Thr\(^{175}\)-phosphorylated tau induces pathologic fibril formation via GSK3\(\beta\)-mediated phosphorylation of Thr\(^{231}\) in vitro

Alexander J. Moszczynski\(^{a,b}\), May Gohar\(^b\), Kathryn Volkening\(^{b,c}\), Cheryl Leystra-Lantz\(^b\), Wendy Strong\(^b\), Michael J. Strong\(^{a,b,c,*}\)

\(^a\) Graduate Program in Neuroscience, Schulich School of Medicine & Dentistry, Western University, London, Ontario, Canada
\(^b\) Robarts Research Institute, Schulich School of Medicine & Dentistry, Western University, London, Ontario, Canada
\(^c\) Department of Clinical Neurological Sciences, Schulich School of Medicine & Dentistry, Western University, London, Ontario, Canada

**A R T I C L E  I N F O**

Article history:
Received 5 October 2014
Received in revised form 11 November 2014
Accepted 3 December 2014

Keywords:
Amyotrophic lateral sclerosis
Microtubule-associated protein tau
Glycogen synthase kinase 3 \(\beta\)
Tauopathy

**A B S T R A C T**

We have previously shown that amyotrophic lateral sclerosis with cognitive impairment can be characterized by pathologic inclusions of microtubule-associated protein tau (tau) phosphorylated at Thr\(^{175}\) (pThr\(^{175}\)) in association with GSK3\(\beta\) activation. We have now examined whether pThr\(^{175}\) induces GSK3\(\beta\) activation and whether this leads to pathologic fibril formation through Thr\(^{231}\) phosphorylation. Seventy-two hours after transfection of Neuro2A cells with pseudophosphorylated green fluorescent protein-tagged 2N4R tau (Thr\(^{175}\)-Asp), phosphorylated kinase glycogen synthase kinase 3 \(\beta\) (active GSK3\(\beta\)) levels were significantly increased as was pathologic fibril formation and cell death. Treatment with each of 4 GSK3\(\beta\) inhibitors or small hairpin RNA knockdown of GSK3\(\beta\) abolished fibril formation and prevented cell death. Inhibition of Thr\(^{231}\) phosphorylation (Thr\(^{231}\)-Ala) prevented pathologic tau fibril formation, regardless of Thr\(^{175}\) state, whereas Thr\(^{231}\)-Asp (pseudophosphorylated at Thr\(^{231}\)) developed pathologic tau fibrils. Ser\(^{205}\) mutations did not affect fibril formation, indicating an unprimed mechanism of Thr\(^{231}\) phosphorylation. These findings suggest a mechanism of tau pathology by which pThr\(^{175}\) induces GSK3\(\beta\) phosphorylation of Thr\(^{231}\) leading to fibril formation, indicating a potential therapeutic avenue for amyotrophic lateral sclerosis with cognitive impairment.

\(\odot\) 2015 Elsevier Inc. All rights reserved.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult onset neurodegenerative disorder of the motor system with a lifetime risk of 1:300 and a survival of 2–5 years after diagnosis (Factor-Litvak et al., 2013). Over 50% of patients with ALS develop a cognitive (ALSci), behavioral or dysexecutive syndrome consistent with that of frontotemporal dysfunction, including a frontotemporal dementia (Ringholz et al., 2005; Strong et al., 2009). The frequent co-existence of ALS and frontotemporal dementia has led to the postulate that both are 2 states along 1 disease continuum (Robberecht and Philips, 2013). Importantly, patients with frontotemporal dysfunction have a reduced survival compared with other ALS cases (Elamin et al., 2011, 2013; Hu et al., 2013; Olney et al., 2005). We have previously shown that ALSci is typically associated with frontotemporal atrophy with superficial linear spongiosis affecting the frontal cortex (Wilson et al., 2001), accompanied by both neuronal and glial inclusions of microtubule-associated protein tau (tau) (Yang and Strong, 2012; Yang et al., 2003). This finding is significantly greater than observed as a function of age (Yang et al., 2005).

Tau is a cytoskeletal-stabilizing protein, which binds to microtubules in the axonal processes, helping to prevent microtubule breakdown and providing structural support by maintaining space between microtubules and other cytoskeletal elements or the cell wall (Chen et al., 1992; Weingarten et al., 1975). In the diseased states known as tauopathies, tau relocates from its normal localization in the axon to the cell body where it forms aggregates (Kowall and Kosik, 1987). In ALSci cases, tau is found in the form of fibrillar inclusions and is phosphorylated at Thr\(^{175}\) (Kosik, 1987). In Alzheimer’s tau inclusions (Strong et al., 2006) and to a much greater extent than that observed in ALS with no cognitive impairment (Yang and Strong, 2012). In solution, tau isolated from ALSci patients has a greater propensity to aggregate, whereas in both HEK293T and Neuro2A cells, tau pseudophosphorylated at Thr\(^{175}\)...
was found to form fibrillar aggregates to a much larger extent than wild-type (WT) tau, regardless of the isoform (Gohar et al., 2009).

In these latter experiments, pathologic tau inclusions in ALS cells colocalize with phosphorylated kinase glycogen synthase kinase 3 beta (pGSK3β-the active isoform of GSK3β) (Yang et al., 2008). Because GSK3β is a proline-directed kinase capable of exhibiting primed and unprimed phosphorylation of tau (Cho and Johnson, 2003) and because GSK3β has been strongly implicated as a major contributor to tau pathology (Cho and Johnson, 2003, 2004a, 2004b; Hernandez et al., 2013; Lucas et al., 2001; Pei et al., 1997; Sahara et al., 2008; Sato et al., 2002), we postulated that GSK3β activation would also be key to pathologic tau fibril formation in ALS cells. We have also postulated that phosphorylation of Thr231 will be key to pathologic tau fibril formation.

Thr231 is a tau phosphorylation site which, when phosphorylated, causes a conformational change in which tau’s ability to bind to microtubules is reduced (Lin et al., 2007). Thr231 is a known substrate of GSK3β (Alonso et al., 2010; Cho and Johnson, 2004b; Sahara et al., 2008; Sengupta et al., 1998). It is neighbored by a proline and also fits the Ser/Thr-XXX-pSer/pThr motif required by GSK3β for primed phosphorylation if the site at Ser235 is phosphorylated first. It is therefore a likely site of synergistic modification to tau in its pathology along with phosphorylation of Thr175.

In this study, we demonstrate that phosphorylation of tau at Thr175 leads to the activation of GSK3β, which then phosphorylates tau at Thr231 and which in turn leads to pathologic fibril formation. Inhibition of GSK3β, pharmacologically and by small hairpin RNA (shRNA) knockdown prevents toxic pathologic tau fibril formation and cell death. Phosphorylation at Thr231 is critical to this process, although independent of Ser235 phosphorylation status.

2. Methods

2.1. Cell culture and transfection

Because we had previously shown that Thr175Asp induces pathologic tau fibril formation in Neuro2A and HEK293T cells, we performed all studies using the 2N4R tau isoform in Neuro2A cells (Gohar et al., 2009). Neuro2A cells were grown on 10-cm plates in Dulbecco Modified Eagle medium (DMEM; Gibco, Burlington, Ontario, Canada) enriched with 10% fetal bovine serum and containing 50 μg/mL penicillin and/or streptomycin (Gibco). Cells were maintained at 37°C and 5% CO2. Transfections for all survival and aggregation studies were performed using Lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada) with appropriate amounts of DNA for the plate size at a 3:1 ratio (μL Lipofectamine: μg DNA). Liposome-DNA complex was added to cells in serum free medium and transfected for 3 hours at 37°C. Because of the increased number of cells required to yield sufficient amounts of protein for lysate analysis, the calcium phosphate method of transfection (Jordan et al., 1996) was used for Western blot studies. Cells were incubated for 18 hours at 37°C and 5% CO2 in the presence of 10 μg DNA and a mixture of CaCl2 and HEPES-buffered serum. Medium was changed to end all transfections. All analyses were conducted 72 hours after transfections were ended.

2.2. Thioflavin S assay for tau aggregation

GST-fusion variants of WT and Thr175Asp 2N4R tau were generated and expressed in Escherichia coli (E. coli) BL21 cells via pGEX vector using the GST SpinTrap purification module (General Electric Healthcare, NJ, USA). E. coli was grown in YTA medium for 3–5 hours at 37°C with vigorous agitation. E. coli was then pelleted by centrifugation, resuspended in phosphate-buffered saline, and then lysed by sonication. Tau was purified from the resulting homogenate as previously described (Gohar et al., 2009), and the ability to form pathologic fibrils assayed by in vitro thioflavin S assay (Friedhoff et al., 1998; Taniguchi et al., 2005). Purified protein sample (100 μL) containing 0.35–10.0 μg/μL was brought to a total volume of 300 μL with a final concentration of 3 μM thioflavin S (Sigma-Aldrich, Oakville, Ontario, Canada) in 20 mM 3-(N-morpholino) propanesulfonic acid (Sigma-Aldrich, Oakville, Ontario, Canada), pH 6.8. Samples were analyzed with or without 5 μM heparin (Sigma-Aldrich, Oakville, Ontario, Canada). The assembly of tau into fibrils is enhanced in the presence of a polyanion, in this case heparin (Pickhardt et al., 2005; von Bergen et al., 2000). Thioflavin S fluorescence was read at 22°C with a SPECTRMax M5 ROM (Fischer Scientific, Pittsburgh, PA, USA) set at 440 nM excitation and 521 nM emission. Background fluorescence and light scattering of a negative control sample containing only thioflavin S was subtracted from the values obtained. All experiments were performed in triplicate.

2.3. Fibril quantification

Cells were transfected with green fluorescent protein (GFP)-tagged mutant tau and visualized live by fluorescence microscopy on a Zeiss LSM 510 Meta multimorph confocal microscope in confocal microscopy dishes at ×63 magnification. Plates were divided into 4 quadrants and a minimum of 25 transfected cells from random fields in each quadrant were counted and categorized into the following: (1) cells containing fibrillar aggregates or (2) cells containing no fibrillar aggregates. Consistent with our previous studies, fibrillar aggregates were defined as discrete, dense, thickened, curvilinear cytosolic structures in contrast to the fine filamentous threads observed in WT-tau transfected cells (Supplementary Fig. 1) (Gohar et al., 2009). The percentage of cells containing aggregates was defined as the number of GFP-tau expressing cells counted containing fibrils. All experiments were performed in triplicate after plates were blinded to the observer (Alexander J. Moszczynski) by a separate party.

2.4. GSK3β inhibitors

Four GSK3β inhibitors that act through different mechanisms were used at their respective IC50 values in fibril and survival experiments. These included lithium chloride (LiCl; Sigma-Aldrich, St. Louis MO, USA), IC50 5 mM, which acts through a Mg2+ competitive mechanism; AR-A014418 (Sigma-Aldrich, St. Louis MO, USA), IC50 104 nM (Bhat et al., 2003), which acts through an ATP competitive mechanism; Tidegusib (Sigma-Aldrich, St. Louis MO, USA), IC50 60 nM (Dominguez et al., 2012), which acts through a non-ATP competitive mechanism; and TWS-119 (BioVision, Milpitas CA, USA), IC50 30 nM (Ding et al., 2003), which acts through a non-ATP competitive mechanism. Toxicity was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay before use in fibril studies to demonstrate that the inhibitors would not be lethal to Neuro2A cells at their IC50. Inhibitors were administered in fresh medium at their IC50 concentration at the end of transfection when medium was changed.

2.5. Site-directed mutagenesis and mutant constructs used

Site-directed mutagenesis was used to create mutant GFP-tagged Tau protein from plasmid constructs in pEGFP-C1 vector as previously described (Gohar et al., 2009). Based on our previous observations, all studies performed here utilized the 2N4R
tau isoform (with a green fluorescent protein [GFP] tag on the N-terminus) as the template for all double mutants. To assess the effect of phosphorylation of Thr^{175}, the following constructs were used wild-type 2N4R tau, a Thr^{175}Ala mutant (cannot be phosphorylated at Thr^{175}), and a Thr^{175}Asp mutant (mimics phosphorylation at Thr^{175}). Agilent technologies QuickChange lightening site-directed mutagenesis kit (Agilent Technologies, Mississauga Ontario, Canada) was used to generate 6 double mutant GFP-tagged tau constructs containing Thr^{231} mutants (1: WT Thr^{175}/Thr^{231}Ala, 2: WT Thr^{175}/Thr^{231}Asp, 3: Thr^{175}Ala/Thr^{231}Ala, 4: Thr^{175}Ala/Thr^{231}Asp, 5: Thr^{175}Asp/Thr^{231}Ala, 6: Thr^{175}Asp/Thr^{231}Asp). Full length primers for Thr^{231}Ala mutants were forward (5'-CGA GTG GTC CGT CCT CCA CCC AAG TCG-3') and reverse (5'-CGA CTG TGG AGG AGC AGC AAC GAC CAC TGC-3'). Full length primers for Thr^{231}Asp mutations were forward (5'-GCA GTG GTC CGT CAT CCA CCC AAG TCG-3') and reverse (5'-CGA CTG TGG AGG AGC AGC AAC GAC CAC TGC-3'). An additional series of 6 GFP-Tau mutants containing Ser^{235} mutations were created to test the requirement of a primed phosphorylation mechanism at Thr^{231}. These mutants were (1: WT Thr^{175}/Ser^{235}Ala, 2: WT Thr^{175}/Ser^{235}Asp, 3: Thr^{175}Ala/Ser^{235}Ala, 4: Thr^{175}Ala/Ser^{235}Asp, 5: Thr^{175}Asp/Ser^{235}Ala, 6: Thr^{175}Asp/Ser^{235}Asp). Full length primers for Ser^{235}Ala mutants were forward (5'-CCC AAG GGC AGG CGA GAG CGG TGG AAG AGG GAG CAC TGC TGG-3') and reverse (5'-CCC AAG GGC AGG CGA GAG CGG TGG AAG AGG GAG CAC TGC TGG-3'). Full length primers for Ser^{235}Asp mutations were forward (5'-CCC AAG GGC AGG CGA GAG CGG TGG AAG AGG GAG CAC TGC TGG-3') and reverse (5'-CCC AAG GGC AGG CGA GAG CGG TGG AAG AGG GAG CAC TGC TGG-3'). All mutants were sequenced to confirm the presence of mutations of interest.

2.6. MTT survival assay

Cells were grown and transfected in 96-well plates. Seventy-two hours after transfection, 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis MO, USA) was added to each well and then incubated for 1 hour at 37 C and 5% CO2. After incubation, cells from 1 well per treatment group were resuspended in 100 μl fresh DMEM and transferred to a 1.5-ml microcentrifuge tube. Ten microliters was then loaded onto a hemacytometer (improved Neubauer, Hausser Scientific, Horsham, PA) and purple (live) and white (dead) cells were counted. Cell death was expressed as dead cells/total cells counted. All experiments were performed in triplicate.

2.7. Trypan blue assay

Cells were grown and transfected in 96-well plates. Seventy-two hours after transfection, cells from 1 well per treatment group were resuspended in 100 μl fresh DMEM and transferred to a 1.5-ml microcentrifuge tube. One hundred microliters of 0.4% trypan blue stain (Gibco) was then added, and cells were incubated at room temperature for 1 minute. Ten microliters was then loaded onto a hemacytometer and white (live) and blue (dead) cells were counted. Cell death was expressed as dead cells/total cells counted. All experiments were performed in triplicate.

2.8. Western blot

Cells were lysed on ice 72 hours after transfection in NP40 lysis buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% NP40, 10% glycerol) containing protease (Complete, Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitors (Phosstop, Roche Diagnostics). Lysate protein content was quantified using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Samples were suspended in sample buffer (100 mM Tris-HCl, 4% SDS, 0.02% bromophenol blue, 20% glycerol, 200 mM DTT) and denatured for 5 minutes in a hot water bath at 95 C. Protein (20 μg) was run on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane. To assess transfection efficiency, gels were probed for GFP using a rabbit anti-GFP antibody (1:5000 titer; Life Technologies, Eugene, OR, USA). GSK3β activation was assessed using a mouse anti-pTyr^{216} GSK3β (1:10,000 titer; BD Biosciences, Mississauga, Ontario, Canada). To fully investigate activation status relative to overall levels of kinase, total GSK3β was examined by stripping blots (2% SDS, 62.5 mM Tris-HCl, 100 mM β-mercaptoethanol, pH 6.8) and reprobing with mouse anti-total GSK3β (1:10,000 titer; BD Biosciences). Nitrocellulose membranes were probed with primary antibody overnight at 4 C. Blots were then washed in Tris-buffered saline with 0.2% Tween (TBS-T) before probing with horseradish peroxidase tagged secondary antibody (Goat anti-Mouse IgG [1:5000 titer; Bio-Rad] or Swine anti-rabbit [1:1000 titer; Dako, Burlington, Ontario, Canada]) for 1 hour at room temperature. Densitometry was conducted using an open source ImageJ software v 1.48 (NIH). GSK3β activation was normalized for the overall expression and transfection efficiency by the equation (pTyr^{216}/GSK3β)/(Total GSK3β)/GFP). All experiments were performed in triplicate.

For shRNA knockdown efficacy studies, total GSK3β was normalized to α-tubulin by densitometry using mouse anti-α-tubulin (1:2500 titer, Abcam, Toronto, Ontario, Canada). GSK3α was assessed using mouse anti-GSK3α (1:1000 titer, Abcam) and normalized to α-tubulin by densitometry.

2.9. Small hairpin RNA

A shRNA specific to GSK3β was designed according to a previously reported sequence (Yu et al., 2003) shown to specifically knockdown GSK3β in Neuro2A cells while leaving GSK3α unaffected (Garrido et al., 2007). The shRNA sequence was modified to have a hairpin sequence specific to the pSuper plasmid vector into which it was inserted via BglII and HindIII restriction digest and ligation with T4 DNA ligase. Sequence primers were as described by Yu et al., 2003: forward (5'-GAT CCG CGA CTT CTG GAA AAG AAG AGA AGG AGC AGG ACC TCC AGA TCT AGT ACT TA-3') and reverse (5'-ACC TAA AAA AGA TCT GCA GCT CTC GGT TCT TTC CGA AGA ACC GAG AGC TCC AGA TCG GG-3').

shRNA plasmid was transfected into Neuro2A cells with lipofectamine 2000 and GSK3β expression analyzed at 24 and 96 hours after transfection to investigate the efficacy and sustainability of GSK3β knockdown.

For knockdown studies, 24 hours before transfection with GFP-tau plasmid, shRNA, or pSuper vector was transfected into cells so that expression of GSK3β would be reduced at the time of tau plasmid transfection. Fibril quantification was then conducted as mentioned previously.

2.10. Nocodazole experiments

Cells were transfected with GFP-tagged wild-type tau or Thr^{175}Asp tau with Lipofectamine as mentioned previously in confocal dishes. Seventy-two hours after transfection cells were exposed to 500 nM nocodazole (Sigma-Aldrich, Oakville, Ontario, Canada) for 1 hour which has previously been reported to effectively reduce microtubule dynamics (Vasquez et al., 1997). Cells were imaged live by confocal imaging after 1 hour incubation at 37 C.
2.11. In vitro β-tubulin co-localization

N-terminal mCherry-tagged β-tubulin constructs were created by inserting β-tubulin (isolated from human muscle tissue) into a pmCherry-N1 vector (Clontech, Mountain View, CA, USA) using HindIII and SalI cleavage sites incorporated into forward and reverse primers, respectively: forward (5’-CGA AGT TTA TGA GGC AAATC-3’) and reverse (5’-AAG TCG ACC GGT CCT CTT CTT CGG C-3’).

Neuro2A cells were co-transfected with both GFP-tagged Thr175Asp tau and pmCherry-tagged β-tubulin. Live cell confocal imaging was conducted at 72 hours after transfection. To compare the differential effects on tau and tubulin formations, half of the plates were exposed to 500 nM nocodazole for 1 hour before live cell confocal imaging.

2.12. Statistical analysis

Statistics were conducted using Sigmastat 10.0 software. Following a Shapiro-Wilk test for normality, a 1-way analysis of variance (ANOVA) was conducted (or Kruskal-Wallis ANOVA on ranks for z nonnormal data) and Tukey post hoc test was conducted. Results were considered to be significant when \( p < 0.05 \).

3. Results

3.1. Thr175 phosphorylation alone is insufficient to induce fibril formation

WT, Thr175Asp, and Thr175Ala 2N4R tau was isolated from E. coli and the extent to which each would form fibrils, in the presence or absence of heparin, determined using the Thioflavin S assay (Fig. 1). No difference was detected between the individual constructs. This suggested that phosphorylation at Thr175 is not sufficient to induce pathologic fibril formation by itself.

3.2. GSK3β activation is increased in cells transfected with Thr175Asp tau

Given our previous observation of colocalization between tau aggregates and activated GSK3β in ALSci, we characterized GSK3β activation status in Neuro2A cells transfected with each tau construct. In cells transfected with Thr175Asp tau, the level of pTyr216 was elevated relative to all other transfection groups (Fig. 2). Relative to the GFP control, WT-tau transfected cells had 1.13 ± 0.16 times as much pGSK3β (mean ± standard error of the mean), and Thr175Ala tau had 1.12 ± 0.13 times as much pGSK3β. Neither was significantly different relative to each other or to the GFP control. Only Thr175Asp-tau transfected cells had a significant increase in pGSK3β (1.81 ± 0.14) relative to GFP-transfected cells after Kruskal-Wallis 1-way ANOVA on ranks (\( p = 0.002, F = 8.684 \)). This indicates that phosphorylated at Thr175 tau induces increased expression of the active form of GSK3β. This in turn may further modify tau protein, giving rise to pathologic changes necessary for tau fibril formation.

3.3. Fibril formation is abolished by pharmacologic inhibition of GSK3β

In keeping with previous studies (Gohar et al., 2009), GFP-tau fibril formation occurred to some extent in all tau construct transfection groups but to a much greater extent in Thr175Asp-tau transfected cells (Fig. 3).

Before pharmacologic studies, all 4 inhibitors were tested on untransfected Neuro2A cells over a range of concentrations focused around their respective IC50. Survival was assessed by MTT assay (described in the following) after 72 hours of exposure. None of the inhibitors was toxic to the cells at their reported IC50.

On analysis of fibril formation, Thr175Asp-tau transfected cells exhibited increased levels of fibril formation relative to all other groups (\( p < 0.001 \) Tukey post hoc test after 1-way ANOVA with \( F = 7.905 \)) (Fig. 3, Supplementary Table 1).

All 4 inhibitors administered at their respective IC50 concentrations were able to reduce fibril formation in Thr175Asp-tau transfected cells to baseline levels (Supplementary Table 1, Fig. 3). This indicates that the increased GSK3β activity observed in Thr175Asp mutant tau is necessary for the increased fibril formation observed.

3.4. shRNA knockdown of GSK3β abolishes fibril formation

Although the evidence thus far suggests GSK3β is the downstream kinase responsible for further tau protein phosphorylation and toxicity leading to fibril formation, a separate isoform (GSK3α) shares 95% similarity in its catalytic domain and 85% similarity overall (Woodgett, 1990). Although there are shared substrates, GSK3α and GSK3β are encoded by 2 separate genes; they are differentially expressed in different tissues (Lau et al., 1999; Uzbekova et al., 2009); they are differentially regulated in the brain (Takahashi et al., 1994) with GSK3β having a higher overall expression in the brain (Lau et al., 1999; Yao et al., 2002); and, they show different substrate affinity (Wang et al., 1994). Therefore, a shRNA was developed to selectively knockdown GSK3β. Western blots for GSK3β and GSK3α were quantified by densitometry and standardized relative to that of an untransfected control. At 24 hours, levels of GSK3β expression (standardized against untransfected control) were pSuper vector 76.9% ± 8.8% and shRNA 50.8% ± 3.0% (\( p < 0.05 \) after significant ANOVA with \( p = 0.002 \) and \( F = 21.177 \)). At 96 hours, relative levels of GSK3β expression were pSuper vector 123.5% ± 3.3% and shRNA 67.7% ± 6.7% (\( p < 0.05 \) after significant ANOVA on ranks with \( p = 0.004 \)). GSK3α levels were unaffected by shRNA transfections.

Using this shRNA, tau construct transfected cells were assessed for fibril formation as in previous experiments (Fig. 4). As previously noted, Thr175 Asp-tau transfected cells showed a significant
increase in fibril formation when contrasted to WT-tau transfected cells (52% ± 2% vs. 26% ± 2%, Thr175 vs. WT-tau, respectively). Cotransfection with pSuper vector had no effect on fibril formation (54% ± 4% vs. 25% ± 2%, Thr175 vs. WT-tau, respectively). In contrast, cells cotransfected with GSK3β-specific shRNA showed a complete inhibition of fibril formation (24% ± 0% vs. 27% ± 1%, Thr175 vs. WT-tau, respectively) (p < 0.001 after significant ANOVA with p < 0.001 and F = 50.339).

3.5. Thr175Asp-tau induced cell death is prevented by GSK3β inhibition

After 72 hours, Thr175Asp transfected cells showed increased death relative to GFP, WT-tau, or Thr175Ala-tau transfected cells, consistent with previous studies (Fig. 5, Supplementary Table 2) (Gohar et al., 2009). This was also increased relative to all GSK3β inhibitor treatment groups (p < 0.05 Tukey post hoc test after
1-way ANOVA). The same observations were made using the Trypan blue experiments across all inhibitors (Supplementary Fig. 2). These data suggest that fibril formation is accompanied by cell death and inhibiting fibril formation prevents cell death.

### 3.6. Thr231 phosphorylation is necessary for Thr175-mediated fibril formation

To test if Thr231 is a downstream mediator of Thr175Asp-induced pathologic fibril formation, we constructed GFP-tagged double mutant tau with either a Thr231Ala or Thr231Asp mutation, with or without Thr175Asp. A total of 6 constructs were tested. All constructs formed fibrils to a baseline extent as previously observed in other constructs (Supplementary Table 3, Fig. 6). However, those containing the Thr231Ala mutation did not form increased fibrils, regardless of Thr175 phosphorylation state, whereas all Thr231Asp mutants formed increased fibrils relative to baseline regardless of Thr175 phosphorylation status (increased relative to others with \( p < 0.001 \) Tukey post hoc test after 1-way ANOVA with \( p < 0.001 \) and \( F = 60.087 \)). This suggests that phosphorylation at Thr231 is key to pathologic fibril formation.

### 3.7. Thr175 Asp-mediated phosphorylation of Thr231 is not dependent on primed phosphorylation at Ser235

To test if Ser235 phosphorylation is necessary to prime phosphorylation of Thr231 in response to Thr175Asp, site-directed mutagenesis was used to create GFP-tagged tau with Ser235Ala and Ser235Asp mutations. A total of 6 mutants were made, each having a combination of Thr175 mutations as discussed previously. Ser235Ala mutations did not prevent fibril formation in Thr175Asp-mutant transfected cells (increased relative to all others with \( p < 0.001 \) Tukey post hoc test after 1-way ANOVA with \( p < 0.001 \) and \( F = 70.537 \)), indicating that the mechanism of primed Thr231 phosphorylation is not necessary for the downstream pathology after Thr175 phosphorylation (Fig. 7, Supplementary Table 4). Ser235Asp mutations did not increase fibril formation in the absence of Thr175 phosphomimic, indicating that its presence is not permissive to fibril formation either.

### 3.8. Tau fibrils persist after nocodazole exposure

After 1 hour exposure to nocodazole, live cell confocal imaging showed that GFP-tau fibril structures were still present in cells transfected with both wild-type GFP-tau and Thr175Asp tau (Supplementary Fig. 3). This effect was observed in co-transfected cells even when β-tubulin structures were lost, and there was a lack of co-localization with tau fibrils (Supplementary Fig. 4).

### 3.9. Tau fibrils are not composed of β-tubulin

Co-localization of β-tubulin and tau was observed in cellular processes extending from cell bodies. This was in stark contrast to the fibrils within the soma of the cell, which were visibly composed...
of tau protein, but lacked β-tubulin in their composition (Fig. 8). This effect was especially clear when nocodazole-treated cells showed the persistence of tau fibrils, but not the extending processes consisting of both proteins (Supplementary Fig. 4).

4. Discussion

We have demonstrated that phosphorylation of Thr175 induces pathologic fibril formation by inducing GSK3β activation, which in turn leads to unprimed Thr231 phosphorylation. The latter step is both necessary and sufficient for the formation of pathologic fibrils. Inhibition of this event using any of a panel of GSK3β inhibitors resulted in reduced fibril formation and cell death. To address the inability of these inhibitors to differentiate between GSK3α and GSK3β, we used shRNA studies to confirm that GSK3β knockdown was able to prevent fibril formation.

The role of Thr231 in mediating a conformational change in tau protein has been previously described (Lin et al., 2007) and shown to have functional implications on tau’s microtubule-binding ability. Of note, its proximity to a bend in what has been proposed as tau’s soluble global hairpin structure (Jeganathan et al., 2006). In this structure, the N-terminus of tau folds over the C-terminus, effectively shelters it from further modification and interaction with other proteins. It is possible that phosphorylation at this site opens up the hairpin, exposing normally sheltered sections of tau, conferring the ability to self-interact, effectively seeding insoluble aggregates with itself beginning with dimerization through cross linking of α pleated sheets located within the microtubule-binding domains which are normally sheltered by the C- and N-termini (von Bergen et al., 2000).
neurodegeneration in transgenic mice (Lucas et al., 2001). Of specific importance to this study, GSK3β activity is also associated with phosphorylation at Thr231 (Cho and Johnson, 2004b; Sato et al., 2002; Sun and Gamblin, 2009) in cell culture models. It has also been suggested that early, but not late administration of GSK3β inhibitors such as LiCl may be able to prevent tauopathy (Hernandez et al., 2013), as done in this study. Further investigations using this model can be used to assess the efficacy to abolish fibrils after they have formed.

GSK3β has been heavily implicated as a kinase responsible for tau protein phosphorylation, and its expression profile in the central nervous system through development has been shown to closely follow that of tau protein phosphorylation status (Takahashi et al., 1994). Although closely related, GSK3β and GSK3α have differences in substrate specificity (Wang et al., 1994). Although there is evidence for tau phosphorylation by GSK3α (Maurin et al., 2013), we have established that in our model, there is no role for GSK3α in the pathologic phosphorylation of tau protein downstream of Thr175 phosphorylation. This is in keeping with previous reports of the modulation of GSK3α and GSK3β activity. Although GSK3β can be highly upregulated, with increases in expression and activity, GSK3α is relatively consistently expressed across the life span (Takahashi et al., 1994) and therefore may not be tied as closely to the disease state. In fact, GSK3β has been shown to follow a level of expression proportional to the level of tau phosphorylation in normal development, as well as increased expression in the diseased state when tau phosphorylation is increased (Pei et al., 1997; Yang et al., 2008).

In this study, only the 2N4R tau isoform of tau protein was assessed. Of note, it has been shown that differential phosphorylation patterns can have different or even opposite effects on different tau isoforms with regards to aggregation propensity. This phenomenon has been suggested to explain differential isoform expression in insoluble aggregates in different tauopathies (Combs et al., 2011). One example of this differential isoform expression is that the triplet isoform motif (1N4R, 1N3R, and 0N3R) in the Western blot of the sarkosyl-insoluble fraction in brain tissue from Alzheimer’s disease (Buee et al., 2000; Strong et al., 2006). This is in stark contrast with the inclusion of all 6 tau isoforms in the insoluble fraction from ALSci brains (Strong et al., 2006). The expression of all 6 isoforms in ALSci insoluble tau is consistent with the observation that Thr175-Asp-tau induced aggregation in cells transfected with all 6 tau isoforms equally (Gohar et al., 2009). Therefore, the analysis of only the longest tau isoform in this study may be justified, and these results extended to the other 5 isoforms found in the human brain, although further studies would have to confirm this.

The finding that Thr231 phosphorylation is necessary and sufficient for the induction of fibril formation after Thr175 phosphorylation does

Because GSK3β activity is enhanced by tau priming where an amino acid at the n + 4 site has already been phosphorylated and because Ser235 is commonly found to be phosphorylated in conjunction with Thr231 (Cho and Johnson, 2004b), it was expected that Ser235 phosphorylation would enhance the observed pathology. However, we observed no impact of Ser235 phosphorylation on the extent of tau fibril formation, suggesting that GSK3β acts on Thr231 in the absence of priming in response to phosphorylated at Thr175. This is not completely unexpected as GSK3β is known to phosphorylate tau through unprimed mechanisms as well (Cho and Johnson, 2003), and activated GSK3β is known to be capable of phosphorylating substrates through both primed as well as unprimed mechanisms (Doble and Woodgett, 2003). That GSK3β does not depend on any other kinases to phosphorylate Thr231 is consistent with the finding that other kinases known to phosphorylate tau protein do not show increased activity in the ALSci brain (Yang et al., 2008).

The increased GSK3β activation induced tau pathology is consistent with previous reports of GSK3β overexpression-induced Tau mutant transfection group

![Fig. 7](image-url) Ser235 phosphorylation is not required for Thr175-mediated fibril formation. Asterisk denotes p < 0.001 post hoc after 1-way ANOVA. Values are representative of 3 independent experiments. Abbreviations: ANOVA, analysis of variance; Thr175Ala, unphosphorylated mutant; Thr175Asp, phosphomimic; Thr175/Ser235Ala, phosphorylation inhibited only at Ser235; Thr175/Ser235Asp, phosphomimic only at Ser235; Thr175Ala/Ser235Asp, phosphorylation prevented at Thr175 and Ser235; Thr175Ala/Ser235Ala, phosphorylation prevented at Thr175 but phosphomimic at Ser235; Thr175Asp/Ser235Ala, phosphomimic at Thr175 but phosphorylation prevented at Ser235; Thr175Asp/Ser235Asp, phosphomimic at both Thr175 and Ser235; WT, wild-type tau.

![Fig. 8](image-url) β-Tubulin does not co-localize with tau protein in fibrils. Representative confocal micrograph depicting tau fibril in cell body (arrowhead) lacking tubulin co-localization. Co-localization occurs in the processes extending out from the cell (arrow). Images shown are live cells transfected with both tau and β-tubulin constructs. Images taken at >63 magnification. Abbreviations: GFP-Thr175Asp, tau protein phosphomimic; pmCherry β-tubulin, β-tubulin; overlay, both tau and tubulin channels.
not rule out the possibility that other sites are also being phosphorylated downstream, or that this may be part of a series of sites that may all be critically phosphorylated and work synergistically for this process to occur, as suggested by others (Alonso et al., 2010; Sengupta et al., 1998; Sun and Gamblin, 2009). However, it does imply that without this site, the others are not capable of inducing fibril formation in this model.

To further characterize the fibrils observed in these studies, β-tubulin co-localization studies and nocodazole studies were conducted to determine if these observations were a result of tubulin bundling, a commonly described artifact of tau protein over-expression (Liu et al., 2012). The persistence of fibrils in the presence of nocodazole (a tubulin polymer destabilizing agent), paired with the lack of co-localization of tubulin with tau protein in these structures suggests that this is an independent phenomenon from tubulin bundling, and that it is in fact a result of pathologic tau protein modification in these experiments.

5. Conclusions

These findings represent the first time a cascade-like sequence of phosphorylation events underpinning the induction of pathologic tau aggregates in ALS has been described. The focus of these studies was on the downstream effects of Thr175 phosphorylation. Although how this site is phosphorylated will be the subject of future investigations, sequence analysis searches using the kinase phosphorylation prediction tool KinasePhos has suggested that likely candidates are MAPK and cdc2. Our studies also suggest a potential therapeutic avenue through the inhibition of GSK3β activation. Further studies using in vivo models of Thr175Asp expression are currently in progress.

Disclosure statement

The authors have no conflicts of interest to disclose.

Acknowledgements

Research supported by the Ontario Neurodegenerative Disease Research Initiative of the Ontario Brain Institute and by the McFeat Family Fund.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2014.12.001.

References


Sun, Q., Gambin, T.C., 2009. Pseudohyperphosphorylation causing AD-like changes in tau has significant effects on its polymerization. Biochemistry 48, 6002–6011.


