Published in final edited form as:

Neurosci Lett. 2019 January 23; 692: 187–192. doi:10.1016/j.neulet.2018.11.011.

Phosphorylation of Serine 305 in Tau Inhibits Aggregation

Kevin H. Strang^{1,2}, Zachary A. Sorrentino^{1,2}, Cara J. Riffe^{1,2}, Kimberly-Marie M. Gorion^{1,2},
Niran Vijayaraghavan^{1,2}, Todd E. Golde^{1,2,3}, and Benoit I. Giasson^{1,2,3}

¹Department of Neuroscience, University of Florida, Gainesville, FL 32610, USA.

²Center for Translational Research in Neurodegenerative Disease, University of Florida, Gainesville, FL 32610, USA.

³McKnight Brain Institute, University of Florida, Gainesville, FL 32610, USA.

Abstract

Alzheimer's disease and other tauopathies are characterized by the brain accumulation of hyperphosphorylated aggregated tau protein forming pathological inclusions. Although elevated tau phosphorylated at many amino acid residues is a hallmark of pathological tau, some evidence suggest that tau phosphorylation at unique sites, especially within its microtubule-binding domain, might inhibit aggregation. In this study, the effects of phosphorylation of two unique residues within this domain, serine 305 (S305) and serine 320 (S320), were examined in the context of established aggregation and seeding models. It was found that the S305E phosphomimetic significantly inhibited both tau seeding and tau aggregation in this model, while S320E did not. To further explore S305 phosphorylation *in vivo*, a monoclonal antibody (2G2) specific for tau phosphorylated at S305 was generated and characterized. Consistent with inhibition of tau aggregation, phosphorylation of S305 was not detected in pathological tau inclusions in Alzheimer's disease brain tissue. This study indicates that phosphorylation of unique tau residues can be inhibitory to aggregate formation, and has important implications for potential kinase therapies. Additionally, it creates new tools for observing these changes *in vivo*.

Keywords

Aggregation; Antibody; Inhibition; Phosphorylation; Seeding; Tau

1. Introduction

Aggregation of the microtubule (MT) associated protein tau (MAPT) into hyperphosphorylated inclusions is characteristic of a number of phenotypically diverse diseases known as tauopathies. These diseases include Alzheimer's disease (AD),

Address correspondence to Benoit I Giasson (bgiasson@ufl.edu). Phone: 1-352-273-9363.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest

TEG is a co-founder of Lacerta Therapeutics Inc.

corticobasal degeneration, progressive supranuclear palsy, Pick's disease, chronic traumatic encephalopathy, as well as frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17t)[1]. Tau binds to, stabilizes and assembles MTs, among a host of other diverse functions[1,2]. Tau is normally expressed in the CNS as six distinct isoforms due to alternative mRNA splicing with approximately half of the isoforms containing three MT binding repeats (3R) and the other half containing four repeats (4R)[3]. Though natively a soluble and intrinsically disordered protein, tau can aggregate as β -sheet folded, amyloidogenic paired helical filaments that coalesce to form pathological inclusions[1,4,5]. How this protein undergoes this transformation is not fully elucidated, but post-translational modifications, namely phosphorylation, have been implicated[1,6–11]. Additionally, growing evidence suggests that tau can aggregate intracellularly and spread between cells throughout functionally connected areas of the brain by a process known as “seeding,” in which tau monomers can conformationally template onto existing nucleated tau polymers, which can further expand into longer tau fibrils[12,13].

Tau MT binding and stabilization is a process that is negatively regulated by phosphorylation[1,2,10,14]. There are over 80 serine, threonine and tyrosine residues on the longest isoform of tau that are potential sites of phosphorylation, many of which are phosphorylated normally in the human brain[10]. These are modified by many different kinases and several phosphatases, not all of which have been identified[11]. There are several phosphorylation sites that are particularly associated with AD and other tauopathies[15], and hyperphosphorylation of these sites often precedes, and potentially may lead to, aggregation[6,16]. Indeed, hyperphosphorylated tau from AD brain extracts can self-aggregate into fibrils *in vitro*[17], and proline-directed pseudo-phosphorylation of the disease-associated epitopes of AT8 and PHF1 induces tau to form a pathological structure[7]. Conversely, MT affinity-regulating kinase (MARK) and protein kinase A (PKA) are known to phosphorylate sites of tau within the MT repeats, which decreases tau's affinity for microtubules but also can inhibit aggregation *in vitro*[18]. Thus, not all phosphorylation is necessarily associated with hyperphosphorylated, pathological tau, and phosphorylation of certain sites, particularly within the region of tau important for polymerization, could even be protective against aggregation.

In the studies here, using established cellular models of tau aggregation and seeding[19], we focus on potential tau phosphorylation sites, serine 305 (S305) and serine 320 (S320), within the tau MT region that might influence tau aggregation based on their proximity to the PHF6 motif important for amyloid formation[20,21] and proline 301 (P301) that plays a key role in seeded tau aggregation[19]. In addition, the individual FTDP-17 tau mutations S305N[22,23] and S320F[24], which are sufficient to cause disease, abolishes these potential phosphorylation sites that have both been shown to be phosphorylated by several kinases *in vitro*[18,25–27]. We show that phosphorylation of S305, but surprisingly not S320, can significantly inhibit tau aggregation.

2. Materials and methods

2.1. Tau Mammalian Expression Plasmids

The cDNAs encoding full length 0N4R human tau isoforms were cloned in the mammalian expression vector pcDNA3.1(+). QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA) using mutant-specific oligonucleotides was used to create the different missense tau mutations. DNA sequencing confirmed the correct mutations and absence of errors throughout the entire length of the tau cDNA.

2.2. Expression and Purification of Recombinant 2N4R Human Tau and K18 Tau

cDNAs encoding 2N4R human tau or the human tau K18 fragment (residues Q244-E372 in 2N4R human tau) with an added methionine residue at the N-terminus cloned into bacterial expression plasmid pRK172 were used for expression in BL21 (DE3)/RIL Escherichia coli (Agilent Technologies, Santa Clara, CA). This protein was purified as previously described[28], and concentrations were determined using the bicinchoninic acid assay (BCA; Pierce, Waltham, MA) and bovine serum albumin (BSA) as the standard.

2.3. Assembly of Recombinant K18 Tau Fibrils

K18 tau proteins were fibrillized at 1 mg/mL by shaking at 1050 rpm (Eppendorf Thermomixer R) and incubating at 37°C for at least 48 hours while in sterile PBS containing 50 µM heparin[29,30]. Fibril formation was confirmed via K114 fluorometry as previously described[19,31]. To remove heparin, tau fibrils were centrifuged at 100,000 x g and resuspended in sterile PBS, followed by BCA assay to determine concentration. Bath sonication for 60 minutes was used to fragment tau fibrils into shorter tau “seeds” as previously described[32].

2.4. Cell Culture and Transfection

HEK293T and Neuro 2A cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/100 µg/ml streptomycin at 37°C and 5% CO₂. For transfections, cells were plated on 6-well or 12-well polystyrene plates with 2 or 1 mL of media, respectively. Neuro2A cells were transfected with Lipofectamine 2000 reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. For HEK293T cellular transfection, 1.5 µg of plasmid DNA expressing 0N4R tau was diluted into 18.75 µl of 0.5 M CaCl₂ and stepwise added to an equal volume of 50 mM N, N-bis (2-hydroxymethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 6.96, per 1 ml of cell culture media. This mixture was incubated at room temperature for 15–20 minutes before adding dropwise to the media in each well. For seeding studies, K18 fibrils were added to cells 1 hour following transfection at a final concentration of 1 µM. Cells were harvested either as total lysate or as detergent soluble and insoluble fractions 48 hours after transfection. For phosphorylation studies, cells were treated with 500 nM okadaic acid (Cayman Chemical, Ann Arbor, MI) for 2 hours prior to total cell lysate harvest.

2.5. Biochemical Cellular Fractionation and Total Cell Harvest

Cells were harvested in 200 μ L of Triton extraction buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 mM NaF) and a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 mg/ml each of pepstatin, leupeptin, N-tosyl-L-phenylalanyl chloromethyl ketone, N-tosyl-lysine chloromethyl ketone and soybean trypsin inhibitor). Samples were sedimented at 100,000 $\times$ g for 30 minutes at 4°C and the supernatants collected as previously described[19]. To ensure supernatant removal, pellets were washed and sedimented again. Supernatants were removed and the pellets were resuspended in 200 μ L of Triton extraction buffer. 5X SDS sample buffer (final concentration of 10 mM Tris, pH 6.8, 1 mM EDTA, 40 mM DTT, 0.005% Bromophenol Blue, 0.0025% Pyronin Yellow, 1% SDS, 10% sucrose) was added to the collected supernatants and resuspended pellets, referred to as the Triton-soluble and Triton-insoluble fractions. These samples were heated at 100°C for 10 minutes and the Triton-insoluble samples were probe-sonicated. For total cell harvest, samples were collected in 1X SDS sample buffer and boiled for 10 minutes at 100°C.

2.6. Antibodies

Total tau antibodies used in these studies were rabbit polyclonal H150 (Santa Cruz, Dallas, TX) and 3026[33]. H150 was raised against amino acids 1–150 of human tau, while 3026 was raised against full length recombinant 0N3R human tau. 81A11 is a mouse monoclonal antibody specific for the R2 region of tau[34]. Mouse monoclonal 2G2 was generated by immunizing BALB/c mice with phosphopeptide CKHPGGGpSVQIVYKPVDL synthesized and purified by GenScript USA Inc (Piscataway, NJ) and conjugated to inject maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH; Thermo Scientific, Waltham, MA). Mouse immunization injection, spleen harvest, hybridoma fusion and screening, and isotyping were performed as previously described[33]. Hybridoma clones were initially screen by enzyme-linked immunosorbent assay (ELISA)[35] for reactivity for the CKHPGGGpSVQIVYKPVDL phospho-peptide and the lack thereof for the CKHPGGGSVQIVYKPVDL (GenScript USA Inc., Piscataway, NJ) similar but not phosphorylated peptide. All procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and were approved by the University of Florida Institutional Animal Care and Use Committee.

2.7. Human brain tissue and immunohistochemistry analyses

Formalin fixed, paraffin embedded human brain tissue from de-identified AD and control donors was obtained from the University of Florida Neuromedicine Human Brain Tissue Bank. Tissue processing and sectioning as well as immunohistochemical antigen retrieval and staining were performed as previously described[33].

3. Results

3.1. S305E Phosphomimetic Tau Mutation Inhibits Tau Aggregation

We previously showed that FTDP-17 tau mutations at proline 301 (P301L, P301S and P301T) and serine 320 (S320F) can uniquely potentiate tau aggregation in cultured cells[19],

and of note these residues are both found near the PHF6 motif important for β -sheet formation[20,21]. In addition some previous studies suggest that phosphorylation of certain residues near the MT-binding domain, including S320, could be protective against aggregation[18], and the mutation of S320 actually ablates this phosphorylation site. Furthermore, S305 that is located between these two residues has been shown to be phosphorylated by several kinases *in vitro*[18,25–27], and the FTDP-17 mutation S305N[22,23] abolishes this phosphorylation site.

To investigate the possible role of S305 and S320 phosphorylation on seeded aggregation in cultured cells, the S305 and S320 residues, numbered according to the 2N4R tau isoform but within 0N4R tau with the P301L mutation, were individually mutated to a glutamate, which mimics phosphoserine in size and structure[7]. Tau with the P301L mutations was used to promote seeding in cell culture[19]. When compared to P301L for their ability to aggregate when treated with K18 fibrils, it was found that only the S305E and not S320E inhibited P301L tau aggregation with seeding (Fig. 1A). To test the effects of S305E on aggregation independently of exogenous seeding, the S305E phosphomimetic was introduced in the P301L/S320F double mutant, which shows robust aggregation without seeding in culture[19]. The presence of the S305E phosphomimetic significantly reduced aggregation of even this robust self-aggregating tau double mutant (Fig. 1B).

3.2. Monoclonal antibody 2G2 is Specific for Tau Phosphorylated at S305 and Phosphorylated S305 is Not Detected in Pathological Tau Inclusions.

S305 is reportedly phosphorylated by several kinases *in vitro*[18,25–27,36], but to explore if this site could be phosphorylated *in vivo*, we generated the monoclonal antibody 2G2 specific against tau phosphorylated at S305. The specificity of the antibody was initially characterized by ELISA with synthetic peptides and recombinant tau proteins showing that it only reacted with the epitope when phosphorylated at Ser305 (Fig. 2A). To further demonstrate the specificity of the 2G2 antibody, WT, S305N, P301L and S320F tau were expressed in HEK293T cells that were also treated with the cell permeable phosphatase inhibitor okadaic acid[37] to promote phosphorylation (Fig. 2B). Antibody 2G2 only recognized WT and S320F mutant tau in cells treated with okadaic acid, showing that in HEK293 cells, the basal level of Ser305 phosphorylation is low. Antibody 2G2 did not react with S305N tau in cells treated with okadaic acid, showing the specificity of the antibody for tau phosphorylated at Ser305 (Fig. 2B). The presence of the P301L mutation, which is in close proximity to S305, appeared to completely block 2G2 antibody binding (Fig. 2B), although the paucity of the immunoreaction of 2G2 for P301L tau could also be due to an inhibitory effect on S305 phosphorylation. The specificity of antibody 2G2 was further determined with similar studies using Neuro 2A cells (Fig. 2C) Consistent with an inhibitory effect of S305 phosphorylation on tau aggregation, inspection of brain tissues from several AD patients with abundant tau pathology as shown by phospho-tau antibody 7F2[33] revealed that tau pathology is not reactive with antibody 2G2 (Fig. 3).

4. Discussion

The studies presented here aimed to provide further insight on the complex role of phosphorylation in tauopathy. While hyperphosphorylation of tau is associated with and potentially causative of disease[1,7,15,16], specific residues have been found to be phosphorylated within the normal brain but not in AD brains[10]. Additionally, phosphorylation of multiple residues near the MT-binding region, including S320, can potentially inhibit aggregation *in vitro*[18]. To test this in a model of tau seeding and aggregation, mutations at the S305 and S320 sites, both within the MT-binding domain and close to the PHF6 site important for aggregation[21,38], were mutated to a glutamate, whose structure and charge resembles that of a phosphorylated serine. S305E and S320E phosphomimetics were created within the P301L tau mutant, since it has been shown to robustly aggregate with seeding[19,39]. Seed-induced aggregation of P301L tau was compared with P301L/S305E and P301L/S320E tau in our cellular seeding model. While P301L/S320E tau aggregated with seeding at levels similar to P301L tau, P301L/S305E tau aggregation was inhibited. This difference is likely due to the S305 residue's closer proximity to the PHF6 site[20,21], which forms the beginning of the proposed PHF core[40], and where the phosphorylated serine may inhibit β -sheet formation or fibrillar stacking. In addition to inhibiting P301L tau seed-induced aggregation, the S305E substitution also inhibited the robust self-aggregation of the P301L/S320F double mutant. Thus, it is likely that the S305E substitution directly inhibits the propensity of amyloid formation.

Although *in vitro* phosphorylation of S305 by several kinases was previously reported[18,25–27,36], we generated a monoclonal antibody (2G2) to tau phosphorylated at S305 to be able to monitor this modification *in vivo*. We showed using S305N tau (S305 phosphorylation-dead mutant) that the 2G2 antibody is specific for phosphorylated S305 and that, at least in HEK293T cells, phosphorylation of S305 was greatly increased by cellular phosphatase inhibition. Consistent with an inhibitory effect of S305 phosphorylation on tau aggregation, this modification could not be observed in tau inclusion pathology in AD brain tissue.

The finding that the S320E substitution had little impact on aggregation likely indicates that the conformation of these aggregates does not conform to one of the proposed models elucidated in recent cryo-electron microscopy studies of AD PHF tau, in which S320 resides in a hydrophobic pocket within the a key amyloid fold[40] that would be destabilized by S320 phosphorylation. However, phosphorylation of S320 may not be sufficient to impact tau aggregation due to the various different folding structures that amyloidogenic tau can present that can still polymerize into fibrils[40], and S320E would only impact some of these structures. This finding also suggests that the S320F mutation that promotes tau aggregation[19] may have a greater impact on destabilizing the native global hairpin structure of tau[41] or reducing MT assembly[42], thus allowing for tau aggregation, rather than stabilizing tau amyloid structure. However, it is still possible that the S320F mutation can directly promote tau aggregation by promoting one of several tau amyloidogenic folds[40], which initiates and potentiates tau aggregation.

5. Conclusions

We have shown using an established model of tau seeding and aggregation that pseudo-phosphorylation of a specific residue of tau can have inhibitory effects. This phosphomimetic mutant at S305 mitigates tau's ability to aggregate with seeding, which was not seen with a similar mutation at S320. To further explore the importance of this site, we created an antibody specific to phosphorylated S305, and showed that, consistent with this inhibitory effect, this site is not phosphorylated in pathological tau inclusions found in AD. These studies have implications both in potential kinase and phosphatase-based therapies as well as in better identifying post-translationally modified or conformationally distinct populations of tau protein as therapeutic targets.

Acknowledgments

This work was supported by grants from the National Institutes of Health (NS089022 and AG047266) and the Florida Department of Health (7AZ25).

References

- [1]. Guo T, Noble W, Hanger DP, Roles of tau protein in health and disease, *Acta Neuropathol.* 133 (2017) 665–704. doi:10.1007/s00401-017-1707-9. [PubMed: 28386764]
- [2]. Cleveland DW, Hwo SY, Kirschner MW, Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly, *J. Mol. Biol.* 116 (1977) 227–247. doi: 10.1016/0022-2836(77)90214-5. [PubMed: 146092]
- [3]. Liu F, Gong C-X, Tau exon 10 alternative splicing and tauopathies, *Mol. Neurodegener* 3 (2008) 8. doi:10.1186/1750-1326-3-8. [PubMed: 18616804]
- [4]. Wischik CM, Novak M, Thøgersen HC, Edwards PC, Runswick MJ, Jakes R, Walker JE, Milstein C, Roth M, Klug A, Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease, *Proc. Natl. Acad. Sci. U. S. A* 85 (1988) 4506–4510. doi:10.1073/pnas.85.12.4506. [PubMed: 3132715]
- [5]. Mukrasch MD, Bibow S, Korukottu J, Jeganathan S, Biernat J, Griesinger C, Mandelkow E, Zweckstetter M, Structural polymorphism of 441-residue Tau at single residue resolution, *PLoS Biol.* 7 (2009) 0399–0414. doi:10.1371/journal.pbio.1000034.
- [6]. Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI, Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology, *Proc. Natl. Acad. Sci. U. S. A* 83 (1986) 4913–4917. doi:10.1073/pnas.83.13.4913. [PubMed: 3088567]
- [7]. Jeganathan S, Hascher A, Chinnathambi S, Biernat J, Mandelkow EM, Mandelkow E, Proline-directed pseudo-phosphorylation at AT8 and PHF1 epitopes induces a compaction of the paperclip folding of tau and generates a pathological (MC-1) conformation, *J. Biol. Chem* 283 (2008) 32066–32076. doi:10.1074/jbc.M805300200. [PubMed: 18725412]
- [8]. Min SW, Cho SH, Zhou Y, Schroeder S, Haroutunian V, Seeley WW, Huang EJ, Shen Y, Masliah E, Mukherjee C, Meyers D, Cole PA, Ott M, Gan L, Acetylation of tau inhibits its degradation and contributes to tauopathy, *Neuron.* 67 (2010) 953–966. doi:10.1016/j.neuron.2010.08.044. [PubMed: 20869593]
- [9]. Cohen TJ, Guo JL, Hurtado DE, Kwong LK, Mills IP, Trojanowski JQ, Lee VMY, The acetylation of tau inhibits its function and promotes pathological tau aggregation, *Nat. Commun* 2 (2011) 252–259. doi:10.1038/ncomms1255. [PubMed: 21427723]
- [10]. Šimić G, Babić Leko M, Wray S, Harrington C, Delalle I, Jovanov-Milošević N, Bažadona D, Buée L, de Silva R, Di Giovanni G, Wischik C, Hof PR, Tau protein hyperphosphorylation and aggregation in Alzheimer's disease and other tauopathies, and possible neuroprotective strategies, *Biomolecules.* 6 (2016) 2–28. doi:10.3390/biom6010006. [PubMed: 26751489]

- [11]. Hanger DP, Anderton BH, Noble W, Tau phosphorylation: the therapeutic challenge for neurodegenerative disease, *Trends Mol. Med* 15 (2009) 112–119. doi:10.1016/j.molmed.2009.01.003. [PubMed: 19246243]
- [12]. Goedert M, Masuda-Suzukake M, Falcon B, Like prions: The propagation of aggregated tau and α -synuclein in neurodegeneration, *Brain*. 140 (2017) 266–278. doi:10.1093/brain/aww230. [PubMed: 27658420]
- [13]. Ayers JI, Giasson BI, Borchelt DR, Prion-like spreading in tauopathies, *Biol. Psychiatry* 83 (2018) 337–346. doi:10.1016/j.biopsych.2017.04.003. \ [PubMed: 28506438]
- [14]. Drechsel DN, Hyman AA, Cobb MH, Kirschner MW, Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau., *Mol. Biol. Cell* 3 (1992) 1141–1154. doi:10.1091/mbc.3.10.1141. [PubMed: 1421571]
- [15]. Wang Y, Mandelkow E, Tau in physiology and pathology, *Nat. Rev. Neurosci* 17 (2015) 22–35. doi:10.1038/nrn.2015.1. [PubMed: 26656254]
- [16]. Noble W, Hanger DP, Miller CCJ, Lovestone S, The importance of tau phosphorylation for neurodegenerative diseases, *Front. Neurol* 4 (2013) 1–11. doi:10.3389/fneur.2013.00083. [PubMed: 23355832]
- [17]. Alonso AC, Zaidi T, Novak M, Grundke-Iqbal I, Iqbal K, Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments, *Proc. Natl. Acad. Sci* 98 (2001) 6923–6928. doi:10.1073/pnas.121119298. [PubMed: 11381127]
- [18]. Schneider A, Biernat J, Von Bergen M, Mandelkow E, Mandelkow EM, Phosphorylation that detaches tau from microtubules S262 and S214 protects it against aggregation into Alzheimer paired helical filaments, *Biochemistry*. 38 (1999) 3549–3558. doi:10.1021/bi981874p. [PubMed: 10090741]
- [19]. Strang KH, Croft CL, Sorrentino ZA, Chakrabarty P, Golde TE, Giasson BI, Distinct differences in prion-like seeding and aggregation between Tau protein variants provide mechanistic insights into tauopathies, *J. Biol. Chem* 293 (2018) 2408–2421. doi:10.1074/jbc.M117.815357. [PubMed: 29259137]
- [20]. von Bergen M, Friedhoff P, Biernat J, Heberle J, Mandelkow EM, Mandelkow E, Assembly of tau protein into Alzheimer paired helical filaments depends on a local sequence motif ((306)VQIVYK(311)) forming beta structure, *Proc. Natl. Acad. Sci. U. S. A* 97 (2000) 5129–5134. doi:10.1073/pnas.97.10.5129. [PubMed: 10805776]
- [21]. Ganguly P, Do TD, Larini L, Lapointe NE, Sercel AJ, Shade MF, Feinstein SC, Bowers MT, Shea JE, Tau assembly: The dominant role of PHF6 (VQIVYK) in microtubule binding region repeat R3, *J. Phys. Chem. B* 119 (2015) 4582–4593. doi:10.1021/acs.jpcc.5b00175. [PubMed: 25775228]
- [22]. Hasegawa M, Smith MJ, Iijima M, Tabira T, Goedert M, FTDP-17 mutations N279K and S305N in tau produce increased splicing of exon 10, *FEBS Lett.* 443 (1999) 93–96. doi:10.1016/S0014-5793(98)01696-2. [PubMed: 9989582]
- [23]. Iijima M, Tabira T, Poorkaj P, Schellenberg GD, Trojanowski JQ, Lee VMY, Schmidt ML, Takahashi K, Nabika T, Matsumoto T, Yamashita Y, Yoshioka S, Ishino H, A distinct familial presenile dementia with a novel missense mutation in the tau gene, *Neuroreport*. 10 (1999) 497–501. doi:10.1097/00001756-199902250-00010. [PubMed: 10208578]
- [24]. Rosso SM, Van Herpen E, Deelen W, Kamphorst W, Severijnen LA, Willemsen R, Niermeijer RRMF, Dooijes D, Smith MJ, Goedert M, Heutink P, Van Swieten JC, A novel tau mutation, S320F, causes a tauopathy with inclusions similar to those in Pick's disease, *Ann. Neurol* 51 (2002) 373–376. doi:10.1002/ana.10140. [PubMed: 11891833]
- [25]. Virdee K, Yoshida H, Peak-Chew S, Goedert M, Phosphorylation of human microtubule-associated protein tau by protein kinases of the AGC subfamily, *FEBS Lett.* 581 (2007) 2657–2662. doi:10.1016/j.febslet.2007.05.009. [PubMed: 17512525]
- [26]. Inoue M, Konno T, Tainaka K, Nakata E, Yoshida HO, Morii T, Positional effects of phosphorylation on the stability and morphology of tau-related amyloid fibrils, *Biochemistry*. 51 (2012) 1396–1406. doi:10.1021/bi201451z. [PubMed: 22304362]
- [27]. Hanger DP, Byers HL, Wray S, Leung KY, Saxton MJ, Seereeram A, Reynolds CH, Ward MA, Anderton BH, Novel phosphorylation sites in tau from Alzheimer brain support a role for casein

- kinase 1 in disease pathogenesis, *J. Biol. Chem* 282 (2007) 23645–23654. doi:10.1074/jbc.M703269200. [PubMed: 17562708]
- [28]. Giasson BI, Forman MS, Higuchi M, Golbe LI, Graves CL, Kotzbauer PT, Trojanowski JQ, Lee VM, Initiation and synergistic fibrillization of tau and alpha-synuclein, *Science*. 300 (2003) 636–640. doi:10.1126/science.1082324. [PubMed: 12714745]
- [29]. Goedert M, Jakes R, Spillantini MG, Hasegawa M, Smith MJ, Crowther RA, Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans, *Nature*. 383 (1996) 550–553. doi:10.1038/383550a0. [PubMed: 8849730]
- [30]. Hasegawa M, Crowther RA, Jakes R, Goedert M, Alzheimer-like changes in microtubule-associated protein tau induced by sulfated glycosaminoglycans. Inhibition of microtubule binding stimulation of phosphorylation filament assembly depend on the degree of sulfation, *J. Biol. Chem* 272 (1997) 33118–33124. doi:10.1074/jbc.272.52.33118. [PubMed: 9407097]
- [31]. Crystal AS, Giasson BI, Crowe A, Kung MP, Zhuang ZP, Trojanowski JQ, Lee VMY, A comparison of amyloid fibrillogenesis using the novel fluorescent compound K114, *J. Neurochem* 86 (2003) 1359–1368. doi:10.1046/j.1471-4159.2003.01949.x. [PubMed: 12950445]
- [32]. Waxman EA, Giasson BI, Induction of intracellular tau aggregation is promoted by α -synuclein seeds and provides novel insights into the hyperphosphorylation of tau, *J. Neurosci* 31 (2011) 7604–7618. doi:10.1523/JNEUROSCI.0297-11.2011. [PubMed: 21613474]
- [33]. Strang KH, Goodwin MS, Riffe C, Moore BD, Chakrabarty P, Levites Y, Golde TE, Giasson BI, Generation and characterization of new monoclonal antibodies targeting the PHF1 and AT8 epitopes on human tau, *Acta Neuropathol. Commun* 5 (2017) 58. doi:10.1186/s40478-017-0458-0. [PubMed: 28760159]
- [34]. Croft CL, Moore BD, Ran Y, Chakrabarty P, Levites Y, Golde TE, Giasson BI, Novel monoclonal antibodies targeting the microtubule-binding domain of human tau, *PLoS One*. 13 (2018). doi: 10.1371/journal.pone.0195211.
- [35]. Hamm M, Bailey R, Shaw G, Yen SH, Lewis J, Giasson BI, Physiologically relevant factors influence tau phosphorylation by leucine-rich repeat kinase 2, *J. Neurosci. Res* 93 (2015) 1567–1580. doi:10.1002/jnr.23614. [PubMed: 26123245]
- [36]. Schwalbe M, Biernat J, Bibow S, Ozenne V, Jensen MR, Kadavath H, Blackledge M, Mandelkow E, Zweckstetter M, Phosphorylation of human tau protein by microtubule affinity-regulating kinase 2, *Biochemistry*. 52 (2013) 9068–9079. doi:10.1021/bi401266n. [PubMed: 24251416]
- [37]. Cohen P, Klumpp S, Schelling DL, An improved procedure for identifying and quantifying protein phosphatases in mammalian tissues, *FEBS Lett*. 250 (1989) 596–600. doi: 10.1016/0014-5793(89)80803-8. [PubMed: 2546812]
- [38]. von Bergen M, Barghorn S, Biernat J, Mandelkow EM, Mandelkow E, Tau aggregation is driven by a transition from random coil to beta sheet structure, *Biochim. Biophys. Acta* 1739 (2005) 158–166. doi:10.1016/j.bbadis.2004.09.010. [PubMed: 15615635]
- [39]. Guo JL, Lee VMY, Seeding of normal tau by pathological tau conformers drives pathogenesis of Alzheimer-like tangles, *J. Biol. Chem* 286 (2011) 15317–15331. doi:10.1074/jbc.M110.209296. [PubMed: 21372138]
- [40]. Fitzpatrick AWP, Falcon B, He S, Murzin AG, Murshudov G, Garringer HJ, Crowther RA, Ghetti B, Goedert M, Scheres SHW, Cryo-EM structures of tau filaments from Alzheimer's disease, *Nature*. 547 (2017) 185–190. doi:10.1038/nature23002. [PubMed: 28678775]
- [41]. Jeganathan S, Von Bergen M, Brutlach H, Steinhoff HJ, Mandelkow E, Global hairpin folding of tau in solution, *Biochemistry*. 45 (2006) 2283–2293. doi:10.1021/bi0521543. [PubMed: 16475817]
- [42]. Combs B, Gamblin TC, FTDP-17 tau mutations induce distinct effects on aggregation and microtubule interactions, *Biochemistry*. 51 (2012) 8597–8607. doi:10.1021/bi3010818. [PubMed: 23043292]

Highlights

- The S305E tau phosphomimetic inhibits aggregation and seeding in established cell culture models.
- Novel monoclonal antibody 2G2 specific for tau phosphorylated at S305 was established and characterized.
- Consistent with an inhibitory effect of phosphorylated S305 on tau aggregation antibody 2G2 does not react with pathological tau inclusions in Alzheimer's disease brains.

Figure 1. The S305E phosphomimetic inhibits tau seeding and aggregation.

HEK293T cells were transfected with plasmids expressing mutant tau as indicated above each lane and treated with K18 fibril when indicated (+). Cells were biochemically fractionated into Triton-soluble and Triton-insoluble as described in “Materials and Methods” as indicated above each blot. Blots were analyzed by the use of total tau antibody H150. Mobilities of molecular mass markers in kDa are indicated on the right. (A) Comparison of tau aggregation with (+) or without seeding (–) between 0N4R P301L and P301L/S305E tau (top). Comparison of tau aggregation with (+) or without (–) seeding between 0N4R P301L and P301L/S320E tau (bottom). Quantification of Triton-insoluble tau expressed as a percentage of total tau for P301L, P301L/S305E, and P301L/S320E when K18 seeds were added (right). (B) Aggregation without seeding of the P301L/S320F double mutant and P301L/S305E/S320F 0N4R tau (left). Quantification of Triton-insoluble tau expressed as a percentage of total tau (right). Percentage of total tau = [insoluble/(soluble + insoluble)*100], N = 4 for A. N = 3 for B. Statistical analysis by Student’s t-test. **** = p < 0.0001. ns = no significance.

Figure 2. New monoclonal antibody 2G2 is specific for tau phosphorylated at S305.

(A) ELISA analysis of antibody specificity using synthetic peptides with non-phosphorylated or phosphorylated Ser305 and recombinant K18 tau fragment or 2N4R tau. ELISA was performed with antibodies 2G2, 81A11 and 3026 as indicated above each graph. (B) HEK293T cells or Neuro 2A cells (C) were transfected with plasmids expressing 0N4R WT or mutant tau as indicated above each lane, along with a negative control (non-transfected cells). Some cells (+) were treated with okadaic acid (500 nM) for 2 hours before the cells were harvested. Total cell lysate was collected as described in “Materials and Methods,” and equal amounts of proteins were loaded in each lane. Blots were analyzed with total tau antibody 3026 or monoclonal antibody 2G2 raised against phospho-S305 peptide. Mobilities of molecular mass markers in kDa are indicated on the right.

Figure 3. Monoclonal antibody 2G2 does not detect pathological tau inclusions. Immunocytochemistry of representative tau pathology in a human AD brain and a human control brain with the new monoclonal antibody 2G2 and the previously characterized phospho-tau antibody 7F2. Bar = 100 μ m.