C11 at 1/1000 ($p = .003$, 0.04). D. A competitive ELISA was performed by incubating each antibody with increasing concentrations of soluble PHF prior to addition to the plate. A two-way ANOVA revealed significant antibody and interaction effects ($p < .0001$ and $p = .002$). Of the four, only 4E6 and Tau-5 showed a significant decrease in binding. 4E6 had significantly reduced binding to the plate at the two highest PHF concentrations (50 and 47% of control, $p = .03$ and $p = .02$) and Tau-5 at the highest concentration (34% of control value, $p \leq .03$) indicating that these two may preferentially bind soluble PHF species. All points and columns are mean ± SD. $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$, $^{****}p \leq .0001$.

($p < .0001$ for all) and 1/1000 dilutions ($p < .0001$ for 4E6 and 2C11, $p = .012$ for Tau-5, respectively, Fig. 4A). Tau-5 showed the strongest binding to solubilized PHF with significantly increased signal relative to 4E6, 1B9 and 2C11 from 1/200–1/25 k ($p < .0001$ for all, Fig. 4B). Results from the sarkosyl insoluble tau were similar to those seen with the sarkosyl soluble fraction. 1B9 had significantly higher binding than 4E6, 2C11 and Tau-5 at the 1/200 ($p < .0001$) and 4E6 and 2C11 at the 1/1000 dilution ($p = .003$ and $p = .04$, Fig. 4C).

To further test whether antibodies bind to soluble or insoluble PHF species, we conducted a competition ELISA assay. Solubilized PHF was used to coat the plates, and each antibody was incubated with increasing concentrations of tau from this same fraction prior to addition. 1B9, 2C11 and Tau-5 were used at the 1:1000 dilution. The absorbance with no added PHF was set at 100% (0.213 for 4E6, 0.312 for 1B9, 0.292 for 2C11 and 1.069 for Tau-5), and the change from that value calculated for each PHF concentration. A two-way ANOVA revealed significant antibody and interaction effects ($p < .0001$, $p = .002$). Only 4E6 and Tau-5 showed any decrease in binding with pre-incubation. At the two highest PHF concentrations, 4E6 had significantly lower binding to the plate (50 and 47% of control, $p = .03$ and $p = .02$), with Tau-5 having significantly lower binding at the highest concentration (34% of control, $p = .03$, Fig. 4D).

In previously published experiments using 4E6, we have shown that binding to soluble PHF tau in competitive ELISA assays, rather than the overall affinity for tau, or its various coated fractions, is a better predictor of efficacy [8]. Of the antibodies newly tested herein, only Tau-5 preferentially binds to soluble PHF (Fig. 4D). In contrast, both 1B9 and 2C11 show no differences in their individual binding to any of the

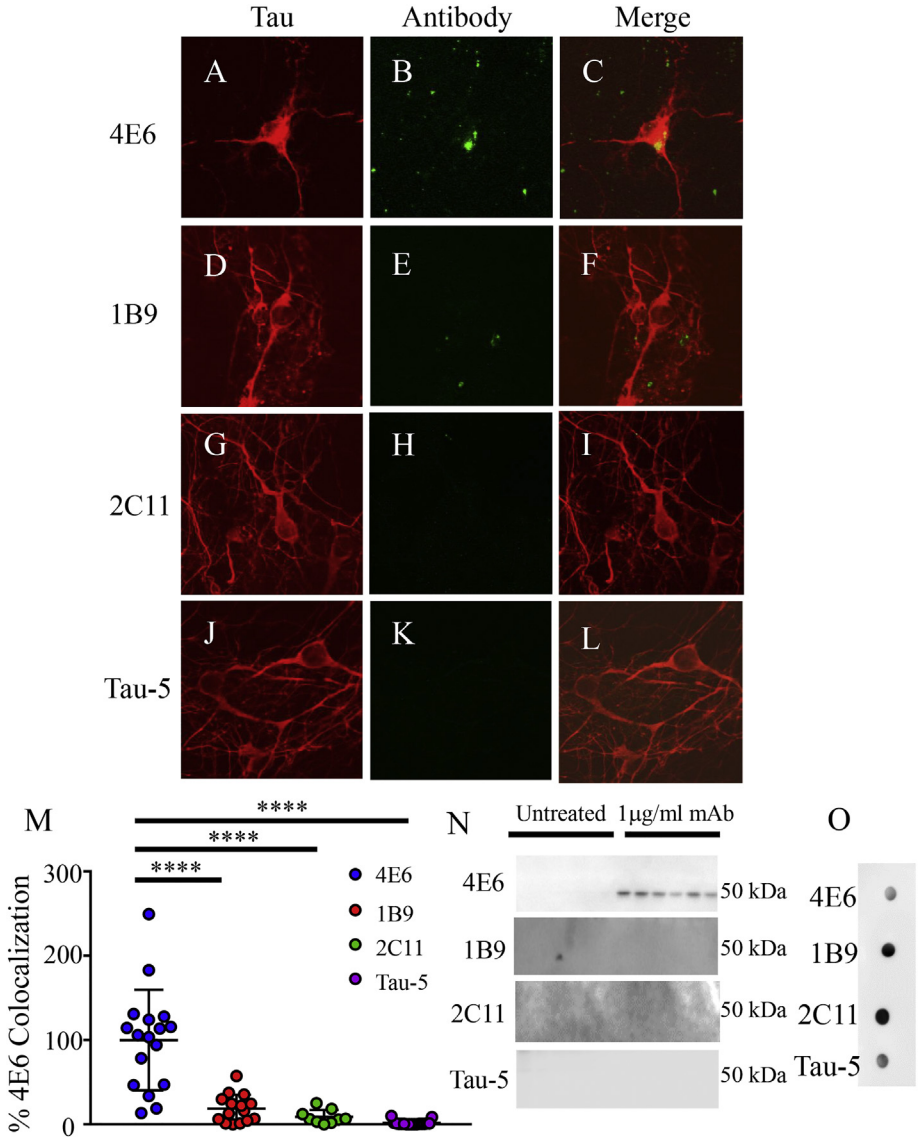

Fig. 5. Antibodies differ in their uptake into neurons. Primary neurons prepared from day 0 JNPL3 pups were incubated with 1 μg/ml tau monoclonal antibodies 4E6, 1B9, 2C11, or Tau-5 for 24 h. Following this period, cells were fixed and stained with a rabbit pan tau antibody (Dako) to visualize the cells. 4E6, 1B9, 2C11 and Tau-5 were detected using a fluorescent anti-mouse secondary. A-C. Confocal images showing uptake of 4E6. The antibody is readily taken up by the neurons. D-L. Confocal images showing uptake of 1B9, 2C11, and Tau-5. Few cells show antibody positive puncta inside the cells. M. Quantitation of antibody uptake for each of the antibodies examined. Multiple images were collected and the percentage of colocalization between the cell stain and the antibody puncta per image were calculated using Image J. Colocalization analysis showed an overall significant difference in antibody uptake (One-way ANOVA, $p < .0001$). All three antibodies showed significantly reduced neuronal uptake with colocalization at 18%, 9% and 2% of the values seen for 4E6 ($p < .0001$ for all, with 4E6 values set at 100%). N. Uptake results were confirmed using immunoblotting. Neurons were incubated for 24 h with 1 μg/ml of 4E6, 1B9, 2C11 or Tau-5. Cell lysate was then collected and run on a western blot. Membranes were probed with mouse secondary antibody to detect tau mAbs. Bands were visible in cells treated with 4E6, but not in blots made from those treated with other tau mAbs. O. A dot blot was then used to compare binding of mouse secondary to each of the antibodies. Samples were diluted to 200 μg/ml and 1 μl of each mAb was spotted onto the membrane, which was then incubated with HRP-conjugated mouse secondary and developed. 4E6 shows the lowest signal, with Tau-5 higher and 1B9 and 2C11 the strongest. All data points are included in the graph with mean ± SD. ****$p \leq .0001$.

fractions tested, or any inhibition in binding when preincubated with soluble PHF. However, 1B9 bound more strongly to all of the coated fractions than 2C11. Hence, 1B9 may be neutralized by its binding to tau fractions that do not convey toxicity, which in turn may explain the efficacy of 2C11 in the PHF + Ab dosing paradigm, since less of it should be neutralized because of its lower affinity for the same tau fractions. These differences may also in part be epitope-dependent.

3.6. Uptake into neurons varies between antibodies

As mentioned above, the differences in antibody efficacy under the two dosing paradigms, suggested that at least two of them, 2C11 and Tau-5, may have difficulties entering the neurons. To test antibody uptake, neuronal cultures were incubated with 1 μg/ml of each of the antibodies for 24 h. Coverslips were stained with a pan-tau antibody (Dako), and an Alexa488-conjugated mouse secondary (Fig. 5A-L). The percentage of pixels per image that were positive for both pan-tau and experimental tau antibodies was then determined. For these experiments, we included tau antibody 4E6 as a positive control. We have previously reported that it is readily taken up by neurons and is efficacious in preventing PHF toxicity and seeding in both dosing conditions [7,8]. Colocalization analysis showed an overall significant difference in antibody uptake when compared to 4E6 (One-way ANOVA, $p < .0001$). All three antibodies showed significantly reduced neuronal uptake with colocalization at 18%, 9% and 2% of the values seen for 4E6 ($p < .0001$ for all, with 4E6 values set at 100%, Fig. 5M).

To further confirm these results, we utilized western blotting to directly detect antibody levels in cell lysate. Neuronal cultures were incubated for 24 h with 1 μg/ml of each antibody. Cells were then collected and their lysate was subjected to western blotting. However, rather than probing with a primary antibody, blots were incubated with mouse secondary antibody alone to detect intracellular IgG. Reduced IgG bands (50 kDa) were visible in cells incubated with 4E6, but not in those treated with any of the other antibodies (Fig. 5N). To verify that these apparent differences in antibody uptake seen in confocal and western blot could not be explained by differences in secondary antibody detection, a dot blot assay was conducted. An equal concentration of each antibody was spotted onto nitrocellulose membrane, then incubated with an HRP-conjugated mouse secondary and developed. The strongest and comparable signal was seen for 1B9 and 2C11, but it was substantially less for Tau-5 and lowest for 4E6. (Fig. 5O). These results show that the higher intracellular signal seen in confocal images from cells incubated with 4E6, and western blotting using cell lysate, are not the result of its having higher binding with the mouse secondary. Because reactivity to the secondary antibody is lower with 4E6, differences in antibody uptake may in fact be underestimated.

3.7. Isoelectric point differs between antibodies

Antibody charge has been shown to influence antibody uptake and retention in cells and may account for the variable intraneuronal levels of the tau mAbs. To assess this, antibodies were run on an isoelectric focusing gel and stained to expose the bands (Fig. 6). 4E6, which readily enters neurons and is effective in preventing pathology [7,8], has an IEP of 6.5. In contrast, Tau-5 is acidic with an IEP near 5.0, while 2C11 and 1B9 are somewhat more basic than 4E6 (close to 7.0 and 8.0 respectively). These results suggest that the lower neuronal uptake of 1B9, 2C11 and Tau-5 compared to 4E6 may be due to charge differences, with a slightly acidic IEP value being optimal and variation in either direction impairing antibody uptake and/or retention. The different isotype of 2C11 (IgG2aκ), compared to the other antibodies (IgG1κ) may also have some influence.

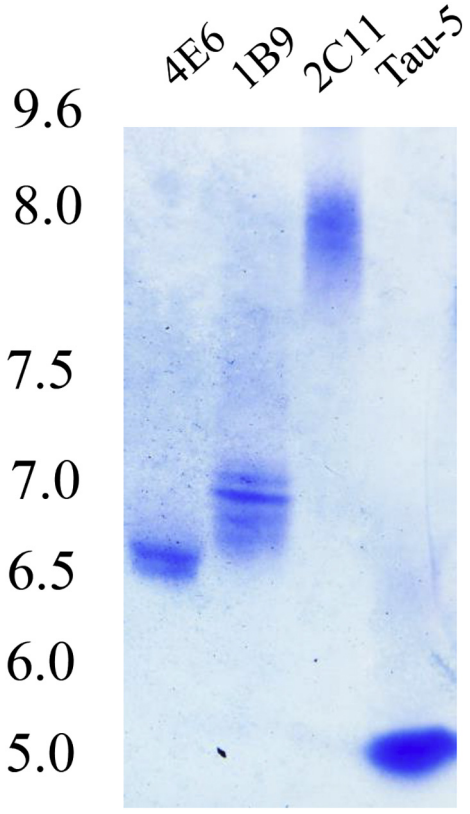

Fig. 6. Tau antibodies have differing isoelectric points. Tau monoclonal antibodies 4E6, 1B9, 2C11 and Tau-5 were run on an isoelectric focusing gel. 4E6 has an IEP of 6.5. 1B9 has a neutral IEP and 2C11 is more basic (7.0 and approximately 8.0 respectively), while Tau-5 is acidic with an IEP near 5.0.

3.8. Increasing the IEP of 4E6 reduces its uptake without altering binding to tau peptides

To determine if altering antibody IEP would change its neuronal uptake, an aliquot of 4E6 was cationized using hexamethylene diamine (HMD), resulting in multiple bands with IEPs ranging from 6.5–7.0 (Fig. 7A). Increasing antibody charge chemically, either with HMD or other compound such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) has been utilized for decades in a variety of model systems and has been shown to improve uptake into tissue and crossing of the blood-brain barrier through association with cell membranes [61–64]. Further this can be achieved without additional changes to antibody structure or promoting aggregation [62]. Cationized F(ab')2 fragments to Aβ showed similar binding to the target at low concentrations, but also increased non-specific binding at higher concentrations, as well as an overall higher non-specific binding to other proteins [63]. In a model of thrombocytopenia, cationization produced significantly increased tissue uptake, but no change in efficacy over unmodified antibody [63].

Subsequently, uptake of both unmodified and cationized 4E6 (cat4E6) was measured using immunoblotting. Primary neuronal cultures were incubated with 10 and 20 μg/ml of 4E6 or cat4E6 for 24 h. Cell lysate was then collected and subjected to western blotting. Blots were incubated with an HRP-conjugated mouse secondary and visible reduced IgG bands were quantified. Cationized 4E6 showed significantly reduced uptake at both 10 and 20 μg/ml (23.6 and 16.3% lower than unmodified 4E6, $p = .04$ and 0.01 respectively, Fig. 7B). To determine that the differences seen via western blotting were not related to altered antibody detection, a dot blot was performed. When probed with mouse secondary antibody, no differences between 4E6 and cat4E6 were seen in reactivity using a dot blot assay (Fig. 7C).

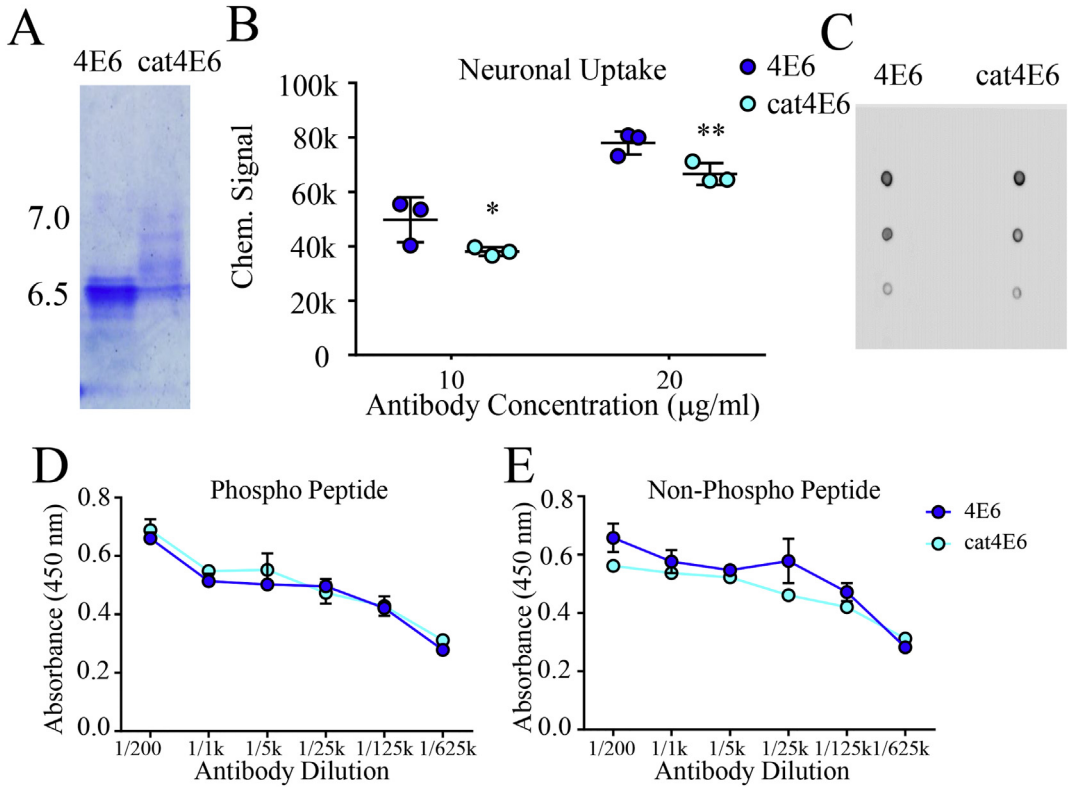

Fig. 7. Increasing IEP reduces antibody uptake without affecting tau peptide binding. Because the tau mAbs used recognize different epitopes, and in the case of 2C11 are of a different IgG isotype, we examined the effects of direct modification of a single antibody. An aliquot of 4E6 was cationized with hexamethylenediamine (HMD) to raise its IEP. A. Cationization resulted in several bands ranging from 6.5–7.0 on an isoelectric focusing gel. B. JNPL3 neurons were incubated with 10 or 20 μg/ml of 4E6 or the cationized 4E6 (cat4E6 in the figure) for 24 h. Intraneuronal antibody levels were detected using Western blotting. At both 10 and 20 μg/ml, a 0.5 change in IEP significantly reduced the uptake of 4E6 into the neurons (23% and 16%, $p = .04$ and $p = .01$ respectively). C. Serial dilutions of 4E6 and cat4E6 (1, 0.25, 0.0625 mg/ml) were spotted onto a nitrocellulose membrane and detected with HRP-conjugated mouse secondary (1:5000). Similar reactivity was seen with both antibodies. D, E. ELISA plates were coated with tau peptides representing the C-terminal either phosphorylated at Ser396/404 or non-phosphorylated. Cationization did not significantly alter antibody binding. All data points are included in panel B with mean ± SD, graphs in D and E show mean ± SD. $^{*}p \le .05$, $^{**}p \le .01$.

Further, cationization did not significantly affect antibody binding to its phospho- or non-phospho peptide epitope. (Fig. 7D, E).

3.9. Cationized 4E6 prevents toxicity and pathological tau seeding

Cationized 4E6 (cat4E6) was assayed for efficacy against PHF-induced toxicity and tau seeding (Supplemental Fig. 4A). As with the other antibodies, primary neuronal cultures prepared from JNPL3 mice were either left untreated for 7 days, exposed to 10 μg/ml PHF alone, or PHF in combination with cat4E6 using the dosing methods described above. Also as above, a set of cells from the same animal served as a control. An additional set of 7 day untreated and PHF alone samples were generated in these tests. Because there were no significant differences between these samples, and the untreated and PHF alone samples used in earlier experiments, the data was pooled for analysis.

PHF-induced toxicity was assessed via immunoblotting for NeuN. A two-way ANOVA revealed a significant effect of treatment group ($p < .0001$, Supplemental Fig. 4B). As in other experiments, PHF alone induced significant toxicity (8.1% of Day 0 control, $p < .0001$). Unmodified 4E6 was effective using both dosing methods ($p < .0001$ for both). cat4E6 also prevented this toxicity in both the PHF + Ab and PHF → Ab paradigms (121 and 84% control values, $p < .0001$ for both.) There were no significant differences seen between groups treated with unmodified 4E6 vs. cat4E6 in either the PHF + Ab or PHF → Ab dosing paradigm.

Intracellular tau levels were also assessed, with a two-way ANOVA indicating a significant effect of treatment group ($p < .0001$, Supplemental Fig. 4C). PHF alone exposure increased total tau levels to 7.8-fold above those in control cells ($p < .0001$). Unmodified 4E6 prevented the PHF-induced increase in both dosing conditions ($p < .0001$ for both). cat4E6 also prevented pathological seeding in both dosing paradigms (Tau/NeuN ratio 1.02, $p < .0001$ for both). Similar results were seen with phospho-tau levels. A two-way ANOVA revealed a significant effect of treatment group ($p < .0001$, Supplemental Fig. 4D). PHF alone significantly increased pSer199 tau (7.8 fold above control, $p < .0001$). As with total tau, unmodified 4E6 prevented this increase under both dosing conditions ($p < .0001$ for both). Similarly, cat4E6 was effective in preventing increased phospho-tau levels in the PHF + Ab and PHF → Ab paradigms (pSer199/NeuN ratio 0.91 and 1.36, $p < .0001$ for both). Post hoc testing from the two-way ANOVAs showed no differences between total and phospho tau results in using 4E6 or cat4E6 in either PHF + Ab or PHF → Ab paradigms. These results show that while cationization significantly reduces uptake, the change in charge and internalization is not sufficient to affect efficacy.

3.10. Human chimerization does not alter the tau binding region of 4E6

Because the ultimate goal of these studies is to develop an antibody that can be administered to patients, we also examined the effects of human chimerization on the IEP, uptake and efficacy of 4E6. The heavy and light chains of the variable domain were retained while the remaining part of the mouse IgG1 was replaced with human IgG1 domain. In order to ensure that this process did not introduce errors into the binding region, we used tandem mass spectrometry to sequence the Fab regions of both the unmodified and human chimeric (h4E6) antibody. Fragments from both antibodies were run on an SDS-PAGE gel and then digested. Peptides were separated and subjected to liquid chromatography tandem-mass spectrometry (Q-Exactive) to obtain

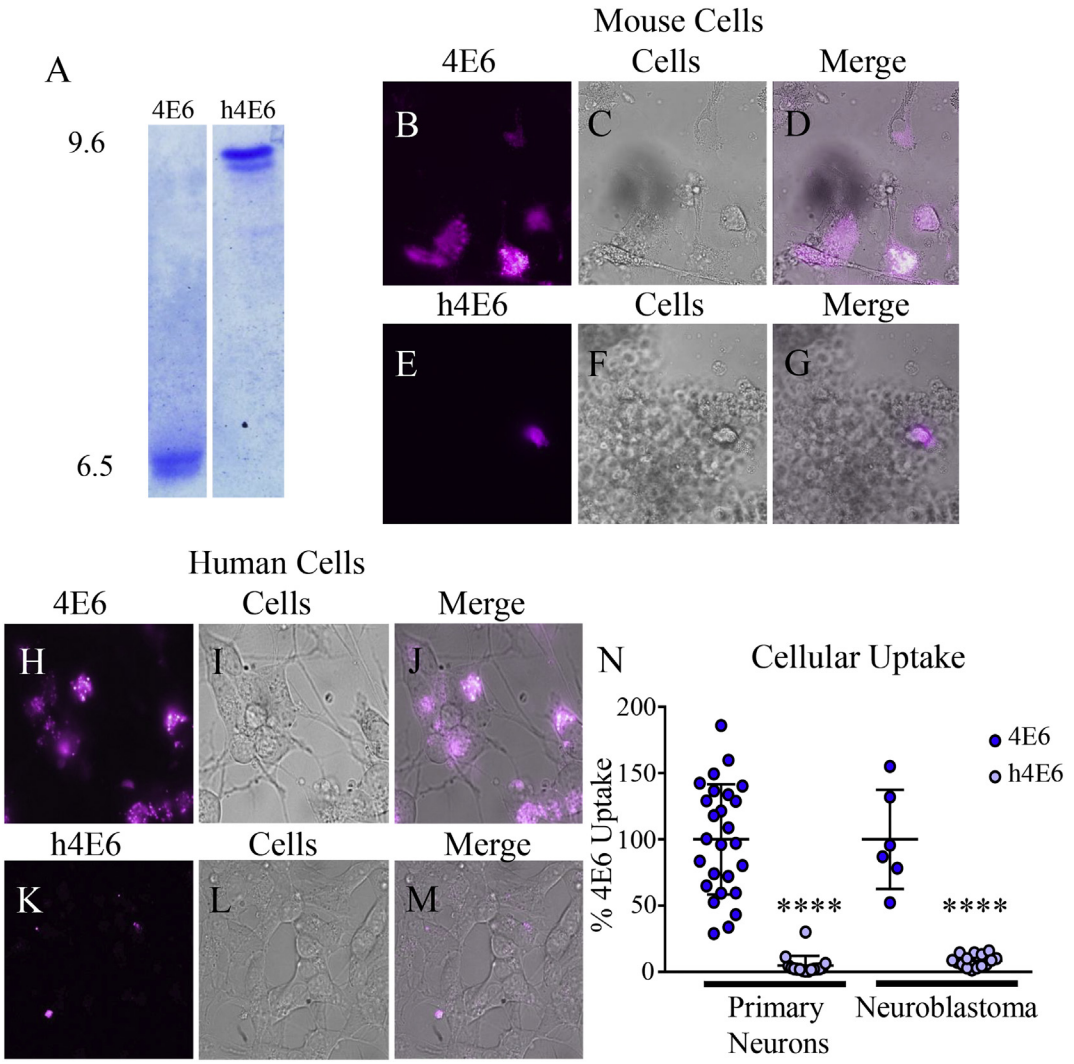

Fig. 8. Human chimerization reduces 4E6 uptake and increases IEP. A. When run on an IEF gel, unmodified 4E6 has an IEP of 6.5, whereas human chimerization increases this to 9.6. Samples were run on the same gel and images cropped to remove unrelated samples. B-D. Primary neurons prepared from JNPL3 mice were incubated with 1 μg/ml CypHer 5 labeled 4E6 for 24 h. In live cell images the antibody is visible as bright puncta which fill the cell bodies, indicating that neurons readily take up the antibody. E-G. However, in JNPL3 neurons incubated with 1 μg/ml CypHer 5 labeled human chimeric 4E6 (h4E6) show substantially reduced uptake via live cell imaging. Few cells contain fluorescent puncta and the overall signal is dimmer. H-J. Human neuroblastoma cells (SH-SY5Y) were incubated with 20 μg/ml CypHer 5 labeled 4E6 for 3 h. Results were similar to those seen in neurons, with high levels of uptake in live cell images. K-M. Uptake was greatly reduced using CypHer 5 labeled h4E6 in the neuroblastoma cells, with relatively few puncta visible. N. Neuronal uptake of the human chimeric antibody was quantified by calculating the percentage of each image containing fluorescent signal using Image J. This signal was significantly reduced in h4E6 treated neurons compared to unmodified 4E6 (95% reduction in mouse primary neurons, 91% in human neuroblastoma ($p < .0001$). All data points are included in the graph with mean ± SD. ****$p \leq .0001$.

the amino acid sequence (See Supplemental Tables 2–5 for individual peptide sequences, and Supplemental Fig. 5). Using these data as well as DNA sequence information provided by GenScript, we were able to determine that chimerization had not altered the sequence of the tau binding portion of the antibody Fab region.

3.11. Human chimerization increases IEP of 4E6, reduces its uptake, and alters tau binding

Since antibody chimerization retains only the antibody binding regions, the IEP of the engineered antibody may be very different from the parent mouse monoclonal antibody. Indeed, substitution of the mouse Fc and parts of its non-binding Fab with its human counterpart changed the IEP from 6.5 to 9.6 (Fig. 8A). To clarify if this dramatic change in antibody charge affected antibody uptake, JNPL3 primary neurons were incubated with either the original mouse 4E6 or human chimeric 4E6 (h4E6) at 1 μg/ml for 24 h (Fig. 8B-G). Antibodies were labeled with a CypHer 5, a pH sensitive dye which fluoresces only in acidic compartments, such as endosomes and lysosomes, where we typically see tau antibodies following neuronal uptake. Hence, the antibody can only emit signal when within the cell. Following incubation, live cell imaging was used to detect the presence of intracellular antibody. This method removes the need for fixing and staining and reduces the cell loss associated with the multiple washings used in immunohistochemistry. In addition, the tag used provides greater certainty that any signal seen is genuine.

To control for possible species differences, a second set of uptake experiments was performed using differentiated SH-SY5Y human neuroblastoma cells. Cultures were incubated with 4E6 and h4E6 at 20 μg/ml for 3 h (Fig. 8H-M) and live cell imaging was conducted as with the mouse neurons.

For both types of cultures, the percentage of pixels in each picture containing antibody signal was determined to quantify antibody uptake. Because the cells are clear and may overlap each other, the boundaries can be difficult to determine. Using the total image therefore reduces a potential source of experimenter bias even though the cell densities may not be identical in each picture. Neuronal uptake of the human chimeric antibody was significantly reduced compared to

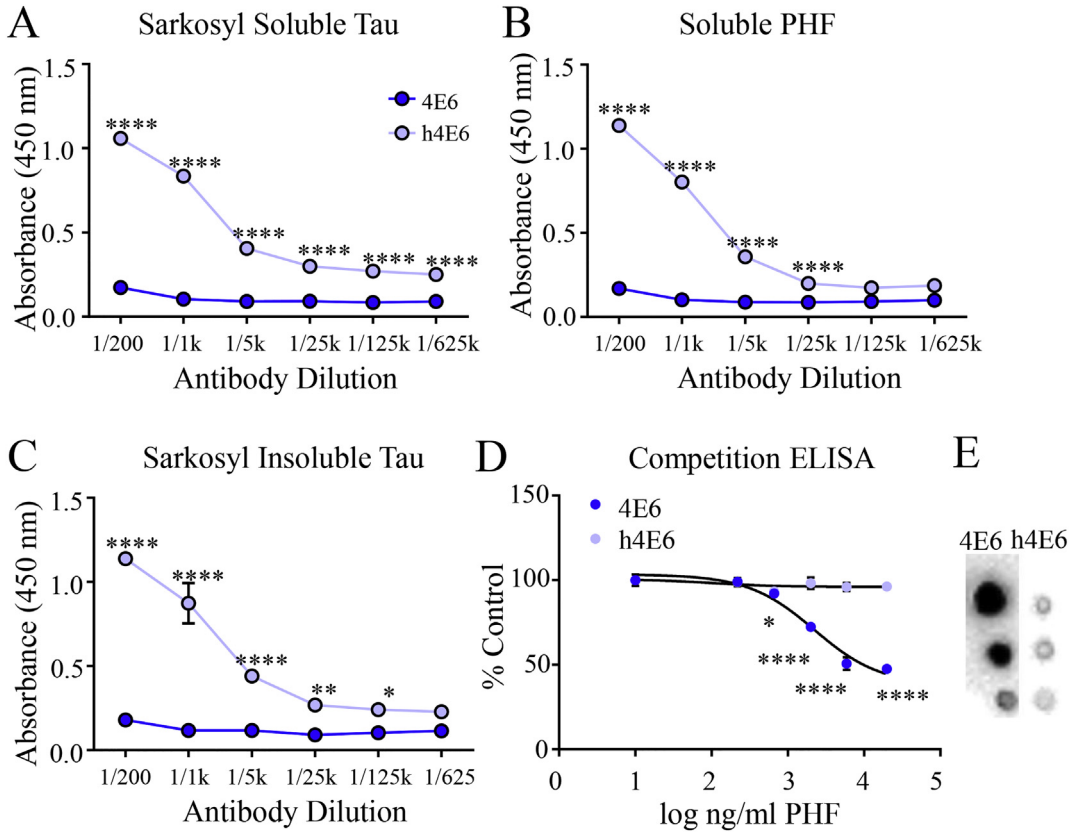

Fig. 9. Human chimerization of 4E6 alters its tau binding. A-C. ELISA plates were coated with sarkosyl soluble, solubilized PHF and sarkosyl insoluble tau. Serial dilutions of unmodified and human chimeric 4E6 were added and binding to each fraction quantified. h4E6 showed similar levels of binding to each fraction, and was significantly higher than mouse 4E6 at every dilution with sarkosyl soluble tau and soluble PHF ($p < .0001$). h4E6 also had significantly higher binding to sarkosyl insoluble tau at every dilution except 1/625 k ($p \leq .0001$-0.02). D. A competitive ELISA was performed to assess binding to soluble PHF. Significant antibody, PHF dose and interaction effects were seen using a two-way ANOVA ($p < .0001$ for all). Reduced binding to the plate was seen at all but the lowest PHF concentration with 4E6 (8–53% reduction, $p \leq .03$–0.0001). In contrast, h4E6 did not show a reduction in binding to the assay plate when pre-incubated with PHF, indicating that it preferentially binds insoluble species. E. Serial dilutions of 4E6 and h4E6 (1, 0.25, 0.062 mg/ml) were spotted onto nitrocellulose membrane and incubated with the same HRP-conjugated mouse and human secondary antibodies used in the ELISA assay at equal concentrations. The h4E6 samples showed lower reactivity, either the result of reduced secondary antibody or ECL developer binding. Thus, the higher signal obtained in ELISA assays with h4E6 is not the result of increased secondary antibody affinity. All points and columns are mean ± SD. $^{*}p \leq .05$, $^{**}p \leq .01$, $^{****}p \leq .0001$.

unmodified 4E6 using *t*-tests (95% reduction in mouse primary neurons, 91% in human neuroblastoma $p < .0001$, Fig. 8N). These similarities between the mouse and human models indicate that the extreme differences in uptake between the mouse and human forms of the antibody cannot be explained by the cellular species.

In addition to changes in uptake, human chimerization also altered tau binding as seen in the two different ELISA assays described above. h4E6 had significantly higher binding to sarkosyl soluble and solubilized PHF fractions coated onto the plate, than unmodified 4E6 at every dilution ($p < .0001$, Fig. 9A, B). With the sarkosyl insoluble fraction, binding of h4E6 was significantly higher at every dilution except 1/625 k ($p \leq .0001$–0.02, Fig. 9C). A competition ELISA was then performed to assess binding to PHF species in solution. A two-way ANOVA showed significant effects of antibody, PHF dose and interaction ($p < .0001$ for all). When incubated with increasing PHF concentrations, 4E6 had an 8–53% reduction in binding ($p \leq .03$–0.0001). Human chimeric 4E6 showed no decrease in binding to the plate coated with solubilized PHF when pre-incubated with increasing concentrations of the same material (Fig. 9D). Because 4E6 and h4E6 require the use of different secondary antibodies for detection, this raises the possibility that variations seen between the two are the result of differences in secondary antibody binding or reactivity to the developing agent. To test this, a dot blot was performed using serial dilutions of 4E6 and h4E6. Two test strips were incubated with equal concentrations of the same commercially available mouse and human secondary antibodies utilized in the ELISA and developed together. In this assay, 4E6 produced a much stronger signal than h4E6, indicating that h4E6 binding to tau is likely even greater than ELISA results indicate (Fig. 9E).

In summary, human chimerization substantially increased antibody binding to various tau fractions coated onto ELISA plates and caused the antibody to lose its binding to soluble pathological PHF tau. Previously, we showed that 4E6 binding to soluble PHF may explain its efficacy in preventing PHF toxicity. Hence, chimerization not only limits antibody access to intracellular tau, which is the largest pool of pathological tau, but also changes its binding characteristics, which likely further diminishes its efficacy.

3.12. Human chimerization impairs antibody efficacy

To directly assess the ability of h4E6 to prevent PHF-induced toxicity, JNPL3 primary cultures were incubated with PHF and h4E6 or 4E6 using the dosing methods described above and assayed by NeuN immunoblotting (Fig. 10A) using a commercially available antibody. As in other experiments, a set of cells prepared from the same animal served as a control. A two-way ANOVA yielded a significant antibody effect ($p < .0001$). As previously reported, mouse 4E6 prevented PHF neuronal toxicity in both the PHF + Ab and PHF → Ab dosing paradigms (NeuN levels at 18 and 25% above control, $p < .0001$, Fig. 10B). However, although h4E6 prevented some toxicity in the PHF + Ab paradigm (36% of control, $p = .0005$) it was ineffective in preventing or reducing PHF toxicity using the PHF → Ab method.

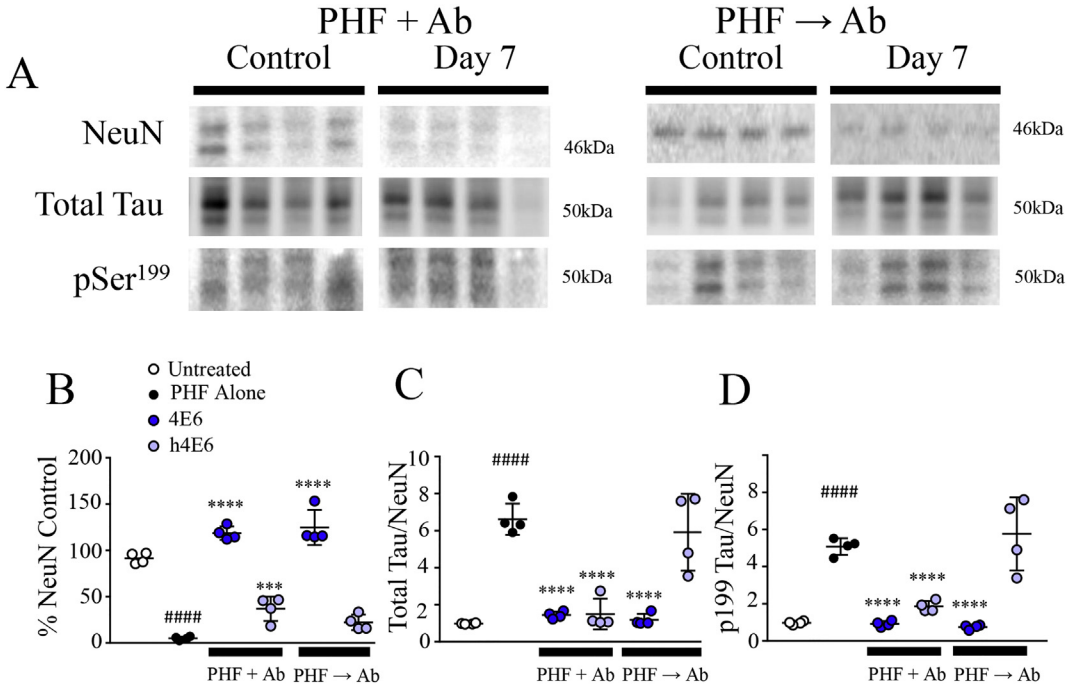

Fig. 10. Chimerization impairs efficacy of 4E6 in preventing PHF induced toxicity and tau seeding. To determine how the changes in IEP resulting from human chimerization affect the efficacy of 4E6, JNPL3 neurons were incubated with 10 μg/ml PHF and 1 μg/ml of antibody in the dosing paradigms described above. A. Samples from day 0 control cells, and those treated with 10 μg/ml PHF and 1 μg/ml h4E6 in the PHF + Ab and PHF → Ab paradigms, were assayed for NeuN, total and phospho-tau. Immunoblots were probed using commercially available antibodies. As above, a set of untreated cells were collected at day 7 and compared to their own day 0 controls (shown in white). B. There was a significant antibody effect by two-way ANOVA ($p < .0001$), when NeuN levels were quantified. Addition of PHF resulted in increased toxicity relative to untreated cells ($p < .0001$). Under both dosing paradigms unmodified 4E6 significantly reduced PHF-induced toxicity ($p < .0001$). Under PHF + Ab dosing conditions, h4E6 was able to prevent some of the PHF toxicity (36% of control, $p = .0005$), but it was ineffective under the PHF → Ab paradigm. C. There were also significant overall antibody, dosing method and interaction effects when corrected total tau levels were quantified using a two-way ANOVA ($p < .0001$, $p = .0003$, and $p < .0001$). As with toxicity data, 4E6 prevented total tau increase in both treatment groups ($p < .0001$). In the PHF + Ab condition, h4E6 prevented the accumulation of tau in the remaining cells (Tau/NeuN ratio 1.49, $p < .0001$). D. A two-way ANOVA using phosphorylated tau levels found an overall significant antibody, treatment group and interaction effects ($p < .0001$, $p = .0016$, and $p < .0001$). Again, 4E6 treated cells had significantly lower phospho-tau levels compared to PHF alone under both conditions ($p < .0001$). Significantly lower corrected phospho-tau levels were recorded using h4E6 in the PHF + Ab paradigm (pSer199/NeuN 1.8, $p < .0001$), but not in the PHF → Ab paradigm. All data points are included in the graph with mean ± SD. ####, ***$p \leq 0.001$, ****$p \leq .0001$, #difference compared to untreated cells, *difference compared to PHF alone treated cells.

Significant antibody, treatment group and interaction effects on tau levels were also observed (two-way ANOVA; $p < .0001$, $p = .0003$, and $p < .0001$). Immunoblots were probed using pan-tau and pSer199 antibodies. Unmodified 4E6 prevented the PHF-induced increase in total tau levels under both dosing conditions ($p < .0001$ for both, Fig. 10C). The h4E6 did retain efficacy in preventing increases in total tau levels when co-administered with PHF (Tau/NeuN ratio 1.49, $p < .0001$). These results are similar to those seen with 1B9 in which an antibody can prevent seeding of tau pathology while having limited or no efficacy with respect to toxicity. However, as in the toxicity assay, h4E6 was ineffective in preventing increased tau levels in the PHF → Ab paradigm (Tau/NeuN 5.9). Similar findings were observed for phospho-tau levels for effects of antibody, treatment group and interaction (two-way ANOVA; $p < .0001$, $p = .0016$, and $p < .0001$). As with total tau, unmodified 4E6 prevented PHF increases in phosphorylated tau levels in the PHF + Ab and PHF → Ab paradigms ($p < .0001$ for both, Fig. 10D). Comparably, h4E6 lowered phospho-tau levels in the remaining neurons in the PHF + Ab paradigm (pSer199/NeuN 1.85, $p < .0001$), but was ineffective under the PHF → Ab condition (pSer199/NeuN 5.7).

In summary, these findings indicate that human chimerization of 4E6 antibody impaired its efficacy, which has important implications for clinical trials. In addition, that h4E6 can prevent seeding of tau pathology with only a limited ability to block tau toxicity cautions against using seeding prevention as the main predictor of therapeutic efficacy of tau antibodies.

4. Discussion

A major finding from our experiments is that antibody efficacy is governed not only by which epitope it recognizes but also by antibody charge, which influences antibody uptake into neurons. Another key finding is that antibody efficacy in preventing toxicity and seeding does not necessarily go hand in hand. A third important finding confirms our previous results that strong affinity for aggregated tau does not correlate with efficacy [8]. All of these outcomes have major implications for ongoing clinical trials on tau immunotherapies, and for the validity of screening approaches to identify clinical candidate drugs. The characteristics of the humanized tau antibodies in clinical trials have not been well described and these findings raise major concerns that their properties may be very different from their mouse counterparts, which can dramatically alter their efficacy. Drug screening assays in culture and in vivo in the amyloid field have in recent years focused more on identifying compounds that prevent seeding of protein aggregation rather than toxicity, which may be misguided. Finally, a promising diagnostic profile for an antibody does not necessarily predict analogous efficacy profile.

As detailed below, there are certain caveats and limitations associated with these findings. These include: 1) the complexity of assessing how efficacy relates to antibody uptake into neurons vs binding to target; 2) these studies are performed in neurons or neuron-like cells, whereas in vivo efficacy of the antibodies may be influenced by other cell types, in particular microglia; 3) antibody charge can directly influence neuronal antibody uptake or indirectly via change in receptor

affinity; 4) human and mouse antibody isotypes are not equivalent in their Fc binding/effector function, and; 5) intraneuronal antibody levels are influenced both by uptake and retention.

Using monoclonal tau antibodies recognizing different epitopes, we analyzed neuronal uptake, tau binding, and efficacy in preventing PHF-induced pathology. 4E6, 1B9, 2C11 and Tau-5 prevented PHF-induced toxicity and seeding when the tau and antibody were extracellular (PHF + Ab). However, the efficacy of 2C11 and Tau-5 was reduced or prevented when the tau was allowed to be internalized prior to antibody administration (PHF → Ab). Interestingly, 1B9 showed a different pattern of results. It was able to prevent tau seeding under both conditions. However, it was unable to prevent toxicity as measured by LDH. Further, its efficacy in preventing NeuN loss in the PHF + Ab paradigm was far less than that seen with 4E6, 2C11 or Tau-5. Of the four antibodies, only 4E6 worked well both extra- and intracellularly.

In previous experiments, efficacy was highly influenced by antibody uptake and binding [8]. For 2C11 and Tau-5, reduced neuronal uptake compared to 4E6 may be sufficient to explain the results seen in efficacy tests. These antibodies readily bind tau extracellularly, but their limited neuronal uptake prevents them from accessing and neutralizing intracellular tau. The varying neuronal uptake of the four tau mAbs may be explained by their different charge, as reflected by their isoelectric points (IEP) ranging from 5.0–8.0. It is well established that antibody charge affects whether and how readily proteins are taken up by neurons, as well as antibody-Fc binding [65–67]. However, the efficacy profile of 1B9 requires further explanation. Under both the PHF + Ab and PHF → Ab conditions, it prevents PHF-induced tau seeding while having a limited effect on the associated toxicity. This may be due to the species of tau that it binds to.

To determine whether differences seen in mAb efficacy preventing PHF-induced toxicity and seeding could be explained by their tau binding profiles, we performed a series of ELISA experiments using sarkosyl soluble tau, soluble pathological PHF, and sarkosyl insoluble tau. 1B9 and 2C11 showed similar binding to each fraction, although with 2C11 the overall level was much lower. Tau-5 bound much more prominently to the coated PHF fraction. More important for efficacy, as we previously reported, is whether the antibodies are capable of binding to the soluble PHF toxic tau species [8]. Only Tau-5 showed reduced binding to the plate in the presence of soluble PHF, indicating that 1B9 and 2C11 have less affinity for soluble toxic tau species. However, 2C11 and Tau-5 showed comparable efficacy, and were more effective than 1B9 in preventing PHF-induced toxicity when acting extracellularly (PHF + Ab). In addition, 1B9 was equally effective as 2C11 and Tau-5 in preventing tau seeding under this condition. For 2C11, its lower overall binding affinity may prevent it from being neutralized by binding to non-toxic aggregates, and thereby it is available in sufficient quantities to block tau toxicity. In contrast, 1B9 may be able to bind to and neutralize species which seed aggregation, but do not induce toxicity in the neurons. Further, we have previously shown that higher binding affinity does not equate to efficacy [8]. Thus, the higher binding seen with 1B9 may reflect the antibody becoming trapped by non-toxic species.

Based on these findings, tau toxicity and seeding do not necessarily go hand in hand, which has important implications for selection of tau antibodies for clinical trials. Furthermore, based on the different properties of these antibodies, how antibodies affect these parameters is likely complex and governed by epitope and affinity for different tau species.

With this in mind, to further clarify the influence of antibody charge on efficacy, the antibodies under study must recognize the same epitope. Hence, we conducted additional experiments directly modifying 4E6. In the first of these tests, mouse monoclonal 4E6 was cationized, resulting in a slight increase in IEP, and concurrent significant decrease in neuronal uptake, but antibody binding and efficacy were not affected. In this case the decrease in uptake while significant, was not severe enough to impact efficacy in this model. More striking, when the human chimeric 4E6 was generated through the substitution of human IgG1 (h4E6), raising the IEP from 6.5 to 9.6, antibody uptake was almost completely prevented in mouse neurons and differentiated human neuroblastoma cells. Because similar results were seen in mouse and human cells, we can conclude that the changes in uptake and efficacy are not the result of using a human IgG in mouse cells.

Chimerization also changed the antibody's affinity to various tau species. Although, h4E6 bound much better to the three coated tau fractions, it did not bind to the more detrimental soluble PHF. Thus, similar to 1B9, h4E6 was able to prevent tau seeding but had limited ability to prevent toxicity. Even in the PHF + Ab paradigm where it showed some efficacy, it was greatly reduced compared to unmodified 4E6. These findings caution against focusing on seeding prevention as a predictor of antibody efficacy. Blocking toxicity is likely a more valuable feature of an antibody that is a candidate for clinical trials.

It can be argued that the efficacy of mouse 4E6 vs. human chimeric h4E6 differs not only because of their different cellular uptake but also because of their different binding to tau. We addressed this issue by examining intracellular (PHF → Ab) vs. extracellular (PHF + Ab) efficacy. In the former approach, h4E6 was completely ineffective because it could not enter the cell. In the latter approach, differences in binding explain efficacy differences.

All the experiments performed suggest that slightly acidic IEP (6.5) is optimal for uptake into neurons under these conditions. Antibodies that were more acidic (Tau-5 at 5.0) or more basic (2C11 at 7.0, 1B9 at 8.0, and h4E6 at 9.6) were taken up to a much lesser extent, which generally correlated with their diminished ability to clear or neutralize intracellular tau. Although 1B9 had some efficacy in preventing increased phospho-tau in the intracellular condition, albeit with limited prevention of toxicity. Quantum dot experiments provide some insight into how molecular charge influences cellular uptake [68,69]. In those studies, neuronal uptake was most prevalent when the dots were negatively charged, shifting to glial cells for positively charged dots. In our cell culture experiments, Neurobasal A media was used with a pH of 7.0. Under these conditions, 1B9, 2C11 and both of the modified 4E6 antibodies would be neutral or positively charged, which may contribute to their decreased neuronal uptake. In contrast, unmodified 4E6 would be negatively charged in culture, suggesting that it should preferentially be trafficked to neurons. Indeed, this is the observed pattern of uptake when brain slice cultures are incubated with 4E6, the majority of the antibody is found in neurons [12], although that may in part be explained by presumed rapid phagocytosis of antibodies in microglia. CSF has a pH of 7.3 indicating that antibodies with a high IEP will be positively charged, thus more likely to be targeted to glial cells. Additionally, antibodies which have a positive charge may have increased non-specific binding to negatively charged surfaces, reducing their availability. Although these experiments may demonstrate that charge affects targeting, they do not fully explain our findings. Tau-5 is also negatively charged under our experimental conditions, but also has significantly lower uptake than 4E6. That may possibly be explained by repulsion to negatively charged cell surfaces because of their high proteoglycan content. It should also be noted that uptake in these quantum dot experiments [68,69] is via bulk-mediated endocytosis, which represents <20% of antibody uptake in our previous experiments with 4E6 [7].

The majority of antibody uptake into neurons occurs via Fc receptors [7]. Affinity for and binding to these receptors is also influenced by antibody charge. Small alterations in antibody charge can greatly affect Fc binding, even in the absence of larger changes in IEP [67]. The presence of a positively charged patch on the cell membrane and slightly elevated IEP has been shown to increase binding to FcRn receptors [67]. Charge variants of single antibodies can also have different Fc binding properties. Hintersteiner et al. (2016) observed that acidic charge variants typically had higher dissociation constants in experiments measuring binding to FcRn and FcγRIIIa [65]. Thus, antibody charges affect whether it binds to cell surface receptors and if it is released into the endosome.

The humanized antibodies in clinical trials for Alzheimer's disease targeting tau (or Aβ) are of the human IgG1 or IgG4 isotype, at least

those with that information readily available (www.alzforum.org/therapeutics), and we used a human IgG1 scaffold to generate the human chimeric h4E6. On the other hand, most of the mouse mAbs that have been tested in AD models are of the mouse IgG1 isotype (including all the tau mAbs examined here except 2C11), presumably because that is the most common isotype recovered from hybridoma screens. It should be noted that human and mouse IgG1 are not equivalent in their Fc binding/effector functions, and to add to the complexity of this issue, the distribution and nomenclature of various Fc receptors differs as well between the species [70]. Future immunotherapy studies in this field should carefully examine how isotype swapping influences efficacy in mouse and human models, and eventually in clinical trials.

It is also important to consider that neuronal antibody levels are influenced both by uptake and retention. Increased clearance may also contribute to the lower intraneuronal levels of the other antibodies compared to 4E6. The literature has conflicting reports on this important issue, which likely reflect the multiple variables in play, and whose degree of influence may not be linear with changes in overall charge. For example, it has been shown that increasing IEPs (from acidic or neutral to basic, or increasing basicity) results in shorter serum half-lives and more rapid tissue uptake than their unmodified counterparts. (reviewed in [71]). However, reducing IEP through antibody modification, can also lead to greater target binding and less non-specific tissue uptake [52,72]. Once the antibody enters the cell, charge affects how it is sorted in the endosome [67]. More basic antibodies may also be more rapidly catabolized [72–74]. Reducing non-specific binding and clearance via bulk endocytosis can also be achieved through lowering antibody IEP [53].

It should also be noted that much of the findings by others discussed herein regarding the effects of antibody charge on uptake and clearance were conducted in cell types other than neurons and glia. This highlights the need for more research using CNS cell types, especially as antibodies for multiple neurodegenerative disorders are in clinical development. With this in mind, our present findings clearly show that antibody charge has a profound effect on the efficacy of tau antibodies, and should be carefully evaluated for all preclinical candidates. Following humanization of an antibody previously tested in cell or animal models, it must be reassessed to ensure that the changes have not impaired functionality. Beside charge differences, higher binding to aggregated tau may not be beneficial as our results indicate. Although such antibodies prevent tau seeding, they do not block tau toxicity. Affinity for soluble PHF pathological tau species may better reflect therapeutic efficacy as supported by our prior and present findings. It is likely that charge modifications will be needed for therapeutic antibody candidates to promote sufficient antibody uptake and efficacy. As more tau antibodies move into clinical trials, ensuring that efficacy is not compromised by humanization will be of utmost importance.

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Declaration of interests

E.M.S. is an inventor on patents on tau immunotherapies and related diagnostics which are assigned to New York University and some of these are licensed to and are being codeveloped with H. Lundbeck A/S.

The authors have no additional financial interests

Author contributions

Conceptualization, E.E.C, E.M.S.; Formal Analysis, E.E.C., J.D.; Investigation, E.E.C, J.E.C., D.B. S., J.D., D. U.; Resources, J.E.C, H.B.R.S, X.P·K.; Data Curation, E.E.C, E.M.S.; Writing-Original Draft, E.E.C.; Writing-Review & Editing, E.E.C., E.M.S., X.P.K. T.N; Visualization, E.E.C, E.M.S.; Supervision, E.E.C, X.P.K, T.N., E.M.S.; Project Administration, E.M.S.; Funding Acquisition, E.E.C, E.M.S.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.03.033.

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