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Phosphorylation of tau protein at Thr175 is a toxic event associated with neurodegeneration

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Abstract:

Aberrant phosphorylation and pathological deposition of the microtubule associated protein tau (tau protein) is associated with toxicity and cellular death in a number of neurodegenerative diseases (tauopathies). Specific phosphorylation sites are of interest in the processes leading to tau protein toxicity. One site of interest on tau protein is Thr^{175} ($pThr^{175}$), which has been identified in diseased brain tissue from individuals with amyotrophic lateral sclerosis with cognitive impairment (ALSci) and Alzheimer's disease. *In vitro*, pseudophosphorylation at this residue has been shown to induce the formation of pathological tau fibrils and, apoptotic cell death.

In my thesis, I have investigated the mechanism of cellular toxicity following phosphorylation of tau protein at Thr^{175}. After showing that Thr^{175} pseudophosphorylation alone is insufficient to initiate tau protein fibrillization, I demonstrated that tau phosphorylation at Thr^{175} directly leads to the induction of kinase glycogen synthase kinase-3β (GSK3β) which in turn phosphorylates tau protein at Thr^{231}. Both of these steps are necessary for the cytotoxicity of $pThr^{175}$ tau to be manifest. I have shown that the pharmacological inhibition of this process leading to Thr^{231} phosphorylation prevents both fibril formation and cell death. To determine the extent to which this pathological process of Thr^{231} phosphorylation was applicable across the tauopathies in general, I characterized the presence of $pThr^{175}$, activated GSK3β, $pThr^{231}$tau and oligomeric tau formation across multiple tauopathies. In doing so, I demonstrated that this pathway may play an integral role in the generation of pathological tau deposition beyond that discovered for ALSci.

I then characterized $pThr^{175}$ tau protein pathology in the trauma-associated neurodegenerative disease chronic traumatic encephalopathy (CTE) and CTE with amyotrophic

lateral sclerosis (CTE-ALS), demonstrating the presence of pThr^{175} tau protein in pathology associated with these diseases as well. In order to determine whether the induction of pathological pThr^{175} tau was a primary event in the induction of this neuropathology, I used a rat model of moderate traumatic brain injury in which I demonstrated that after a single cortical impact, phosphorylation of endogenous tau protein at Thr^{175} was persistently elevated. pThr^{175} tau was present in axonal pathology as well as tau protein fibrillar neuronal pathology.

In order to definitively prove that pThr^{175}tau was sufficient to induce tau pathology *in vivo*, I undertook somatic gene transfer of a rAAV9 construct expressing pseudophosphorylated human pThr^{175} tau (Thr^{175}-Asp tau) in young adult rat hippocampus. I observed that one year following the stereotactic inoculation of this modified viral vector, rats developed tau pathology in construct-expressing hippocampal neurons along with caspase-3 cleavage. While the construct was similarly expressed in control rats, including empty vector and wild-type human tau, none of these latter rats developed pathology.

These findings indicate that phosphorylation of human tau at Thr^{175} triggers the pathological phosphorylation of tau protein at Thr^{231} through activation of GSK3β, and that this cascade leads to pathological fibril formation *in vitro* and *in vivo*. I have further demonstrated that this pathological process may have broader applicability than to the pathogenesis of ALSci, and includes a broad range of tauopathies in addition to CTE and CTE-ALS.

Keywords: Microtubule associated protein tau, phosphorylation, glycogen synthase kinase 3β, tauopathy, amyotrophic lateral sclerosis, chronic traumatic encephalopathy, frontotemporal dementia, Alzheimer's disease, neurotoxicity.

Co-authorship statement:

Chapter 2: Manuscript entitled "Thr^{175} phosphorylated tau induces pathological fibril formation via GSK3β mediated phosphorylation of Thr^{231} *in vitro.*" published in Neurobiology of Ageing. Alexander J. Moszczynski, May Gohar, Kathryn Volkening, Cheryl Leystra-Lantz, Wendy Strong, Michael J. Strong.

1. Moszczynski AJ: Designed and performed all *in vitro* experiments, created constructs by site-directed mutagenesis, analyzed all data, compiled all figures, wrote manuscript.
2. Gohar M: Performed preliminary experiments that formed the basis of all studies in this paper. Contributed to hypothesis that formed the basis of the experiments.
3. Volkening K: Provided guidance on experimental design, assisted in generating plasmid constructs and interpretation of results.
4. Leystra-Lantz C: Generated plasmid constructs and assisted with site-directed mutagenesis.
5. Strong W: Performed all elements of ThS assay.
6. Strong MJ: Supervised all experiments, edited manuscript.

Chapter 3: Manuscript entitled "Threonine^{175}, a novel pathological phosphorylation site on tau protein linked to multiple tauopathies." published in Acta Neuropathologica Communications. Alexander J. Moszczynski, Wencheng Yang, Robert Hammond, Lee Cyn Ang, Michael J. Strong

1. Moszczynski AJ: performed immunohistochemistry and analysis, all co-localization analysis, interpretation of the data and wrote the manuscript.
2. Yang W: performed immunohistochemistry and semi-quantitation analysis.

3. Hammond R: Performed neuropathological examinations on the cohort studied and edited the manuscript.
4. Ang LC: Performed neuropathological examinations on the cohort studied and edited the manuscript.
5. Strong MJ: Supervised all elements of the study and edited the manuscript.

Chapter 4: Manuscript entitled "Chronic traumatic encephalopathy (CTE) and CTE with amyotrophic lateral sclerosis (CTE-ALS): evidence for trauma-induced pathological tau phosphorylation at Threonine^{175}" submitted to Neurology. Alexander J. Moszczynski, Wendy Strong, Kathy Xu, Ann McKee, Arthur Brown, Michael J. Strong.

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2. Strong W: performed tau fractionation experiments and western blot.
3. Xu K: performed animal surgeries.
4. McKee A: contributed tissue to the study, performed pathological diagnoses, edited manuscript.
5. Brown A: supervised and designed animal studies, edited manuscript.
6. Strong MJ: supervised and designed all elements of the studies, edited manuscript.

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neuropathology. Alexander J. Moszczynski, Jason Gopaul, Patrick McCunn, Kathryn Volkening, Madeline Harvey, Robert Bartha, Susanne Schmid, Michael J. Strong

1. Moszczynski AJ: performed surgeries, tissue processing, all elements of immunohistochemical analysis and interpretation histological of data, wrote manuscript.
2. Gopaul J: performed surgeries, all elements of animal behaviour and interpretation of behavioural data, contributed to methods section of manuscript.
3. Mccunn P: performed all neuroumaging analyses and interpreted imaging data.
4. Volkening K: participated in design and organization of the study.
5. Harvey M: assisted with surgeries, all elements of animal care and tissue processing.
6. Bartha R: participated in study design and supervised imaging analyses.
7. Schmid S: participated in study design and supervised behavioural analyses.
8. Strong MJ: Designed and supervised study, edited manuscript.

Epigraph:

We are here to add what we can to life, not to get what we can from life – William Osler

Dedication:

To everyone touched by neurodegenerative disease. The brave patients, loving families and friends, and the relentless caregivers who press on with the hope that we will soon have a cure.

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List of Abbreviations:

AAV9	Adeno-associated virus serotype 9
ACC	Anterior cingulate cortex
AD	Alzheimer's disease
AGD	Argyrophilic grains disease
ALS	Amyotrophic lateral sclerosis
ALSci	Amyotrophic lateral sclerosis with cognitive impairment
ALSbi	Amyotrophic lateral sclerosis with behavioural impairment
ALS-PDC	Amyotrophic lateral sclerosis-Parkinson's disease complex
ANOVA	Analysis of variance
ATF6	Activating transcription factor-6
ATP	Adenosine triphosphate
BG	Basal ganglia
cAMP	Cyclic adenosine monophosphate
CBD	Corticobasal degeneration
Cdk5	Cyclin dependent kinase 5
CK1	Casein kinase-1
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTE	Chronic traumatic encephalopathy
CTE-ALS	Chronic traumatic encephalopathy with amyotrophic lateral sclerosis

DAB	3,3'-Diaminobenzidine
DG	Dentate gyrus
DLBD	Diffuse Lewy body dementia
dlPFC	Dorsolateral prefrontal cortex
DMEM	Dulbecco’s modified eagle medium
DN	Dystrophic neurite
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FAT	Fast axonal transport
FRET	Fluorescence resonance energy transfer
FTD	Frontotemporal dementia
FTDP-17	Frontotemporal dementia with Parkinsonism linked to chromosome 17
FTLD	Frontotemporal lobar degeneration
FUS	Fused in sarcoma
G3BP	Ras GTPase-activating protein-binding protein
GCI	Glial cytoplasmic inclusion
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase 3
GST	Glutathione-S transferase
HEK293T	Human embryonic kidney 293 cells containing the SV40-T antigen

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I_2^{PP2A}	Endogenous inhibitor of protein phosphatase 2A
IC_{50}	Inhibitory concentration 50
IHC	Immunihistochemistry
IP	Immunoprecipitation
IRE1	Inositol requiring enzyme-1
JNK	c-Jun N-terminal kinase
KLC	Kinesin light chain
LRRK2	Leucine-rich repeat kinase 2
MAPK	Mitogen activated protein kinase
MCC	Midcingulate cortex
MRI	Magnetic resonance imaging
mDLBD	Mixed dementia with Lewy bodies
MOPS	3-(N-morpholino) propanesulfonic acid
MPR	Microtubule-binding repeat domain
MSA	Multiple system atrophy
MTOC	Mictotubule organizing centre
MTT	(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	Neuronal cytoplasmic inclusion
NC IR	Diffuse neuronal cytoplasmic immunoreactivity
NFT	Neurofibrillary tangle

NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
PAD	Phosphatase activating domain
PART	Primary age-related tauopathy
PBS	Phosphate buffered saline
PD	Parkinson's disease
PERK	Protein kinase R-like endoplasmic reticulum kinase
PHF	Paired helical filament
PIN-1	Peptide-prolyl *cis/trans* isomerase NIMA-interacting 1
PIP_2	Phosphatidylinositol bisphosphate
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PSP	Progressive supranuclear palsy
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
SAPK	Stress associated protein kinase
SDS	Sodium dodecyl sulfate
Ser	Serine
shRNA	Small hairpin RNA
SN	Substantia nigra

SNP	Single nucleotide polymorphism
SUMO	Small ubiquitin like modifier
Tau protein	Microtubule associated protein tau
TBI	Traumatic brain injury
TDP-43	Transactive response DNA binding protein of 43 kilodaltons
Thr	Threonine
TIA-1	T-Cell-Restricted Intracellular Antigen-1
Tris-HCl	Tris hydrogen chloride
Tyr	Tyrosine
UPR	Unfolded protein response
UV	Ultraviolet
VD	Vascular dementia
WB	Western blot
WT	Wild type

Chapter 1: Introduction

1.1 Tau protein

The microtubule associated protein tau (tau protein) is highly expressed in neurons where it localizes mainly to the axon, acting as a microtubule binding protein. Human tau protein is expressed as 6 isoforms encoded by a single gene encoded by 16 exons on chromosome 17 at band position 17q21. The 6 isoforms of tau protein are the result of alternative splicing of exons 2, 3, and 10 (Andreadis, 2005; Goedert et al., 1989; Himmler, 1989; Himmler et al., 1989). Although exon 1 is transcribed, it encodes for the promoter and is not translated (Andreadis et al., 1996). Exons 2 and 3 encode for 29 amino acid inserts that incorporate a cassette which adds to the N terminus of the protein. While exon 3 cannot be encoded without the inclusion of exon 2, exon 2 can be included in the absence of exon 3 (Andreadis2005). Exon 10 encodes a fourth microtubule-binding repeat (MPR) domain. The nomenclature of tau protein isoforms is based on the number of N terminal inserts (either absent (0N) or encoded by exons 2 and 3 (1N and 2N, respectively) and MPR domains (either 3 or 4 (3R or 4R, respectively), the latter originating from expression of exon 10) expressed in that isoform, giving 0N3R, 1N3R, 2N3R, 0N4R, 1N4R, and 2N4R isoforms (Buee and Delacourte, 1999). These are commonly referred to by group as the 3R or 4R tau proteins.

These tau protein isoforms have characteristic molecular weights and isoelectric points, making them differentiable by Western blot (Figure 1.1 A). In the healthy adult brain, the expressed 3R:4R tau protein ratio is approximately 1:1 (D'Souza and Schellenberg, 2005).

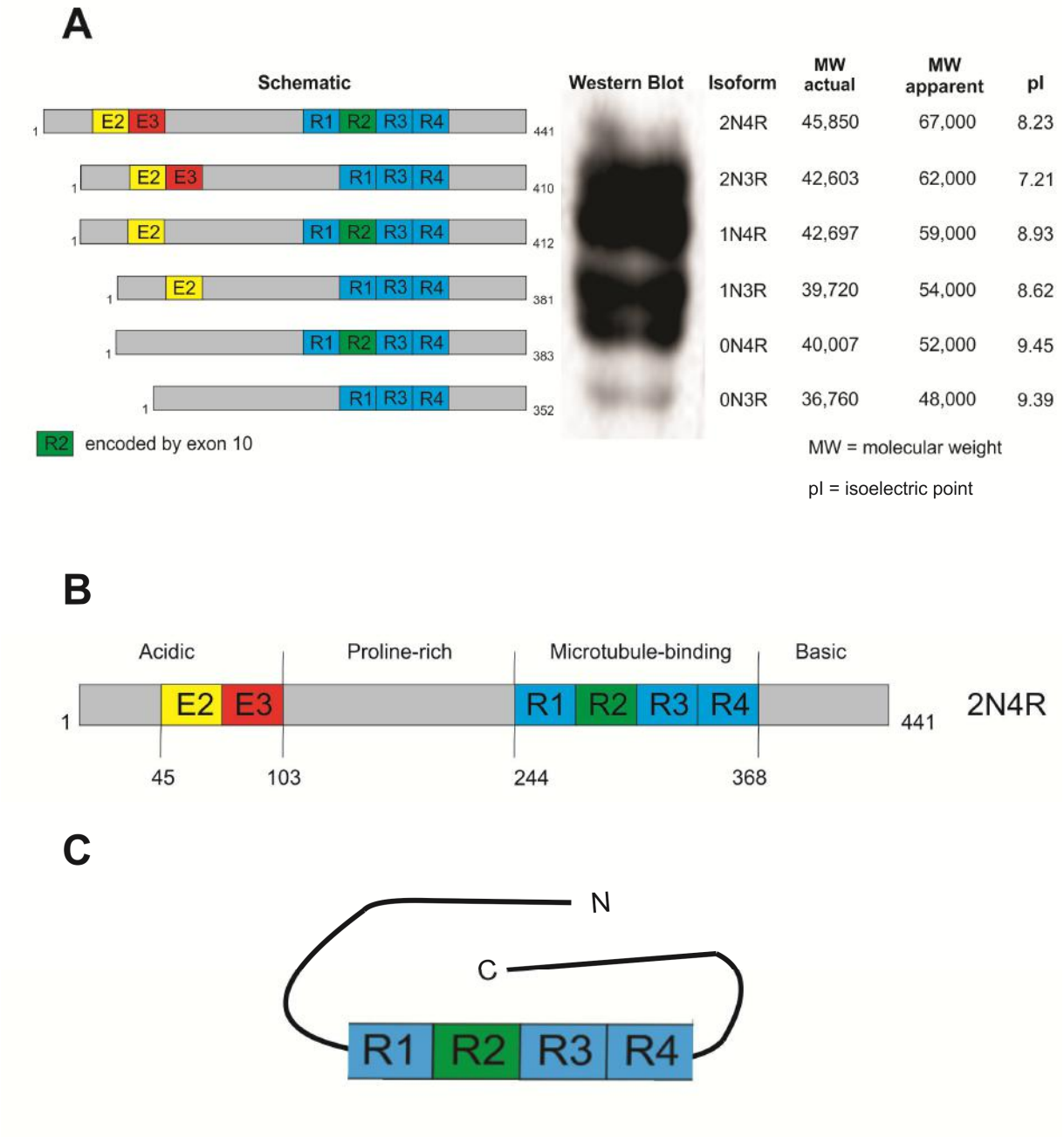

Figure 1.1: Tau protein isoforms and structure. A) Tau protein isoform is composed of 6 distinct isoforms. Western blot for total tau protein isolated from human temporal pole. B) 2N4R tau protein primary structure. C) 2N4R tau protein hairpin conformation.

Tau protein exhibits very little secondary structure and is natively unfolded (Schweers et al., 1994). The primary structure consists of an N-terminal acidic portion with an isoelectric point of 3.8, a proline rich region followed by a series of 3 or 4 imperfect repeat regions which functionally constitute the MPR domains, and a C-terminal basic tail (Figure 1.1 B) with an isoelectric point of 10.8 (Sergeant et al., 2008). This primary structure makes tau protein a dipole and which is crucial for interactions with binding partners and secondary structure. Posttranslational modification of tau protein can modify this dipole, dramatically changing tau protein secondary structure and its interaction with other proteins (Mietelska-Porowska et al., 2014; Sergeant et al., 2008).

Although little secondary structure has been observed, a series of FRET and NMR studies have shown that tau protein does in fact have a preferential secondary structure when in solution in the form of a "global hairpin conformation" whereby the C-terminus folds over the microtubule binding domain and the N-terminus folds over the C-terminus (Figure 1.1 C) (Jeganathan et al., 2006). It is thought that this secondary structure is important in preventing tau protein self-association when soluble such that any alteration to this structure is thought to impact tau protein solubility, predisposing tau protein to self-associate and form fibrillar structures (Bibow et al., 2011; Jeganathan et al., 2008b). Mutations (discussed in section 1.5.2.1) and post translational modifications (section 1.4) have been shown to interfere with tau protein's ability to maintain the hairpin conformation and as a result this may be associated with tau protein dysfunction (Bibow et al., 2011; Jeganathan et al., 2008a).

Functionally, tau protein's N-terminal domain projects off the microtubule allowing tau protein to interact with other proteins such as other cytoskeletal proteins or plasma membranes (Figure 1.2) (Pooler and Hanger, 2010). The most extreme N-terminal domain of tau protein

contains a 17 amino acid segment (aa 2-18) which has been implicated in fast axonal transport through activation of a protein phosphatase-1 (PP1) - GSK3 mediated pathway (Section 1.2.1). This segment has been termed the phosphatase activating domain (PAD) (Kanaan et al., 2011).

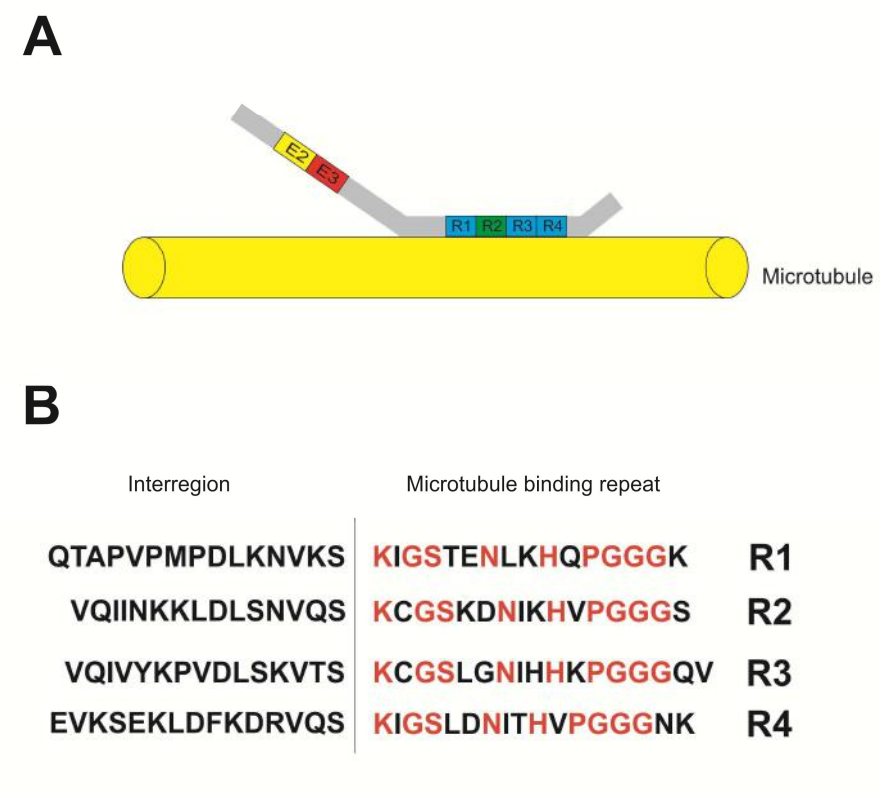

Figure 1.2: Tau protein-microtubule binding. A) Tau protein interacts with microtubules through its microtubule binding domain while the N-terminus projects away from microtubules. B) Imperfect repeat sequence of the microtubule binding domains. Amino acids in red indicate conservation across all four repeats.

The MPR domains of tau protein are encoded by exons 9-12, and consist of imperfectly repeated stretches of 18 highly conserved residues, separated by 13-14 amino acid spacers (Kar et al., 2003) (Figure 1.2). The 18 amino acid repeats bind microtubules through a flexible series of weakly attracted sites distributed through the binding domains (Butner and Kirschner, 1991). 4R tau protein promotes microtubule assembly more actively than 3R tau protein (Goedert and Jakes, 1990). As such, adult brain tau protein, which consists of both 3R and 4R tau protein is more effective at polymerizing microtubules than fetal rat brain tau protein which consists of 0N3R tau protein only (Kosik et al., 1989). The most potent microtubule polymerization region is the inter-region between R1 and R2 (^{275}KVQIINKK^{280}) which exists in 4R tau protein only, making it much more potent than 3R tau protein (Goode and Feinstein, 1994). This inter-region is directly competed for by microtubules and protein phosphatase 2A (PP2A), with the preferred interaction being between microtubules and the inter-region domain. Hence, when bound to microtubules, this domain cannot be dephosphorylated by PP2A (Sontag et al., 1999). Therefore, tau protein phosphorylation on the MPR and microtubule binding are mutually exclusive, as is dephosphorylation of this domain, which requires tau protein to be off the microtubule to take place.

Two *MAPT* gene haplotypes have been described (H1 and H2) (Baker et al., 1999)resulting from an inversion of a 900 kb region spanning the entire MAPT gene (Stefansson et al., 2005) but no change in amino acid sequence. The difference between haplotypes exists in a set of single nucleotide polymorphisms (SNPs) and a 238 bp deletion in intron 9 in H2 (Koolen et al., 2008). The H1 haplotype is associated with increased tau protein expression levels, increased 4R tau protein relative to 3R and tauopathy (Caffrey and Wade-Martins, 2007) while H2 haplotype is associated with decreased tau protein expression levels and is thought to be

protective (Myers et al., 2007). Further analysis has linked a subhaplotype (H1c) to tauopathies (Myers et al., 2007). It has been suggested that pathogenic effects associated with the H1 haplotype may be due to differences in transcription, splicing, posttranscriptional modifications, transcript stability or localization, or rates of translation (Wolfe, 2012).

1.2 Physiological function

1.2.1 Microtubule binding protein

Tau protein was initially described as a microtubule binding protein (Weingarten et al., 1975) and most of the research on its function has focused on this. As a microtubule binding protein, tau protein localization within the neuron is overwhelmingly axonal in healthy adult neurons (Binder et al., 1985). Microtubules are important for cell morphogenesis, cell division, and intracellular trafficking where they provide a road for axonal transport (Morris and Hollenbeck, 1995). Both anterograde (toward the synapse) and retrograde (toward the cell body) axonal transport occur along the microtubule. Transport is carried out by motor proteins such as kinesin (plus end directed) and dynein (minus end directed).

The microtubule is a cytoskeletal component whose function is to maintain neuronal polarity and to act as a scaffold for motor protein based transport of cargo along the axons. Composed of α and β tubulin dimers, the microtubule is itself a polarized structure which undergoes dynamic instability. Microtubules are in constant turnover balanced by regulation of polymerization and depolymerization, reviewed in (Conde and Caceres, 2009). Polymerization occurs in the direction of the “plus end” of the microtubule while depolymerization occurs at the opposite end, termed the “minus end”. The state of more depolymerization than polymerization occurring in the microtubule is termed “catastrophe” while “rescue” is the state of

polymerization occurring at a faster rate than depolymerization (Sept, 2007). Tau protein supports microtubule polymerization by binding with tubulin through the imperfect repeat regions of its microtubule binding domains, stabilizing the microtubule structure itself while inhibiting katanin-induced severing of microtubules (Qiang et al., 2006).

Physiologically, tau protein may detach cargo from kinesin, modulating anterograde transport by regulating the number of kinesin motors attached to cargo (Vershinin et al., 2007). Tau protein has been shown to inhibit anterograde fast axonal transport (FAT) but not retrograde FAT. This function has been shown to require an extreme N-terminal domain of the protein termed the phosphatase activating (PAD) domain (Kanaan et al., 2011). Through a process by which tau protein directly activates a protein phosphatase 1 (PP1) and glycogen synthase kinase-3 (GSK) dependent pathway, tau protein initiates a process contributing to phosphorylation of kinesin light chains (KLCs). GSK3β mediated phosphorylation of KLCs detaches calsyntenin-1, the scaffold protein which binds to the KLCs and vesicles thereby stopping axonal transport (Morfini et al., 2009; Vagnoni et al., 2011).

It has been suggested that abnormal exposure of the N terminus of tau protein leads to a toxic inhibition of axonal transport (Morfini et al., 2009). In fact, tau protein-mediated dysregulation of anterograde transport along microtubules has been shown to slow exocytosis and may also lead to mitochondrial clustering near the microtubule organizing center (MTOC) (Ebneth et al., 1998). The absence of mitochondria in the peripheral regions of axons can cause a decrease in glucose metabolism and ATP synthesis leading to neuronal dysfunction (Schwarz, 2013)

1.2.2 Non-microtubule functions

Beyond its role in the axonal transport and microtubule stabilization, tau protein has also been shown to localize to the cytosol, plasma membrane, dendrites, and nucleus in much lower amounts than its axonal concentration (Loomis et al., 1990; Papasozomenos and Binder, 1987). These other localizations of tau protein suggest that it serves a function in neurons beyond microtubule binding and related functions (Morris et al., 2011). Furthermore, its function may be modified across development as isoform composition has been described to shift in the developing brain where only the ON3R isoform is expressed compared to the adult brain where all 6 isoforms are expressed (Kosik et al., 1989).

Overall, tau protein has been shown to interact with three types of non-microtubule substrates which can be classified as other cytoskeletal elements (F-actin, neurofilament), signaling molecules (Growth factor receptor based protein 2, P85α) and lipids (phosphatidylinositol, phosphatidylinositol bisphosphate) (Flanagan et al., 1997; Reynolds et al., 2008; Surridge and Burns, 1994). The other cytoskeletal element binding exhibited by tau protein plays a role in maintaining a cytoskeletal network consisting of microtubules and other elements such as actin filaments (Farias et al., 2002).

The role of tau protein in signaling could have widespread implications for cell function. For instance, the proline-rich domain of tau protein binds to SH3 domains through any of 7 PXXP motifs contained within this region (Lau et al., 2016). One SH3 domain containing protein is Fyn kinase, which is important in protein trafficking, and may be linked to NMDA receptors (Zhang et al., 2016a). Increased NMDA receptor activation has been linked to increased GSK3β activation, which is associated with widespread signaling changes including toxicity (De et al.,

2006). Additionally, tau protein interaction with Fyn kinase has been shown to promote neurite outgrowth, which is critical in nervous system development (Klein et al., 2002). Tau protein interaction with lipid molecules may also relate to neurite outgrowth, as the association of the N-terminus (including the insert) of tau protein with plasma membrane phospholipids has been shown to enhance growth cone formation in cell culture (Brandt et al., 1995). The role of tau protein in lipid binding likely relates to widespread signaling as well, and many lipid binding partners of tau protein are signaling lipids. For example, tau protein interacts with phosphatidylinositol bisphosphate (PIP_2) which is largely related to cytoskeletal dynamics (van and Jalink, 2002), potassium channel modulation (Huang et al., 1998), as well as exocytosis (Kabachinski et al., 2014). Furthermore, it has been suggested that tau protein may act as a scaffold protein for signaling complexes and that tau protein binding can activate or inhibit several enzymes (Morris et al., 2011).

As tau protein appears to have a role in many cellular molecular networks, dysfunction of tau protein in any way could have widespread effects on the cell through any of these mechanisms. Importantly, many of these functions are likely to occur through N-terminal activities of tau protein, which is increased when global hairpin conformation is lost as observed in several species of phosphorylated or mutated tau protein (Bibow et al., 2011; Jeganathan et al., 2008a).

1.3 Tau protein in the stress response

When exposed to different kinds of physiological stress (ie: heat, osmotic stress, UV radiation), neurons undergo a wide array of changes in transcription, translation and cell signaling known as the stress response. One of the main objectives of the stress response is to maintain homeostasis (Valenzuela et al., 2016). The balance of protein expression in the cell is

closely regulated in a process known as proteostasis. Under stress conditions, the endoplasmic reticulum (ER) can be overloaded by increased translation and misfolding of proteins leading to ER stress, as observed in many neurodegenerative diseases (AD, PD, ALS). Additionally, ER stress is observed after neuronal injury in traumatic brain injury.

To recover proteostasis after ER stress the unfolded protein response (UPR) is activated, This occurs through proteins in the ER lumen, including inositol requiring enzyme-1 (IRE1), protein kinase R-like endoplasmic reticulum kinase (PERK), activating transcription factor-6 (ATF6) (Schroder and Kaufman, 2005). The UPR stops translation of many proteins, increases translation of chaperone proteins to increase proper protein folding, and activates protein degradation pathways. One such protein degradation pathway is the ubiquitin-proteasome pathway which tags and targets specific proteins for degradation and which is implicated in clearing misfolded proteins in many neurodegenerative diseases. Aggregated tau protein has been shown to inhibit proteasome activity through direct inhibitory binding of the 20S core particle of the proteasome, slowing protein clearance and reducing the efficiency of the UPR (Keck et al., 2003). When the UPR is active for a sustained period of time it can induce cellular apoptosis (Fribley et al., 2009).

In addition to protein clearance in the stress response, priority is given to molecular chaperone and repair enzyme synthesis, while translation of most mRNAs is paused until stress conditions have passed. In this state, the mRNA is stored for future use within structures known as stress granules (Anderson and Kedersha, 2006). Several proteins are associated with stress granule formation and maintenance including Staufen, G3BP and TIA-1 (Anderson and Kedersha, 2006) as well as RNA binding proteins such as Tar-DNA binding protein of 43 kDa (TDP-43) and fused in sarcoma (FUS) (Aulas and Vande, 2015). Tau protein has been connected

to the stress granule process through several means. First, tau protein may interact with RNA species themselves and may serve a RNA binding function (Kampers et al., 1996). Second, tau protein has been shown to interact with TIA-1, and may affect TIA-1 distribution in the cell as well as its ability to interact with binding partners affecting stress-response dynamics at the stress granule level (Vanderweyde et al., 2016). Furthermore, it has been shown that TIA-1 and tau protein act synergistically to promote the formation of stress granules as well as the formation of tau protein fibrils in primary hippocampal neurons (Vanderweyde et al., 2016).

There is evidence suggesting that the stress response is capable of inducing pathological tau protein processes. Firstly, osmotic stress has been shown to induce caspase-3 cleavage of tau protein, a process which is observed in AD and is associated with tau protein aggregate formation and toxicity (Chong et al., 2006; Olivera-Santa et al., 2016). Additionally, abnormal tau protein phosphorylation, another change associated with tau protein in disease can be induced by the stress response when ER stress response pathways activate c-Jun N-terminal kinase and other cell signaling cascades which may directly phosphorylate tau protein (Su et al., 2010; Zhang et al., 2016b).

All of these data suggest that tau protein and the stress response are closely related and dependent on each other to some degree for “normal” responses to stress. Abnormally long or severe stress responses may initiate abnormal tau protein metabolism, while abnormal tau protein metabolism may initiate neuronal stress responses.

1.4 Posttranslational modification

Tau protein activity is regulated by post-translational modification, and tau protein is subject to at least 12 distinct types of post-translational modification (summarized in Table 1.1).

These modifications can affect tau protein physiological function, tau protein toxicity to the cell, and tau protein localization. Modifications may compete with one another, enhancing or reducing other types of post-translational modification. These are described in detail below with a specific focus on phosphorylation.

Table 1.1: Tau protein post translational modifications

Modification	Definition	Function to tau	Relation to tau phosphorylation	References
Phosphorylation	Adds a phosphate group to Thr, Ser, or Tyr residue.	Regulates microtubule interaction and cellular processes. Implicated in toxicity and aggregation.	Can increase further phosphorylation.	(Ihara et al., 1986; Lindwall and Cole, 1984)
Glycation	Sugar added to any amino acid.	Reduce tau-microtubule interactions. Enhances fibrillization.	Unknown.	(Wang et al., 1996; Yan et al., 1994)
O-glycosylation	Adds a sugar to oxygen on Ser or Thr (multiple subtypes depending on type of sugar added).	May inhibit tau phosphorylation competing with Ser and Thr.	Reduces phosphorylation.	(Robertson et al., 2004)
N-glycosylation	Sugar added to Asn residue nitrogen.	May promote tau phosphorylation.	Enhances phosphorylation.	(Liu et al., 2002c)
Ubiquitination	Ubiquitin added to protein at Lys residue.	Tag for proteasomal degradation.	Follows pathological phosphorylation. Clears tau.	(Bancher et al., 1991)
SUMOylation	Small Ubiquitin-like Modifier protein covalently attached to the protein at Lys residue.	Nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and progression through the cell cycle. Competes for ubiquitin binding sites.	Follows pathological phosphorylation. Reduces tau clearance.	(Luo et al., 2014)
Nitration	Addition of nitro group to protein.	Conformational changes affecting microtubule binding. May affect aggregation.	Unknown. May occur as consequence of cellular damage.	(Horiguchi et al., 2003)
Methylation	Addition of methyl group to Lys and Arg residues.	Makes protein more alkaline. May prevent/ reduce aggregation.	Unknown.	(Funk et al., 2014)
Acetylation	Addition of acetyl group.	Decreases proteasomal tau degradation.	Reduces tau clearance.	(Min et al., 2010; Min et al., 2015)
Polyamination	Covalently links glutamine and lysine residues. Lysine dependent.	Promotion of protein cross-linking in aggregate formation.	Occurs after phosphorylation.	(Wang et al., 2008)
Prolyl isomerisation	Converts *cis* to *trans* isomers of petide bonds neughbouring Pro residues.	Enables PP2A to dephosphorylate tau at Thr^{231}.	Reduces phosphorylation by making Thr^{231} accessible to phosphatases.	(Nakamura et al., 2012)
Truncation	Cleavage of the protein into smaller fragments.	Involved in degradation but may also produce toxic cleavage products.	Occurs after phosphorylation.	(Ferreira and Bigio, 2011)

1.4.1 Phosphorylation

Tau protein phosphorylation is balanced by kinase activity (addition of phosphates) and phosphatase activity (removal of phosphates). Under physiological conditions tau protein is phosphorylated as a means of modulating its activity, the most well studied being microtubule binding. Sites of phosphorylation are referred to by the amino acid number in the longest tau protein isoform (2N4R, 441 amino acids long).

Tau protein phosphorylation has been its most widely studied posttranslational modification. Tau protein has 85 possible sites of phosphorylation and in the healthy adult brain has a molar ratio of phosphates to tau protein of 3-4:1. In the fetal brain, the molar ratio of phosphates to tau protein is much higher at 8:1 (Kenessey and Yen, 1993), indicating a physiological role of phosphorylation in development, likely related to neurite outgrowth and remodeling/ formation of synaptic connections (Esmaeli-Azad et al., 1994). The phosphorylation of tau protein has an important role in regulating the interactions between it and microtubules, with particularly relevant phosphorylation sites being those sites within the MPR domain and in the adjacent N-terminus region (Jenkins and Johnson, 1999; Lin et al., 2007).

The 8:1 ratio of phosphates to tau protein in the fetal brain is mirrored in pathological tauopathies where it is termed hyperphosphorylation (Kenessey and Yen, 1993). In the fetal brain, many phosphoepitopes that have been considered pathologically associated with tauopathy in the adult brain are observed as well (Hanger et al., 1998; Hanger, 2017; Reynolds et al., 2000). It is important to note that the fetal brain only expresses one isoform of tau protein (0N3R) that lacks the N-terminal inserts. Additionally, at this point the degree of phosphorylation is being directed by developmental processes, so hyperphosphorylation is a physiologically “intended”

state. In pathological states the balance of phosphorylation and dephosphorylation is dysregulated, often with a shift towards the hyperphosphorylation typical of fetal tau protein.

Dysregulated (aberrant) tau protein phosphorylation has been shown to alter tau protein global hairpin conformation. This conformation is thought to protect both the N-terminal domain and the MPR domains from abnormal interactions. For example, the AT8 combination of phosphorylation ($Ser^{199}/Ser^{202}/Thr^{205}$) reduces folding of the N terminus over the rest of tau protein, preventing global hairpin conformation (Jeganathan et al., 2006). This conformational change affects tau protein solubility and interaction behaviour.

Studying the effects of tau protein phosphorylation directly *in vitro* has proven to be a challenge. A common method used to investigate the effects of tau protein phosphoepitopes is the use of pseudophosphorylation (Chang et al., 2011; Haase et al., 2004; Lin et al., 2007). In this process, mutant constructs are generated in which the amino acid (Thr, Ser or Tyr) which would be phosphorylated is mutated to an Asp or Glu, which have large and bulky, negatively charged subgroups, mimicking a permanent, irreversible phosphorylation at this residue. These amino acids only have a total charge of -1 compared to the -3 charge on an actual phosphate group. Additionally, steric forces are different between phosphorylated amino acids and phosphomimics. Despite these differences, a recent study showed that for the investigations of tau protein pathological processes such as aggregation, pseudophosphorylation does in fact mimic phosphorylated tau protein in terms of the aggregate products formed (Prokopovich et al., 2017).

1.4.1.1 Relevant individual sites of phosphorylation

1.4.1.1.1 MPR domain phosphorylation

Although there are 85 possible sites of phosphorylation on tau protein, and hyperphosphorylation is associated with disease in the adult brain, individual sites of phosphorylation may be important physiological regulators and mediators of pathogenicity on their own when phosphorylation is dysregulated. A table of all currently known tau protein phosphorylation sites has been compiled by Diane Hanger (Hanger2017). Here I will highlight several phosphorylation sites that have significance to physiologic function and pathology mediated by tau protein.

An important series of phosphoepitopes associated with a dramatic reduction in tau protein ability to bind microtubules and associated with tau protein fibrillization includes Ser^{262}, Ser^{293}, Ser^{324} and Ser^{356}. These sites are located in equivalent positions in each of the four MPR domains (Drewes et al., 1995). Due to their location on the MPR domain, phosphorylation is likely to directly inhibit tau protein-microtubule interactions, and enhance self-association. Phosphorylation at these sites is predicted to directly impair microtubule binding and mimic tau protein mutations in these regions associated with disease (discussed in section 1.5.2.1).

1.4.1.1.2 Thr^{231}

A physiologically and pathologically relevant phosphoepitope on tau protein is Thr^{231} (Luna-Munoz et al., 2005; Sengupta et al., 1998). Phosphorylation at this site is observed in fetal, adult diseased brains, as well as adult control brain (Hanger et al., 1998; Reynolds et al., 2000). The presence of $pThr^{231}$ in both fetal and adult control brains suggests a physiological role in tau protein regulation. Indeed, it has been shown that phosphorylation at this site alone is capable of

preventing tau protein binding to microtubules, and that phosphorylation at this site causes the N-terminus of the protein to push away from the C-terminus (opening the hairpin), allowing for further access of the MPR domains by tau protein kinases (Lin et al., 2007). More recently, it has been shown that Thr^{231} phosphorylation induces a salt bridge with Arg^{230} which directly inhibits microtubule interaction (Schwalbe et al., 2015). Due to this potent inhibition of tau protein-microtubule interaction, under reversible circumstances phosphorylation of Thr^{231} is a highly effective switch by which to remove tau protein from the microtubule or direct it back.

An effective means of modulating tau protein-microtubule interactions such as $pThr^{231}$ would be crucial in physiological conditions when microtubules must be remodeled such as in situations of neuronal damage, axon growth, or other plastic mechanisms. If enhanced, or locked in place, however, phosphorylation at Thr^{231} could become pathological and has been proposed to result in further phosphorylation of tau protein in the microtubule binding domain (Lin et al., 2007). Furthermore $pThr^{231}$ tau protein has been shown to be toxic in transiently transfected CHO cells (Alonso et al., 2010). The state of phosphorylation at Thr^{231} has been shown to reduce PP2A interaction with tau protein, possibly permitting an increased phosphorylation state of tau protein by reducing phosphate removal (Sontag et al., 2012).

Thr^{231} phosphorylation is commonly observed at the same time as phosphorylation of Ser^{235} which is considered to be a key site which, when phosphorylated, primes tau protein for phosphorylation by GSKβ (Cho and Johnson, 2004). With respect to pathological processes, $pThr^{231}$ tau protein has been shown to be an early phosphoepitope in aggregate formation and may be part of an initiating cascade of phosphorylation sites in this process (Luna-Munoz et al., 2007).

1.4.1.1.3 pThr^{175}

Of particular relevance to this thesis, phosphorylation at Thr^{175} has been observed in both Alzheimer's disease and amyotrophic lateral sclerosis with cognitive impairment (discussed in section 1.5.5) (Hanger et al., 1998; Reynolds et al., 2000; Strong et al., 2006; Yang and Strong, 2012). The observation that pThr^{175} is not present in fetal brain tissue (Hanger et al., 1998; Reynolds et al., 2000) suggests that this phosphoepitope may be a key contributor to adult onset tauopathy. Consistent with this, our lab had previously demonstrated that pThr^{175}, expressed in vitro as a pseudophosphorylation construct, is toxic (Gohar et al., 2009).

Pseudophosphorylation of tau protein on the proline rich domain has been shown to open the global hairpin conformation (Jeganathan et al., 2006). This is relevant for pThr^{175} and pThr^{231} as both sites lie in this region.

1.4.1.2 Kinases involved in tau protein phosphorylation

There are at least 37 kinases that have been implicated in tau protein phosphorylation and tau protein pathology (Hanger et al., 2009). Among these, mitogen activated protein kinase (MAPK), glycogen synthase kinase-3 (GSK3), cyclin dependent kinase-5 (Cdk5), and casein kinase-1 (CK1) have been robustly shown to be involved in tau protein phosphorylation, have different substrate recognition motifs, and have been shown to phosphorylate many residues (Table 1.2) (Hanger2017). GSK3β and Cdk5 have been determined as the likeliest candidate kinases to contribute to pathological tau protein phosphorylation (Flaherty et al., 2000). As the focus of my studies have been primarily amyotrophic lateral sclerosis, GSK3β was the kinase of interest (discussed in section 1.5.5) and will be the only tau protein associated kinase discussed in detail here.

Table 1.2: Main kinases involved in tau protein phosphorylation

Kinase	Preferred recognition motif	No. of phosphorylation sites on tau	$pThr^{175}$?	$pThr^{231}$?
Mitogen activated protein kinase family (MAPK)	Pro-X-Ser/Thr-Pro	22	Yes	Yes
Glycogen synthase kinase 3 (GSK3)	Ser/Thr-XXX-Ser/Thr(p)	37	Yes	Yes
Cyclin dependent kinase 5 (cdk5)	Ser/Thr-Pro-X-Lys/Arg	12	No	Yes
Casein kinase 1 (CK1)	Ser(p)-XX-Ser	39	No	No
Protein kinase A (PKA)	Arg-XX-Ser/Thr	27	No	Yes

Glycogen synthase kinase 3 (GSK3) is a kinase consisting of 2 isoforms encoded by 2 separate genes sharing 85% similarity overall and 95% similarity within the catalytic domain (Woodgett, 1990). The isoforms, GSK3α and GSK3β are regulated differently, expressed in different cell populations and localize to different regions of the cell (Lau et al., 1999; Takahashi et al., 1994; Uzbekova et al., 2009). GSK3β also has a higher expression level in the brain than GSK3α (Lau et al., 1999; Yao et al., 2002). As such, they have different substrates and functions despite their shared structure (Wang et al., 1994). GSK3β is a proline-directed kinase which preferentially phosphorylates a Ser/Thr-XXX-Ser/Thr(p) motif for primed phosphorylation at the former site (Lu et al., 2011). While enhanced by primed phosphorylation, its function does not rely on this. Additionally, GSK3β can phosphorylate tau protein at Ser or Thr residues irrespective of whether or not they are followed by a Pro residue, but prefers those residues located N-terminally to a neighbouring Pro. GSK3β can phosphorylate tau protein at 37 sites including Thr^{231} and Thr^{175}. GSK3β has been implicated in abnormal tau protein phosphorylation in neurodegenerative diseases including Alzheimer's disease. Interestingly, tau protein may be able to activate GSK3β, as the PAD domain activates the PP1-GSK3 pathway (Kanaan et al., 2011). PP1 increases GSK3β activity by removing an inhibitory phosphate at Ser^{9} (McManus et al., 2005). Therefore tau protein can directly induce GSK3 activity.

1.4.1.3 Phosphatases involved in tau protein dephosphorylation

The principal phosphatase involved in tau protein dephosphorylation is protein phosphatase 2A (PP2A). PP2A is able to dephosphorylate abnormally phosphorylated tau protein at residues Ser^{46}, Ser^{199}, Ser^{202}, Ser^{396}, and Ser^{404}, but not Ser^{235} (Gong et al., 1994). Activity of PP2A is inhibited by GSK3β, the activity of which can be enhanced by tau protein directly (Liu

and Gotz, 2013). Additionally, endogenous inhibitor of PP2A (I_2^{PP2A}) is upregulated and translocated from the nucleus to the cytosol in Alzheimer's disease.

1.4.2 Other forms of posttranslational modification

In addition to tau protein phosphorylation, there are a plethora of other posttranslational modifications that tau protein can undertake. These affect tau protein function directly, and many also have implications for tau protein phosphorylation. Notably, some are associated with increased phosphorylation, decreasing phosphorylation, or occur downstream of phosphorylation in pathogenic processes.

1.4.2.1 Posttranslational modifications associated with tau protein phosphorylation

A number of posttranslational modifications have been associated with increased tau protein phosphorylation status. These may act through decreasing tau protein clearance, such as SUMOylation and acetylation, such that there is an increased retention of phosphorylated tau protein which may become further phosphorylated by active kinases (Cohen et al., 2011; Guo et al., 2017; Min et al., 2010). N-glycosylation reduces the rate of dephosphorylation (Liu et al., 2002b) and enhances phosphorylation of tau protein by GSK3β (Liu et al., 2002a). Truncation is associated with phosphorylated tau protein but appears to occur downstream of phosphorylation events (Mondragon-Rodriguez et al., 2008; Rametti et al., 2004).

1.4.2.2 Posttranslational modifications reducing tau protein phosphorylation

A series of modifications are associated with reduced tau protein phosphorylation. These include prolyl isomerisation, in which phosphate group orientation about a Ser/Thr-Pro bond from *cis* to *trans*. Peptide-prolyl *cis/trans* isomerase NIMA-interacting 1 (PIN-1) isomerizes tau protein from *cis* to *trans* conformation at $pThr^{231}$ making tau protein accessible to PP2A for

dephosphorylation (Zhou et al., 2000). Ubiquitination of tau protein results in increased clearance of aberrantly phosphorylated tau protein by the ubiquitin-proteasome system reducing cellular phosphorylated tau protein (David et al., 2002; Keller et al., 2000). Finally, O-glycosylation may be protective against tau protein phosphorylation (Li et al., 2006; Liu et al., 2004). O-glcosylation occurs on Ser and Thr residues sterically hindering the addition of phosphate groups to tau protein (Arnold et al., 1996).

1.4.2.3 Posttranslational modifications with unknown effect on tau protein phosphorylation

There are several types of posttranslational modification with unknown effect on tau protein phosphorylation. Glycation, nitration and polyamination are all enhancers of aggregation (Necula and Kuret, 2004; Wilhelmus et al., 2012; Zhang et al., 2005), while methylation appears to decrease tau protein aggregation (Funk et al., 2014). These modifications may play a role in the disease process but have yet to be described in greater detail.

Table 1.3: Neuropathology of tauopathies (modified from Kovacs 2015)

Disease	Pathology region	Pathology cell type	Pathology phenotype	Isoform composition	Phosphorylation	References
AD	Entorhinal, cortical, hippocampal, deeper subcortical, late stages widespread	Neuronal	NCI, NFT, DNs	68, 64, 60 kDa (major) 72 kDa (minor) PHF motif	45 sites shown	(Braak et al., 2006; Hanger2017; Wang et al., 2013)
FTDP-17	Frontal, temporal, hippocampus, caudate nucleus, putamen	Neuronal, glial	Variable	Variable	Thr^{181}, Ser^{202}, Thr^{205}, Thr^{212}, Ser^{214}, Thr^{231}, Ser^{235}, Ser^{262}, Ser^{356}, Ser^{404}, Ser^{422}	(Ingram and Spillantini, 2002)
PSP	Variable cortical and subcortical	Neuronal, glial	NFT, NCIs, DNs, coiled bodies, tufted astrocytes	64 and 68 kDa bands (4R isoforms)	Ser^{46}, Thr^{181}, Ser^{202}, Thr^{217}, Thr^{231}, Ser^{235}, Ser^{396}, Ser^{400}, Thr^{403}, Ser^{404}	(Dickson et al., 2011; Wray et al., 2008)
CBD	Variable cortical and subcortical	Neuronal, glial	Astrocytic plaques, NFTs, NCIs, DNs, coiled bodies, astrocytic plaques	64 and 68 kDa bands (4R isoforms)	Ser^{202}, Thr^{205}, Ser^{396}, Ser^{404}, Thr^{231}	(Dickson et al., 2011; Feany et al., 1995)
Pick's disease	Frontal and temporal cortex	Neuronal, glial	Pick bodies, ramified astrocytes	60 and 64 kDa bands (3R isoforms)	Thr^{231}, Ser^{235}, Ser^{202}, Thr^{205}, Thr^{181}, Ser^{396}, Ser^{404}	(Buee and Delacourte, 1999; Dickson et al., 2011; Irwin et al., 2016; Probst et al., 1996)
AGD	Temporal, entorhinal cortex, hippocampus, amygdala	Neuronal, glial	NCI, coiled bodies, granular astrocytic immunoreactivity	63, 68 kDa bands (4R isoforms)	Thr^{181}, Ser^{202}, Thr^{205}, Thr^{231}, Ser^{253}, Ser^{356}, Ser^{396}, Ser^{262}, Ser^{404}	(Tolnay et al., 1997; Tolnay and Clavaguera, 2004)
DLBD	Prefrontal, temporal parietal cortices, ACC, subcortical	Neuronal, glial	NFT, thorny astrocytes, coiled bodies	3R+4R (neuronal) 4R (glial)	Ser^{202}, Thr^{205},Thr^{231}, Ser^{235}, Ser^{202}, Ser^{404}, Ser^{396}	(Howlett et al., 2015; Iseki et al., 2003; Ishizawa et al., 2003)
MSA	Internal capsule, putamen	Neuronal, glial	Granular neuronal, glial cytoplasmic inclusions (GCI)	4R	Ser^{202}, Thr^{205}	(Nagaishi et al., 2011)

PART	Temporal cortex, hippocampus basal forebrain, brainstem, olfactory	Neuronal, glial	NFT, DNs, coiled bodies, thorny astrocytes	68, 64, 60 kDa (major) 72 kDa (minor) PHF motif	Ser^{202}, Thr^{205}	(Crary et al., 2014)
ALS (cognitive impaired)	Frontal, temporal, entorhinal cortex, hippocampus, amygdala, ACC, substantia nigra, basal ganglia	Neuronal, glial	NFT, threads, diffuse NC IR, coiled bodies	All 6	$pThr^{175}$, Thr^{231}, Thr^{181}, Thr^{199}, Ser^{202}, Ser^{205}, Thr^{217}, Ser^{396}, Ser^{404}, Ser^{208}, Ser^{210}	(Strong et al., 2006; Yang and Strong, 2012)
CTE	Entorhinal, cortical, perivascular/ periventricular, spinal cord, brainstem	Neuronal, glial	NFT, DNs, NCI, NC IR astrocytic tangles, thorny astrocytes, tufted astrocytes	3R + 4R	Ser^{202}, Thr^{205}, Ser^{396}, Ser^{404}	(McKee et al., 2009; McKee et al., 2010; McKee et al., 2013)

Abbreviations used: NCI= neuronal cytoplasmic inclusion, NFT= neurofibrillary tangles, DNs= dystrophic neurites, NC IR= diffuse neuronal cytoplasmic immunoreactivity, GCI= glial cytoplasmic inclusions, ACC= anterior cingulate cortex, AD= Alzheimer's disease, FTDP-17= frontotemporal dementia with Parkinsonism linked to chromosome 17, PSP= progressive supranuclear pasly, CBD= corticobasal denegeration, AGD= argyrophilic grains disease, DLBD= diffuse Lewy body dementia, MSA= multiple system atrophy, PART= primary age-related tauopathy, ALS= amyotrophic lateral sclerosis, CTE= chronic traumatic encephalopathy

1.5 Tau protein in neurodegenerative diseases

Tau protein pathology has been observed in a host of different neurodegenerative diseases collectively, collectively termed “tauopathies” (Spillantini et al., 1997a). Primary tauopathies such as FTDP-17 are diseases in which tau protein metabolism dysfunction is thought to be the initial insult causing neuronal toxicity whereas secondary tauopathies are neurodegenerative diseases where tau protein metabolism dysfunction is thought to be the result of some other insult leading to tau protein dysfunction. Regardless, tau protein dysfunction appears to be toxic when it occurs, and it is likely that once induced, tau protein pathology is self-perpetuating and contributes to neuronal death (Guo and Lee, 2013).

1.5.1 Alzheimer’s disease

The neurofibrillary tangle (NFT), consisting primarily of phosphorylated tau protein was identified by Alois Alzheimer in his initial description of Alzheimer’s disease (AD) (Alzheimer et al., 1995) and has been a disease defining pathology since (Hippius and Neundorfer, 2003).

Tau protein pathology in AD is primarily neuronal and is observed in the form of neurofibrillary tangles, neuronal cytoplasmic inclusions, threads and dystrophic neurites. By electron microscopy, the ultrastructure of the 8-20 nm wide filamentous inclusions of NFTs in AD forms a regular helical turn at 85-nm intervals known as the paired helical filament (Wischik et al., 1988). A second morphology of the inclusions in AD is the straight filament, which lacks the helical regularity of the PHF and is approximately 15 nm wide (Crowther, 1991). Aggregated tau protein in AD consists of both 3R and 4R tau protein isoforms and when isolated and run on a western blot reveals bands at 68, 64, and 60 kDa with a minor band at 72 kDa. This triplet motif is known as the paired helical filament motif (PHF).

Tau protein phosphorylation has been observed as the major distinguishing modification to tau protein in the AD tauopathic process (Crowther et al., 1989; Goedert et al., 1992; Grundke-Iqbal et al., 1986; Hanger et al., 2009). Indeed, tau protein is hyperphosphorylated in AD, showing a shift in molar ratio from ~2:1 to ~9:1 (Kopke et al., 1993). To date, tau protein has been shown to exhibit phosphorylation at 45 sites in this disease state (Hanger et al., 2009; Hanger2017) many of which are shared with fetal tau protein (Hanger2017; Morishima-Kawashima et al., 1995; Reynolds et al., 2000).

1.5.2 Frontotemporal lobar dementia

Frontotemporal lobar dementia (FTLD) is a neuropathologically diagnosed group of neurodegenerative disorders characterized by neuronal death primarily in the frontal and temporal lobes. FTLD therefore is an umbrella term for a number of neurodegenerative diseases which are defined by the underlying neuropathology. The location of pathology and neuronal death determines the clinical manifestation of the underlying disorder, known as frontotemporal dementia, which may present as behavioural disinhibition, semantic dementia, nonfluent aphasia, Parkinsonism, or motor system dysfunction (Neary et al., 1998). Broadly FTLD is subdivided into 2 main classifications, FTLD-U (50%) and FTLD-Tau (40%). FTLD-U is characterized by tau-negative, ubiquitin positive inclusions composed primarily of TAR DNA binding protein of 43 kDa (TDP-43). Therefore it is referred to as FTLD-TDP. The group of FTLDs comprising FTLD-Tau is further subdivided based on the molecular nature of the tau pathology observed into 3R, 4R and 3R/4R tauopathies. 3R FTLD tauopathies include Pick’s disease and some cases of FTDP-17 while 4R tauopathies include corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), argyrophilic grains disease (AGD) and some cases of FTDP-17. 3R/4R tauopathies include FTDP-17. Depending on the subtype of FTLD-Tau, tau pathology inclusions

are variable in morphology and cell type. The characteristics of tau pathology of the FTLD subtypes are summarized in Table 1.3.

1.5.2.1 Frontotemporal dementia with Parkinsonism linked to chromosome 17

The discovery of causal tau gene mutations in Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) in 1998 (Hutton et al., 1998) lead to an unequivocal link between tau protein metabolism dysregulation induced toxicity and neurodegenerative disease. Clinical manifestations are variable but include dementia, speech or language impairments, behavioural changes, and Parkinsinism (Neary et al., 1998).

To date there have been 53 mutations in tau protein linked to FTDP-17 reviewed in (Ghetti et al., 2015). These mutations can be separated into two main categories: First, mutations reducing microtubule interactions microtubule (R5H, R5L, K257T, G272V, S320F, V337M, K369I, G398R, R406W, P301L and P301S) many of which have a reduced ability to report microtubule polymerization (Hasegawa et al., 1998). Second are mutations affecting exon 10 splicing (Intronic +3, +11, +12, +13, +14, +16, as well as exonic N279K, L284L, N296N, S305N and S305S) which typically display altered ratio of 4R:3R tau protein with a higher proportion of 4R tau protein (Liu and Gong, 2008). Additionally there are three mutations that appear to affect both exon splicing and microtubule binding (N296H, E342V, and ΔK280) (Ingram and Spillantini, 2002).

The tau protein pathology associated with mutations in FTDP-17 is extremely variable and a detailed summery can be found in (Ghetti et al., 2015). Regions frequently affected include frontal and temporal cortices, hippocampus, amygdala, caudate nucleus and putamen (Ghetti et al., 2015). Both neurons and glia can be affected though this appears to be related to the mutation

in question, and glial pathology in the absence of neuronal pathology is not a feature of these disorders (Ghetti et al., 2015). Furthermore, the isoform composition of inclusions and inclusion morphological phenotype varies according to tau protein mutations in these disorders (Ghetti et al., 2015; Ingram and Spillantini, 2002).

1.5.3 Parkinson's related diseases

Parkinson's disease (PD) is not generally associated with tau protein deposition as a major distinguishing neuropathological factor. However, tau protein interaction with α-synuclein, including its presence in Lewy Bodies which consist mainly of α-synuclein (Spillantini et al., 1997b) has shown a strong link between tau protein and Parkinson's disease, particularly when extramotor symptoms are involved, as in the case of Lewy body dementia (Arima et al., 1999; Moussaud et al., 2014). The tau pathology associated with PD is similar to that observed in Lewy body dementia, but less pronounced (Jellinger and Attems, 2006). To this point, dementias associated with PD are generally tau protein positive (Dickson, 2012). In addition to Parkinson's disease and Lewy body dementia, multiple system atrophy (MSA) is another α-synuclein positive neurodegenerative disease characterized by autonomic dysfunction, ataxia and Parkinsonism in which tau protein pathology is a pathological component (Nagaishi et al., 2011).

1.5.4 Primary age-related tauopathy

Primary age related tauopathy (PART) is a recently described neurodegenerative disease associated with cognitive impairment driven by predominantly Alzheimer's- like tau protein pathology in the absence of amyloid-beta pathology (Crary et al., 2014; Jefferson-George et al., 2017). It is notable, however that PART as a disease entity is contentious with some authors

suggesting that it may simply be an early manifestation of preclinical Alzheimer's disease with low levels of amyloid beta pathology (Duyckaerts et al., 2015).

1.5.5 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive upper and lower motor neuron death causing paralysis (Ludolph et al., 2015). While ALS is not typically associated with tau protein pathology there are notable exceptions from several Pacific Island populations and sporadic case reports. The most well-known population of ALS with tau pathology is the Guamanian ALS-Parkinsonism complex (ALS-PDC) also known as Lytico-Bodig. This disease was up to 100 fold higher than the worldwide rate of ALS in the 1950s, but has decreased to similar rates to worldwide of approximately 3/100, 000 presently (Plato et al., 2003). While this was strongly indicative of an environmental factor shifted by cultural changes, leading to several hypotheses of disease origin, the cause remains speculative (Plato et al., 2003). ALS-PDC was characterized by tau protein pathology throughout the central nervous system in the form of neurofibrillary tangles in the temporal lobe especially prominent in the hippocampus and entorhinal cortex (Guiroy et al., 1987; Hirano et al., 1961; Ito et al., 1991; Wakayama et al., 1993). A very unique aspect of this disease was the tau protein deposition in the motor neurons of the anterior horn of the spinal cord (Ito et al., 1991; Schmidt et al., 2001b; Umahara et al., 1994). Tau in ALS-PDC has been shown to be phosphorylated at Thr^{181}, Thr^{231}, Ser^{262}, Ser^{396}, Ser^{404}, and Ser^{422} (Mawal-Dewan et al., 1996).

Similar to the Guamanian ALS-PDC, a set of two separate populations on the Kii peninsula of Japan (Muro disease) (Kuzuhara and Kokubo, 2005) and another population in New Guinea showed a similar disease with an elevated rate of occurrence (Okumiya et al., 2014). Finally, sporadic case reports consistently appear with tau protein deposition throughout the

brain and even in motor neurons (Dobson-Stone et al., 2013; Nakamura et al., 2014; Orrell et al., 1995; Soma et al., 2012). Recently, studies have reported elevated phosphorylated and truncated tau protein in both hippocampus and spinal cord in different ALS populations (Gomez-Pinedo et al., 2016; Vintilescu et al., 2016).

Beyond the aforementioned exceptions, the most consistent observation of tau protein pathology in a Western ALS population have been those observed in the frontal and temporal lobes of patients with ALS with cognitive impairment (ALSci). ALSci is associated with neuronal degeneration in both the frontal and temporal cortex, and is pronounced in the anterior cingulate cortex (Wilson et al., 2001). ALSci is defined as a variant of ALS in which patients perform at or below the 5^{th} percentile on at least 2 cognitive tasks, but not meeting the full criteria for frontotemporal dementia (Strong et al., 2009; Strong et al., 2017). In ALS, cognitive impairment occurs in upwards of 50% of ALS cases (Ringholz et al., 2005).

Tau protein pathology in ALSci was first observed as Gallyas silver staining (Yang et al., 2003). Tau protein was then observed to be phosphorylated at a series of epitopes that had been previously observed in other tauopathies. However tau protein deposits in ALS consisted of all 6 isoforms, suggesting that it is distinct from the Alzheimer's disease associated PHF motif (Strong et al., 2006). Tau protein deposition in ALSci is not simply a function of aging (Yang et al., 2005). It was subsequently demonstrated that the tauopathy of ALSci was associated with an upregulation of active GSK3β activation (Yang et al., 2008). Due to the relatively uninvestigated nature of phosphorylated Thr^{175}, an epitope that had been previously identified in phosphopeptide mapping studies of AD tissue (Hanger et al., 1998; Reynolds et al., 2000), our lab conducted *in vitro* studies that demonstrated that tau protein pseudophosphorylated at Thr^{175} was prone to form fibrils in both HEK293T cells and Neuro2A cells (Gohar et al., 2009). It was

further shown that phosphorylation of Thr^{175} was associated with cell toxicity and apoptotic cell death (Gohar et al., 2009).

Following this a more detailed characterization of tau protein pathology in ALSci was conducted in which tau protein pathology in the anterior cingulate and superior frontal cortices along with entorhinal cortex, hippocampus, amygdala, basal ganglia and substantia nigra was observed (Yang and Strong, 2012). The major distinguishing feature from ALS was tau protein pathology in the frontal cortex and anterior cingulate cortex of ALSci. Pathology was observed in neurons and astrocytes as NFTs, neuropil threads and diffuse neuronal cytoplasmic immunoreactivity. The observations of tau pathology, phosphorylated at Thr^{175} in ALS and ALSci were more recently demonstrated in 50% of cases studied in a separate cohort (Behrouzi et al., 2016). One genetic study of a family in which ALS and FTD were both present, and the only case in which cognitive impairment was present was that driven by a *MAPT* mutation driving tau protein pathology in which the authors suggested that a critical driver of the cognitive elements was tau dysfunction (King et al., 2013).

1.5.6 Chronic traumatic encephalopathy

Chronic traumatic encephalopathy (CTE) is a neurodegenerative disease associated with of repeated head trauma (McKee et al., 2009). CTE is associated with rapid cognitive decline and neurobehavioral disturbances. In about 10% of cases, motor neuron dysfunction develops in a manner consistent with ALS. In this case, it is, termed chronic traumatic encephalomyelopathy with amyotrophic lateral sclerosis (CTE-ALS) (McKee et al., 2010; Meyers et al., 1974).

It is thought that dementia pugilistica (DP), a clinically diagnosed disease associated with neurodegeneration after repeated head trauma (Martland, 1928) is the same disease. However, no

neuropathological assessment under the CTE diagnostic criteria has been conducted on cases prior to 2013 (McKee et al., 2013). As such, this means that much of the literature of dementia pugilistica may or may not represent CTE, as dementia pugilistica may involve different neurodegenerative diseases without the hallmarks of CTE, such as AD, PD, ALS or combinations of these diseases without meeting the criteria for CTE. Notably, one account of dementia pugilistica (Schmidt et al., 2001a) makes the case for AD type tau pathology, but this was undertaken before the detailed classification of CTE was described.

CTE is defined by phosphorylated tau protein pathology located at the depths of the sulci, periventricular and perivascular regions preferentially. In late stages, this pathology is observed in a more widespread manner. CTEM differs from CTE by the additional presence of tau protein pathology in both motor neurons and astrocytes in the ventral and lateral horn of the spinal cord (McKee et al., 2009; McKee et al., 2010; McKee et al., 2013). The isoform composition of insoluble tau protein is in the form of 3R and 4R tau but has not been described in detail (McKee et al., 2013). No known mutations are currently associated with CTE.

1.6 Experimental paradigms used in the study of tauopathy

1.6.1 Human tissue and tau antibodies

The study of tissue from human tauopathy cases has been the gold standard in the understanding and characterization of tau protein abnormalities in neurodegenerative disease. Immunohistochemical and biochemical analysis of human brain tissue have been critical in determining isoform composition, tau protein pathological distinctions, cell type and regional specificity, as well as posttranslational modifications associated with the disease processes themselves. Additionally, any discovery made *in vivo* or *in vitro* must be validated in human

disease to be meaningful for future disease understanding or therapeutic potential. A host of tau protein conformation and phosphorylation specific antibodies have been developed which enable the assessment of human tissue without requiring full sequencing and phosphopeptide mapping by mass spectrometry (Table 1.4). Double labeling experiments are particularly useful for determining more specific information such as dual protein contribution to inclusions, or isoform, truncation product, and phosphoepitpe co-occurrence which can be insightful to validation of disease mechanisms.

Table 1.4: Commonly used tau antibodies and their epitopes

Antibody name	**Epitopes**
AT270	$pThr^{181}$
AT8	$pSer^{202}/pThr^{205}$
AT180	$pThr^{231}/pSer^{235}$
PHF1	$pSer^{396}/pThr^{205}$
AT100	$pSr^{212}/pThr^{214}$
CP13	$pSer^{202}$
CP9	$pThr^{231}$
T22	Soluble tau protein oligomer
TG3	$pThr^{231}$
PG5	$pSer^{404}$
T14	aas 141-149
Tau46	aas 404-441
HT7	aas 159-163

1.6.2 Tau protein polymerization assays

Tau protein is frequently studied for its propensity to form fibrillar aggregates *in vitro*. Pseudophosphorylation studies have shown that in the absence of external modifiers, tau protein phosphorylated or mutated at various sites, especially within the C-terminal can form fibrillar aggregates much more effectively than unmodified tau protein, and in some studies, differential sites of pseudophosphorylation have shown different effects on aggregate propensity in different tau protein isoforms (Abraha et al., 2000; Combs et al., 2011; Sun and Gamblin, 2009). Tau protein pseudophosphorylated at Thr^{175} did not show increased aggregation propensity, but Thr^{231} tau protein did (Haase et al., 2004). Additionally, tau protein phosphorylated at Thr^{231} showed reduced ability to promote microtubule polymerization by this assay (Kiris et al., 2011).

1.6.3 Cell culture

Tau protein has been studied in cell culture models using a variety of immortalized cell lines and primary neuronal cultures. These studies have used human tau protein isolated from disease cases (Santa-Maria et al., 2012), expression of tau protein constructs displaying genetic mutations associated with neurodegenerative disease (Alonso et al., 2010), and tau protein constructs displaying pseudophosphorylation at residues or sets of residues identified in human disease states including Thr^{175} and Thr^{231} (Alonso et al., 2010; Cho and Johnson, 2004; Fath et al., 2002; Lin et al., 2007). In most cases tau protein is observed to be toxic to cells and display reduced microtubule interaction when modified in any way associated with disease, or when expressed at sufficient levels. The primary benefit of cell culture studies is their utility in studying cause and effect of individual protein modifications rapidly and at low cost. They do not, however provide insight into organ-level changes where multiple cell types are present.

1.6.4 Rodent models of tauopathy

Mutant mouse models expressing G272V, P301L, P301S, V337M, K369I and R406W mutatiuons causing FTDP-17 have been studied (Ittner et al., 2008; Santacruz et al., 2005; Schindowski et al., 2006; Tanemura et al., 2001; Zhang et al., 2004). A rat model using adenoviral (AAV9) expression of P301L mutant human tau protein showed that expression of mutant tau protein in adult brain could induce tau protein pathology and behavioural changes (Mustroph et al., 2012). Depending on location, cell type, and the specific mutation or pseudophosphorylated variant expressed, differing behavioural and pathological phenotypes are observed and mimicry of all elements of the disease is never accomplished (Pankevich et al., 2013; Richardson and Burns, 2002). In particular, driving tauopathy appears to be challenging in the absence of tau mutation expression. The cause of this may be in the difference in human and rodent tau protein expression, whereby rodent tau protein is more difficult to drive to pathological processes. Mice only express 3 tau protein isoforms, while rats do express 6 isoforms, however 4R isoforms are expressed at much higher levels (Hanes et al., 2009). In fact, Thr^{175} in the rodent brain is neighboured by a second Thr residue rather than a Pro residue, making phosphorylation by proline directed kinase much more difficult, making Thr^{175} less likely to be phosphorylated and unable to exert toxicity. However, studies have shown that tau protein isolated from neural tissue of neurodegenerative disease cases and injected into rodent brain is able to induce propagating tau protein pathology in the brain which is capable of spreading (Lasagna-Reeves et al., 2012).

1.7 Abnormal tau protein metabolism is a source of neuronal toxicity

Tau protein has been associated with neurodegenerative disease and pathology in many different states and forms, and has been implicated as both a primary cause and a secondary

contributor to these states (Iqbal et al., 2016). The hypothesis of tau protein being causative to disease etiology was confirmed with the discovery of FTDP-17 causing mutations (Hutton et al., 1998). Existing evidence supports both toxic gain of function changes along with loss of function associated toxicity (Trojanowski and Lee, 2005). It is likely however that tau protein loss of function is not sufficient to induce neurodegenerative disease as *MAPT* knockout mice are viable (Dawson et al., 2001; Harada et al., 1994). It is possible that at least in the total absence of tau protein, other microtubule associated proteins are capable of maintaining microtubule function. It is notable, however that when the ability of tau protein to bind to the microtubule is inhibited, it may become toxic to the cell (Patrick et al., 1999). Moreover, microtubule binding may be essential to reduce a pool of unbound tau protein which may contribute to other toxic activities in the cell (Ballatore et al., 2007; Kuret et al., 2005).

The hypothesis that tau protein itself gains toxic function is supported by the observation that expression of FTDP-17-associated *MAPT* mutations or pseudophosphorylation at epitopes observed in tauopathies induces neuronal dysfunction and cell death (Alonso et al., 2010; Combs et al., 2011; Fath et al., 2002; Gohar et al., 2009; Mustroph et al., 2012). Additionally, tau protein isolated from neurodegenerative disease tissue can induce tau protein pathology, neuronal dysfunction, and neuronal death in both cultured cells and rodent brains (Lasagna-Reeves et al., 2012; Santa-Maria et al., 2012). This has lead to hypotheses surrounding the uptake of tau protein by neurons from the interstitium, and tau protein seeding to promote spread of pathology. This is not, however consistent with prion biology in which a pathological prion protein is capable of autocatalyzing a conformational shift from a healthy isoform to pathological prion in another protein of the same type (Baskakov and Breydo, 2007). It is more likely that pathological tau protein exerts toxicity to the cell via cell-wide molecular signaling

changes induced by uncontrolled N-terminal interactions with other proteins including the kinase GSK3β, which then result in cell stress-induced tau protein pathology, introducing a cycle of aberrant stress response ultimately resulting in cellular apoptosis. If tau protein can induce increased activation of tau-phosphorylating kinases, however, it is possible that it could induce a positive feedback loop whereby tau protein becomes increasingly toxic to cells, ultimately inducing neuronal death. This would be consistent with the slowly developing nature of many tauopathies lacking mutations or other external stressors.

The question of what the toxic species may be in tauopathies has also been asked, and whether the fibrillar aggregates themselves are toxic or if some other form of tau protein is toxic. While fibrillar tau protein has been shown be associated with toxicity, this may be a result of the toxic process itself (Cowan and Mudher, 2013). It has been suggested that it is likely the soluble tau protein oligomer that constitutes the toxic species (Lasagna-Reeves et al., 2012) which goes along with the concept that tau protein may exert toxicity through aberrant signaling, which it can do while still soluble rather than in an aggregate sink. Importantly, tau protein oligomerizes on its way to forming aggregates (Cisek et al., 2014), and as such the fibrils themselves are an indicator of toxicity (Bandyopadhyay et al., 2007).

Regardless of the toxic species, aberrant, uncontrolled tau protein phosphorylation is a common factor in many neurodegenerative diseases, leading to tau-mediated toxicity, tau protein fibril formation and neuronal death. That Thr^{175} phosphorylation is unique to pathological disease states, present in 2 tauopathies (uninvestigated in others), and demonstrates toxicity when expressed in cell culture, warrants further investigation. That $pThr^{175}$ tau protein does not form aggregates alone suggests that further modification to tau protein is required for this to occur. Thr^{231} phosphorylation alone has been shown to regulate tau protein structure, and

if driven further than normal may be able to drive tauopathy. Given that this site has been shown to be phosphorylated by GSK3β and that tau protein has been shown to be capable of activating GSK3β-dependent pathways, it is conceivable that these two phospho-epitopes and kinase are closely related to one another as mediators of phospho-tau toxicity.

1.8 Hypothesis

pThr^{175} tau protein is implicated in the neurodegenerative process through a toxic pathway dependent on downstream activation of GSK3β and further phosphorylation at pThr^{231} which is critical for fibril formation and cell death.

1.9 Thesis overview

The focus of the studies presented in this manuscript is the pathogenesis of tau protein phosphorylated at amino acid Thr^{175}.

In Chapter 2, I show that pThr^{175} tau protein when unmodified by further phosphorylation does not have an increased propensity to self-aggregate, but that when further phosphorylated at Thr^{231} in a sequence of events dependent on pThr^{175}-mediated enhancement of GSK3β activation, pThr^{175} tau protein induces fibril formation and cell death. Inhibition of GSK3β reduces tau protein fibril formation and cell death associated with pThr^{175} tau protein expression.

In Chapter 3, I show that pThr^{175} tau protein pathology is not unique to ALSci or Alzheimer's disease but that it can be observed in 8 other tauopathies, and that it is associated with Thr^{231} phosphorylation and tau protein oligomerization in pathological inclusions. I also show that pThr^{175} tau protein is not observed in non-pathological human brain tissue.

In Chapter 4, I show that pThr^{175} tau protein pathology is present in chronic traumatic encephalopathy and in chronic traumatic encephalomyelopthy in both hippocampal and spinal cord neuronal pathology. This was associated with pThr^{231} and activated GSK3β in human tissue. In a rat model of moderate traumatic brain injury I show that after a single head trauma pThr^{175} tau protein can be induced along with tau protein pathology.

In Chapter 5, I show that pThr^{175} tau protein induces tau pathology in adult rat hippocampus when expressed by an adenoviral vector.

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Chapter 2: Thr^{175} phosphorylated tau induces pathological fibril formation via GSK3β mediated phosphorylation of Thr^{231} *in vitro*

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2.1 Abstract:

We have previously shown that amyotrophic lateral sclerosis with cognitive impairment (ALSci) can be characterized by pathological inclusions of microtubule associated protein tau (tau protein) phosphorylated at Thr^{175} ($pThr^{175}$) in association with GSK3β activation. We have now examined whether $pThr^{175}$ induces GSK3β activation and whether this leads to pathological fibril formation through Thr^{231} phosphorylation. 72 hours after transfection of Neuro2A cells with pseudophosphorylated GFP-tagged 2N4R tau protein (Thr^{175}Asp), pGSK3β (active GSK3β) levels were significantly increased as was pathological fibril formation and cell death. Treatment with each of 4 GSK3β inhibitors or shRNA knockdown of GSK3β abolished fibril formation and prevented cell death. Inhibition of Thr^{231} phosphorylation (Thr^{231}Ala) prevented pathological tau protein fibril formation, regardless of Thr^{175} state while Thr^{231}Asp (pseudophosphorylated at Thr^{231}) developed pathological tau protein fibrils. Ser^{235} mutations did not affect fibril formation, indicating an unprimed mechanism of Thr^{231} phosphorylation. These findings suggest a mechanism of tau protein pathology by which $pThr^{175}$ induces GSK3β phosphorylation of Thr^{231} leading to fibril formation, indicating a potential therapeutic avenue for ALSci.

2.2 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), behavioural (ALSbi) or dysexecutive syndrome consistent with that of frontotemporal dysfunction, including a frontotemporal dementia (FTD) (Ringholz et al., 2005; Strong et al., 2009). The frequent co-existence of ALS and FTD has led to the postulate that both are two states along one disease continuum (Robberecht and Philips, 2013). Importantly, patients with frontotemporal dysfunction have a reduced survival compared to other ALS cases (Elamin et al., 2011; Elamin et al., 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 protein) (Yang et al., 2003; Yang and Strong, 2012). This finding is significantly greater than observed as a function of age (Yang et al., 2005).

Tau protein 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 protein relocates from its normal localization in the axon to the cell body where it forms aggregates (Kowall and Kosik, 1987). In ALSci cases, tau protein is found in the form of fibrillar inclusions and is phosphorylated at Thr^{175} ; a phenomenon not observed in Alzheimer's tau protein 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 protein isolated from ALSci patients has a greater propensity to aggregate, while in both HEK293T and Neuro2A cells, tau protein pseudophosphorylated at Thr^{175} was found to form fibrillar aggregates to a much larger extent than wild type (WT) tau protein, regardless of the isoform (Gohar et al., 2009). In these latter experiments, pathological fibril formation was associated with increased cell death. We also observed that pathological tau protein inclusions in ALSci co-localize 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 protein (Cho and Johnson, 2003) (Cho and Johnson, 2003)and because GSK3β has been strongly implicated as a major contributor to tau protein pathology (Cho and Johnson, 2003; Cho and Johnson, 2004a; Cho and Johnson, 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 pathological tau protein fibril formation in ALSci. We have also postulated that phosphorylation of Thr^{231} will be key to pathological tau protein fibril formation.

Thr^{231} is a tau protein phosphorylation site which, when phosphorylated, causes a conformational change in which tau protein's ability to bind to microtubules is reduced (Lin et al., 2007). Thr^{231} 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 Ser^{235} is phosphorylated first. It is therefore a likely site of synergistic modification to tau protein in its pathology along with phosphorylation of Thr^{175}.

In this study, we demonstrate that phosphorylation of tau protein at Thr^{175} leads to the activation of GSK3β, which then phosphorylates tau protein at Thr^{231} and which in turn leads to pathological fibril formation. Inhibition of GSK3β, pharmacologically and by small hairpin RNA (shRNA) knockdown prevents toxic pathological tau protein fibril formation, and prevents cell death. Phosphorylation at Thr^{231} is critical to this process, although independent of Ser^{235} phosphorylation status.

2.3. Methods:

2.3.1. Cell culture and transfection:

Because we had previously shown that Thr^{175}Asp induces pathological tau protein fibril formation in Neuro2A and HEK293T cells, we performed all studies using the 2N4R tau protein isoform in Neuro2A cells (Gohar et al., 2009). Neuro2A cells were grown on 10 cm plates in Dulbecco's Modified Eagle medium (DMEM; Gibco, Burlington ON, Canada) enriched with 10% fetal bovine serum (Gibco, Burlington On. Canada) and 50 µg/mL Penicillin/Streptomycin (Gibco, Burlington, On. Canada). Cells were maintained at 37˚C and 5% CO_2. Transfections for all survival and aggregation studies were performed using Lipofectamine 2000 (Invitrogen, Burlington On. 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. Due to 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% CO_2 in the presence of 10 µg DNA and a mixture of $CaCl_2$ and HEPES buffered serum.

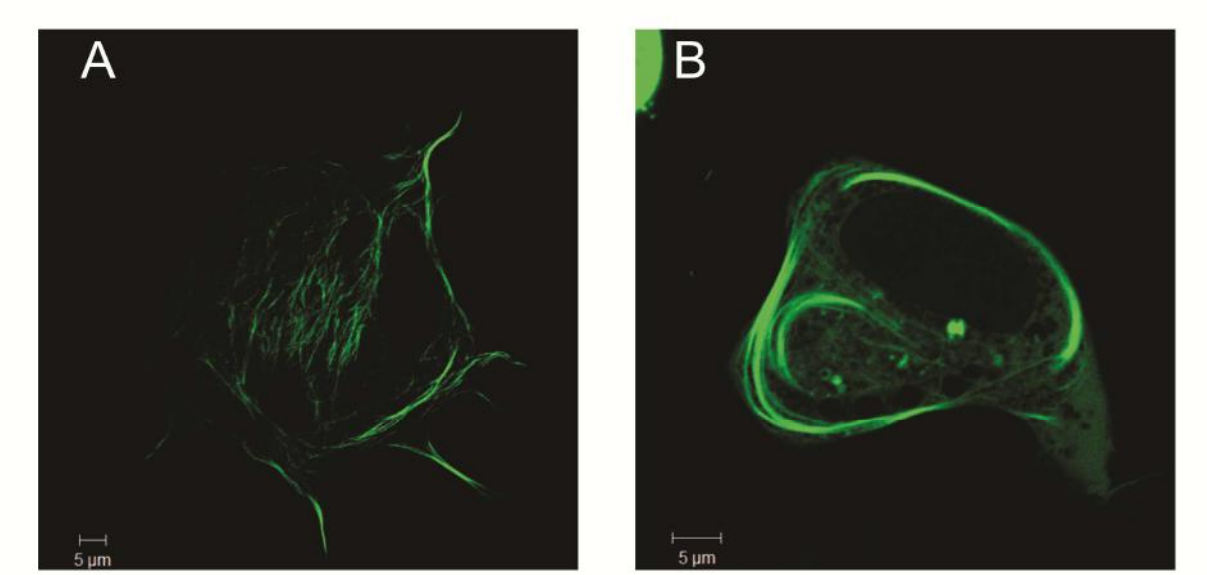
Medium was changed to end all transfections. All analyses were conducted 72 hours after transfections were ended.

2.3.2. Thioflavin S assay for Tau aggregation:

GST fusion variants of WT and $Thr^{175}Asp$ 2N4R tau protein 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 PBS and then lysed by sonication. Tau protein was purified from the resulting homogenate as previously described (Gohar et al., 2009), and the ability to form pathological fibrils assayed by *in vitro* thioflavin S assay (Friedhoff et al., 1998; Taniguchi et al., 2005). 100 µL purified protein sample containing 0.35 to 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, ON, Canada) in 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS; Sigma-Aldrich, Oakville, ON, Canada), pH 6.8. Samples were analyzed with or without 5 µM heparin (Sigma-Aldrich, Oakville, ON, Canada). The assembly of tau protein into fibrils is enhanced by heparin, a polyanion, which acts as a positive control for this assay (Pickhardt et al., 2005; von Bergen M. 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.

2.3.3. Fibril quantification:

Cells were transfected with GFP-tagged mutant tau protein and visualized live by fluorescence microscopy on a Zeiss LSM 510 Meta NLO multiphoton confocal microscope in confocal microscopy dishes at 63x 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 a) cells containing fibrillar aggregates or b) 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 protein transfected cells (Supplemental figure 2.1) (Gohar et al., 2009). The percentage of cells containing aggregates was defined as the number of GFP- tau protein expressing cells counted containing fibrils. All experiments were performed in triplicate after plates were blinded to the observer (AJM) by a separate party.



Supplemental figure 2.1: Phenotype of Thr^{175}Asp tau fibrils in Neuro2A cells.
GFP-tagged tau protein was imaged by live cell confocal microscopy 72 hours after transfection. A) Both wild-type and Thr^{175}Ala transfected cells demonstrated fine, filamentous fibril formation typical of that expected for cytosolic tau protein. B) Fibrillar tau protein pathology in Thr^{175}Asp tau protein transfected cells. In contrast to the wild-type and Thr^{175}Ala transfected cells, cells transfected with Thr^{175}Asp formed thick, curvilinear tau protein inclusions. Additional bundling along the periphery of the cell was also commonly observed. Images taken at 63x magnification.

2.3.4. GSK3β Inhibitors:

Four GSK3β inhibitors that act through different mechanisms were used at their respective IC_{50} values in fibril and survival experiments. These included: lithium chloride (LiCl; Sigma-Aldrich, St. Louis MO, USA), IC_{50} 5 mM, which acts through a Mg^{2+} competitive mechanism; AR-A014418 (Sigma-Aldrich, St. Louis MO, USA), IC_{50} 104 nM (Bhat et al., 2003), which acts through an ATP competitive mechanism; Tideglusib (Sigma-Aldrich, St. Louis MO, USA), IC_{50} 60 nM (Dominguez et al., 2012), which acts through a non-ATP competitive mechanism; and, TWS-119 (BioVision, Milpitas CA, USA), IC_{50} 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 prior to use in fibril studies to demonstrate that the inhibitors would not be lethal to Neuro2A cells at their IC_{50}. Inhibitors were administered in fresh medium at their IC_{50} concentration at the end of transfection when medium was changed.

2.3.5. 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 protein 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 protein, 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, Mississauge On, Canada) was used to generate 6 double mutant GFP-tagged tau protein 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'-GCA GTG GTC CGT **G**CT CCA CCC AAG TCG-3') and reverse (5'-CGA CTT GGG TGG AG**C** ACG GAC CAC TGC-3'). Full length primers for Thr^{231}Asp mutations were: Forward (5'-GCA GTG GTC CGT **G**AT CCA CCC AAG TCG-3') and reverse (5'-CGA CTT GGG TGG A**TC** ACG GAC CAC TGC-3').

An additional series of 6 GFP-tau protein mutants containing Ser^{235} mutations was 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}Ala /Ser^{235}Asp). Full length primers for Ser^{235}Ala mutants were: Forward (5'-CCC AAG **G**CG CCG TCT TCC GCC-3') and reverse (5'-GGC GGA AGA CGG CG**C** CTT GGG-3'). Full length primers for Ser^{235}Asp mutants were: Forward (5'-CCC AAG **GAC** CCG TCT TCC GCC-3') and reverse (5'-GGC GGA AGA CGG **GTC** CTT GGG-3').

All mutants were sequenced to confirm presence of mutations of interest.

2.3.6. MTT survival assay:

Cells were grown and transfected in 96 well plates. 72 hours post transfection, 20µL 5mg/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% CO_2. After incubation, cells from one well per treatment group were resuspended in 100 µL fresh DMEM, and transferred to a 1.5 mL microcentrifuge tube. 10 µL was then loaded onto a hemocytometer (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.

2.3.7. Trypan blue assay:

Cells were grown and transfected in 96 well plates. 72 hours post transfection, cells from one well per treatment group were resuspended in 100 µL fresh DMEM and transferred to a 1.5 mL microcentrifuge tube. 100 µL 0.4% trypan blue stain (Gibco, Burlington, On. Canada) was then added and cells were incubated at room temperature for 1 min. 10µL was then loaded onto a hemocytometer 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.

2.3.8. 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, Indiapolis, IN, USA) and phosphatase inhibitors (Phosstop, Roche Diagnostics, Indiapolis, IN, USA). 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. 20 µg protein 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, On. 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 re-probing with mouse anti-total GSK3β (1:10 000 titer; BD Biosciences, Mississauga, On. Canada). 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, Hercules, CA, USA) or Swine anti-rabbit (1:1000 titer; Dako, Burlington, On. Canada) for 1 hour at room temperature. Densitometry was conducted using open source ImageJ software (NIH). GSK3β activation was normalized for overall expression and transfection efficiency by the equation ($pTyr^{216}$/Total GSK3β)/(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, On. Canada). GSK3α was assessed using mouse anti-GSK3α (1:1000 titer, Abcam, Toronto, On. Canada), and normalized to α-tubulin by densitometry.

2.3.9. shRNA:

A small hairpin RNA (shRNA) specific to GSK3β was designed according to a previously reported sequence (Yu et al., 2003) shown to specifically knock down 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 2013: Forward (5'-GAT CCC CGA TCT GGA GCT CTC GGT TCT TTC AAG AGA AGA ACC GAG AGC TCC AGA TCT TTT TA-3') and Reverse (5'-AGC TTA

AAA AGA TCT GGA GCT CTC GGT TCT TCT CTT GAA AGA ACC GAG AGC TCC AGA TCG GG-3').

shRNA plasmid was transfected into Neuro2A cells with lipofectamine 2000 and GSK3β expression analysed at 24 and 96 hours post 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 above.

2.3.10. Nocodazole experiments:

Cells were transfected with GFP-tagged wild type tau or Thr^{175}Asp tau with Lipofectamine as above in confocal dishes. 72 hours after transfection cells were exposed to 500 nM nocodazole (Sigma-Aldrich, Oakville, ON, 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.

2.3.11. *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 AGC TTA TGA GGG AAA TC-3') and Reverse (5'-AAG TCG ACC CGG CCT CCT CTT CGG C-3').

Neuro2A cells were co-transfected with both GFP-tagged Thr^{175}Asp tau protein and pmCherry-tagged β-tubulin. Live cell confocal imaging was conducted at 72 hours post transfection. To compare the differential effects on tau protein and tubulin formations, half of the plates were exposed to 500 nM nocodazole for 1 hour before live cell confocal imaging.

2.3.12 Statistical analysis:

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

2.4. Results:

2.4.1. Thr^{175} phosphorylation alone is insufficient to induce fibril formation:

WT, Thr^{175}Asp, and Thr^{175}Ala 2N4R tau protein 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 (Figure 2.1). No difference was detected between the individual constructs. This suggested that phosphorylation at Thr^{175} is not sufficient to induce pathological fibril formation by itself.

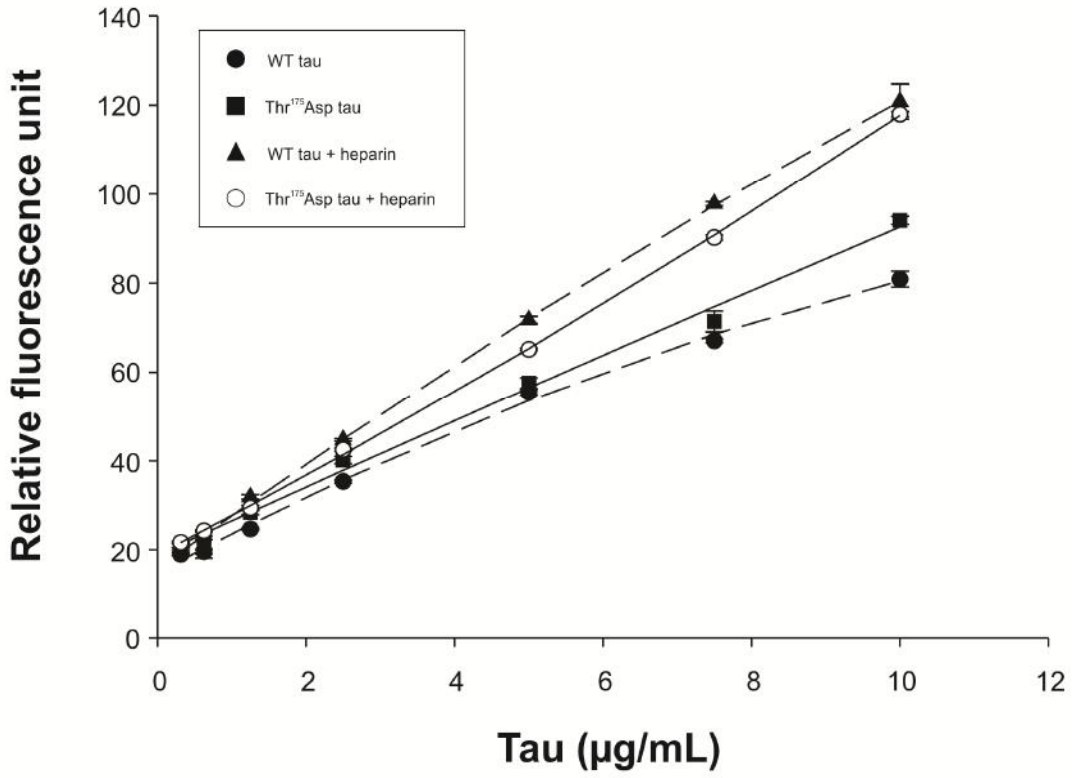


Figure 2.1: Phosphorylation at Thr^{175} alone is not sufficient to induce aggregation. GST fusion variants of WT and Thr^{175}Asp 2N4R tau protein was isolated from E. coli BL21 cells. Extent of fibril formation was then assayed by *in vitro* Thioflavin S assay with and without heparin. No difference was detected between Thr^{175}Asp GST-fusion tau protein compared to WT GST-fusion tau protein with or without heparin. Values are representative of three independent experiments.

2.4.2. GSK3β activation is increased in cells transfected with Thr^{175}Asp tau:

Given our previous observation of co-localization between tau protein aggregates and activated GSK3β in ALSci, we characterized GSK3β activation status in Neuro2A cells transfected with each tau protein construct. In cells transfected with Thr^{175}Asp tau protein, the level of $pTyr^{216}$ was elevated relative to all other transfection groups (Figure 2.2). Relative to the GFP control, WT tau protein transfected cells had 1.13±0.16 times as much pGSK3β (mean±SEM), and Thr^{175}Ala tau protein had 1.12±0.13 times as much pGSK3β. Neither were significantly different relative to each other, or to the GFP control. Only Thr^{175}Asp tau protein transfected cells had a significant increase in pGSK3β (1.81±0.14) relative to GFP transfected cells after Kruskal-Wallis one-way ANOVA on ranks ($p=0.002$, $F=8.684$). This indicates that $pThr^{175}$ tau protein induces increased expression of the active form of GSK3β. This in turn may further modify tau protein, giving rise to pathological changes necessary for tau protein fibril formation.

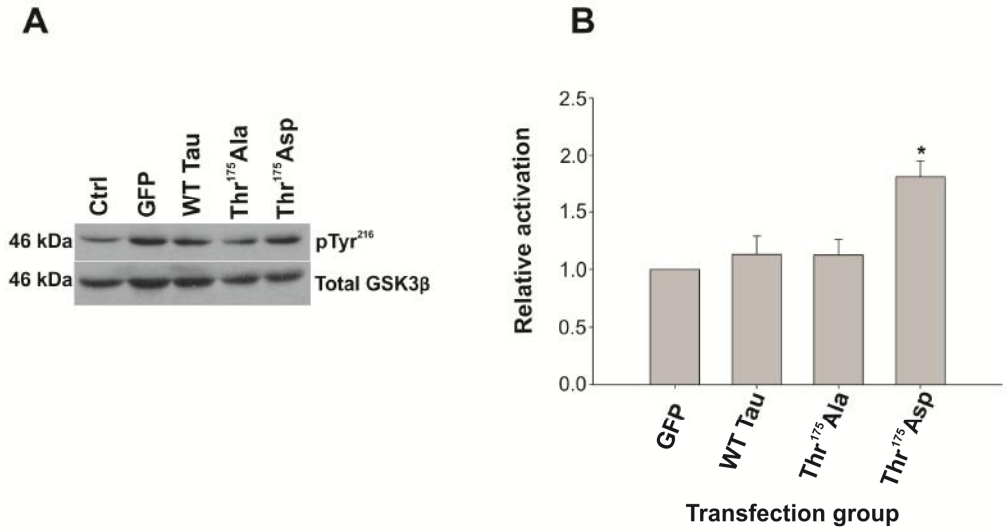


Figure 2.2: GSK3β activation increases in Neuro2A cells transfected with Thr^{175} phosphomimic tau protein. A) Representative western blot for active GSK3β (pTyr^{216}) in untransfected (Ctrl), wild type tau protein (WT), unphosphorylated (Thr^{175}Ala) and phosphomimic (Thr^{175}Asp) transfected Neuro2A cells 72 hours after transfection. B) Densitometric analysis of western blots probed for pTyr^{216} GSK3β and then normalized against total GSK3β, and transfection efficiency by the equation (pTyr^{216}/Total GSK3β)/(GSK3β/GFP). Values are representative of three independent experiments.

2.4.3. Fibril formation is abolished by pharmacologic inhibition of GSK3β:

In keeping with previous studies (Gohar et al., 2009), GFP-tau protein fibril formation occurred to some extent in all tau construct transfection groups but to a much greater extent in $Thr^{175}Asp$ tau transfected cells (Figure 2.3).

Prior to pharmacological studies, all four inhibitors were tested on untransfected Neuro2A cells over a range of concentrations focused around their respective IC_{50}. Survival was assessed by MTT assay (described below) after 72 hours of exposure. None of the inhibitors was toxic to the cells at their reported IC_{50}.

Upon analysis of fibril formation, $Thr^{175}Asp$ tau transfected cells exhibited increased levels of fibril formation relative to all other groups ($p<0.001$ Tukey's post-hoc test after one way ANOVA with $p<0.001$, and $F=7.905$) (Figure 2.3, Supplemental table 2.1).

All four inhibitors administered at their respective IC_{50} concentrations were able to reduce fibril formation in $Thr^{175}Asp$ tau transfected cells to baseline levels (Supplemental table 2.1, Figure 2.3). This indicates that the increased GSK3β activity observed in $Thr^{175}Asp$ mutant tau protein is necessary for the increased fibril formation observed.

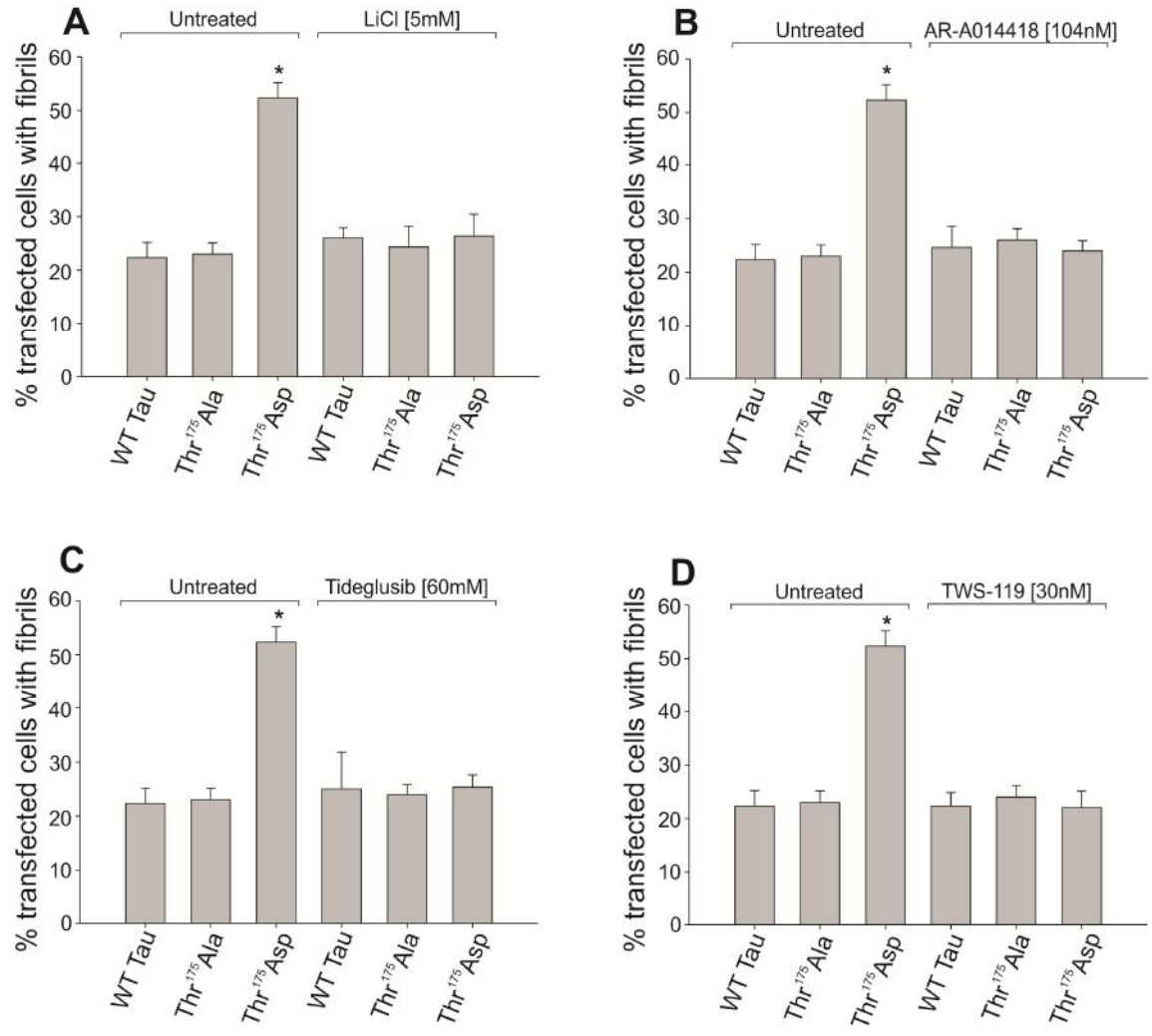


Figure 2.3: Fibril formation is reduced to baseline when GSK3β is pharmacologically inhibited. A) 5 mM LiCl. B) 104 nM AR-A014418. C) 60 nM Tideglusib. D) 30 nM TWS-119. GFP= GFP transfected group, WT= wild type tau, $Thr^{175}Ala$= unphosphorylated mutant, $Thr^{175}Asp$= phosphomimic. TWS= TWS119 treated group, Tide= Tideglusib treated group, LiCl= LiCl treated group, ARA= AR-A014418 treated group. * denotes $p<0.05$ compared to all other groups by post hoc test after one way ANOVA. Values are representative of three independent experiments.

Supplemental Table 2.1: Thr^{175}Asp tau fibril formation is increased compared to all treatment groups and constructs. Untreated= no GSK3β inhibitor used, WT= wild type tau, Thr^{175}Ala= unphosphorylated mutant, Thr^{175}Asp= phosphomimic. TWS= TWS119 treated group, Tide= Tideglusib treated group, LiCl= LiCl treated group, ARA= AR-A014418 treated group. Values represent mean±SEM % of GFP-tau protein expressing cells exhibiting fibrils. * denotes increased fibril formation in comparison to all groups (treated and untreated) $p<0.001$ Tukey's post-hoc test after one way ANOVA ($p<0.001$, $F=7.905$).

Group	**Untreated**	**5 mM LiCl**	**104 nM AR-A014418**	**60 nM Tideglusib**	**30 nM TWS-119**
WT	22±2	26±1	25±3	25±6	22±2
Thr^{175}Ala	23±2	24±3	26±2	24±1	24±2
Thr^{175}Asp	52±2 *	24±3	24±1	25±2	25±2

2.4.4. 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 knock down 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 (Figure 2.4). As previously noted, Thr^{175}Asp tau transfected cells showed a significant increase in fibril formation when contrasted to WT-tau transfected cells (52±2% vs. 26±2%, Thr^{175} vs. WT-tau respectively). Cotransfection with pSuper vector had no effect on fibril formation (54±4% vs. 25±2%, Thr^{175} vs. WT-tau respectively). In contrast, cells cotransfected with GSK3β specific shRNA showed a complete inhibition of fibril formation

(24±0% vs. 27±1, Thr^{175} vs. WT-tau respectively) ($p<0.001$ after significant ANOVA with $p<0.001$ and $F=50.339$).

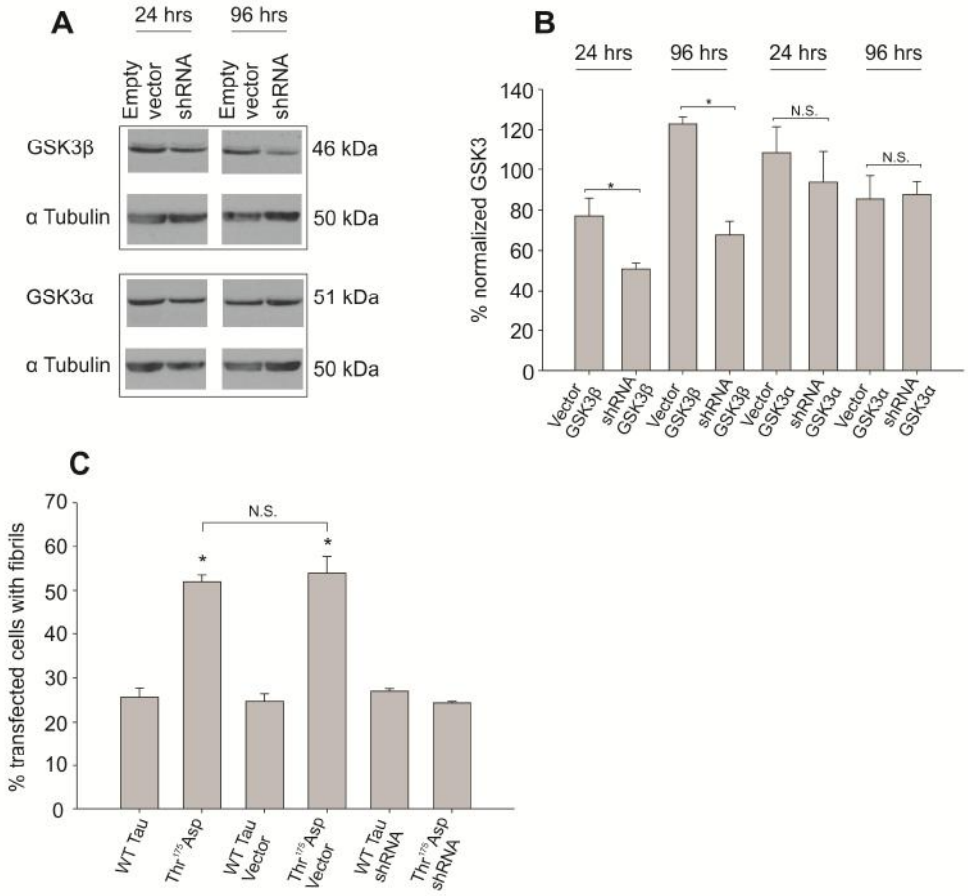


Figure 2.4: shRNA knockdown of GSK3β abolishes pathological fibril formation. A) Representative western blots for GSK3β and GSK3α at 24 and 96 hours post-transfection. B) Densitometric quantification of GSK3β and GSK3α expression relative to untransfetced control cells. Values were expressed as the ratio of GSK:α-tubulin before comparison. C) Fibril formation in shRNA expressing cells was abolished relative to empty vector (pSuper) and cells only transfected with tau protein. WT= wild type tau, $Thr^{175}Asp$= phosphomimic * denotes $p<0.001$ compared to all other groups by post hoc test after significant one way ANOVA. All values are representative of three independent experiments.

2.4.5. Thr^{175}Asp tau induced cell death is prevented by GSK3β inhibition:

After 72 hours, Thr^{175}Asp transfected cells showed increased death relative to GFP, WT tau, or Thr^{175}Ala tau transfected cells, consistent with previous studies (Figure 2.5, Supplemental table 2.2) (Gohar et al., 2009). This was also increased relative to all GSK3β inhibitor treatment groups ($p<0.05$ Tukey's post-hoc test after one way ANOVA). The same observations were made using the Trypan blue experiments across all inhibitors (Supplemental figure 2.2). These data suggest that fibril formation is accompanied by cell death, and inhibiting fibril formation prevents cell death.

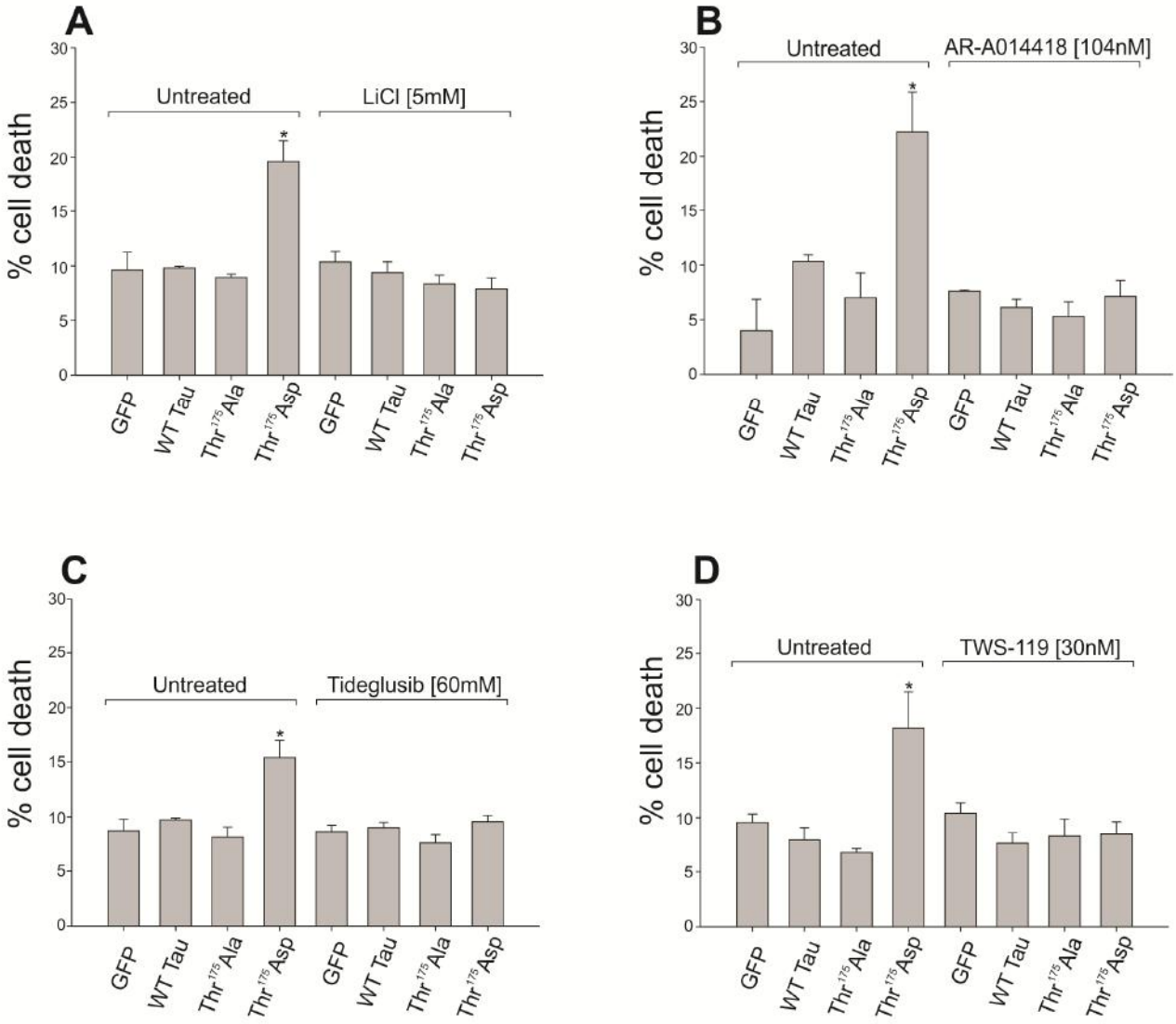
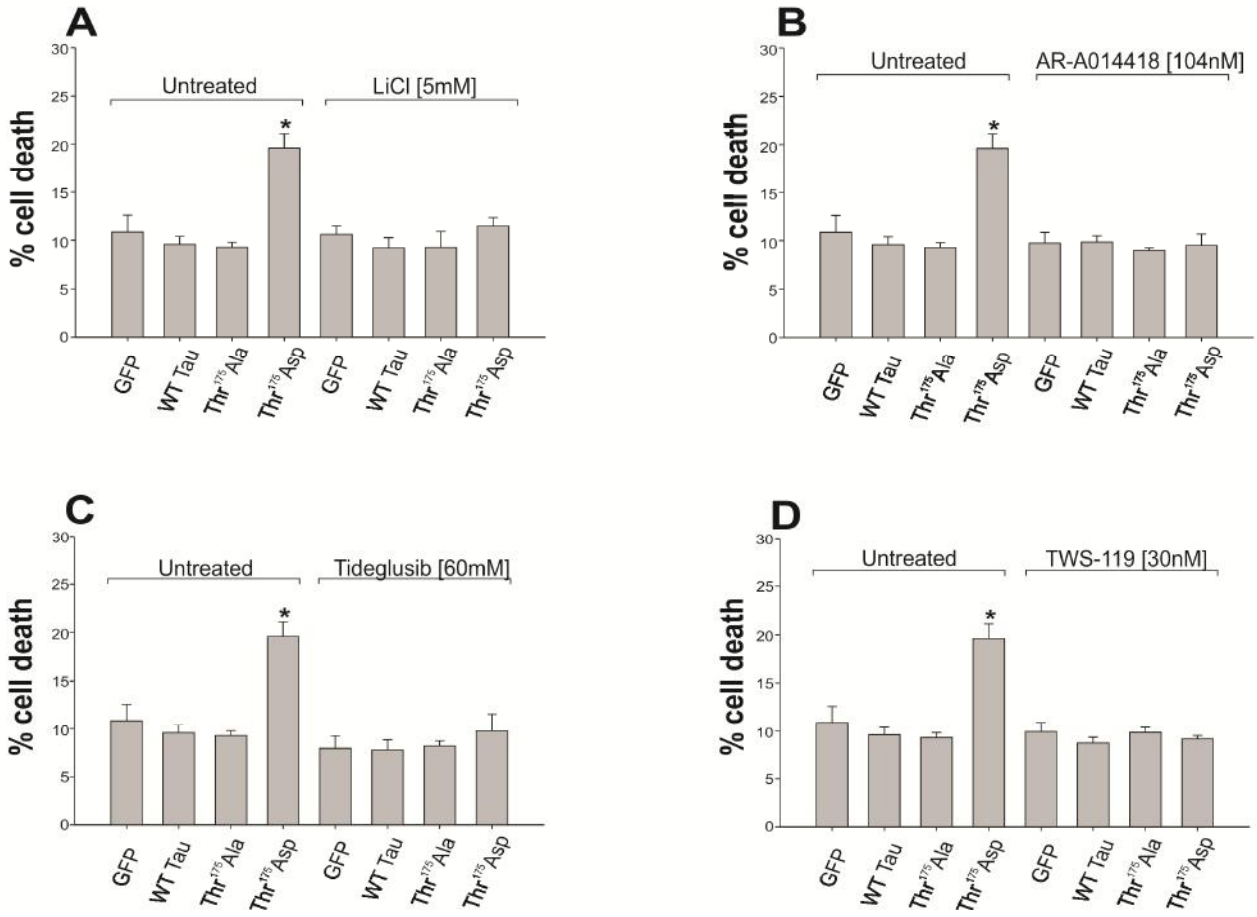


Figure 2.5: Thr^{175}Asp induced tau cell death is alleviated by GSK3β inhibition. A) 5 mM LiCl. B) 104 nM AR-A014418. C) 60 nM Tideglusib. D) 30 nM TWS-119. GFP= GFP transfected group, WT= wild type tau, Thr^{175}Ala= unphosphorylated mutant, Thr^{175}Asp= phosphomimic. TWS= TWS119 treated group, Tide= Tideglusib treated group, LiCl= LiCl treated group, ARA= AR-A014418 treated group. * denotes $p<0.05$ compared to all other groups by post hoc test after one way ANOVA. Values are representative of three independent experiments.



Supplemental figure 2.2: $Thr^{175}Asp$ induced tau cell death is prevented by GSK3β inhibition as determined by trypan blue assay. A) 5 mM LiCl. B) 104 nM AR-A014418. C) 60 nM Tideglusib. D) 30 nM TWS-119. GFP= GFP transfected group, WT= wild type tau, $Thr^{175}Ala$= unphosphorylated mutant, $Thr^{175}Asp$= phosphomimic. TWS= TWS119 treated group, Tide= Tideglusib treated group, LiCl= LiCl treated group, ARA= AR-A014418 treated group. * denotes $p<0.05$ compared to all other groups by post hoc test after one way ANOVA. Values are representative of three independent experiments.

Supplemental Table 2.2: pThr^{175} induced cell death is prevented by GSK3β inhibition. Untreated= no GSK3β inhibitor used, Treated= GSK3β inhibitor administered. GFP= GFP transfected group, WT= wild type tau, Thr^{175}Ala= unphosphorylated mutant, Thr^{175}Asp= phosphomimic. TWS= TWS119 treated group, Tide= Tideglusib treated group, LiCl= LiCl treated group, ARA= AR-A014418 treated group. Values represent mean±SEM % cell death. * indicates p=0.002, ** indicates p<0.001 compared to all other groups in each treatment row after significant ANOVA.

Treatment	**GFP**		**WT**		**Thr^{175}Ala**		**Thr^{175}Asp**	
	Untreated	**Treated**	**Untreated**	**Treated**	**Untreated**	**Treated**	**Untreated**	**Treated**
5 mM LiCl	10±2	10±1	10±0	9±1	9±0	8±1	20±2**	8±1
104 nM AR-A014418	4±3	8±0	10±1	6±1	7±2	5±1	22±4**	7±1
60 nM Tideglusib	9±1	9±1	10±0	9±0	8±1	8±1	15±2**	10±1
30 nM TWS-119	10±1	10±1	8±1	8±1	7±0	8±2	18±3*	8±2

2.4.6. Thr^{231} phosphorylation is necessary for Thr^{175} mediated fibril formation:

To test if Thr^{231} is a downstream mediator of Thr^{175}Asp induced pathological fibril formation, we constructed GFP-tagged double mutant tau protein with either a Thr^{231}Ala or Thr^{231}Asp mutation, with or without Thr^{175}Asp. A total of 6 constructs were tested. All constructs formed fibrils to a baseline extent as previously observed in other constructs (Supplemental table 2.3, Figure 2.6). However, those containing the Thr^{231}Ala mutation did not form increased fibrils, regardless of Thr^{175} phosphorylation state, while all Thr^{231}Asp mutants formed increased fibrils relative to baseline regardless of Thr^{175} phosphorylation status (increased relative to others with $p<0.001$ tukey's post-hoc test after one way ANOVA with $p<0.001$, and $F=60.087$). This suggests that phosphorylation at Thr^{231} is key to pathological fibril formation.

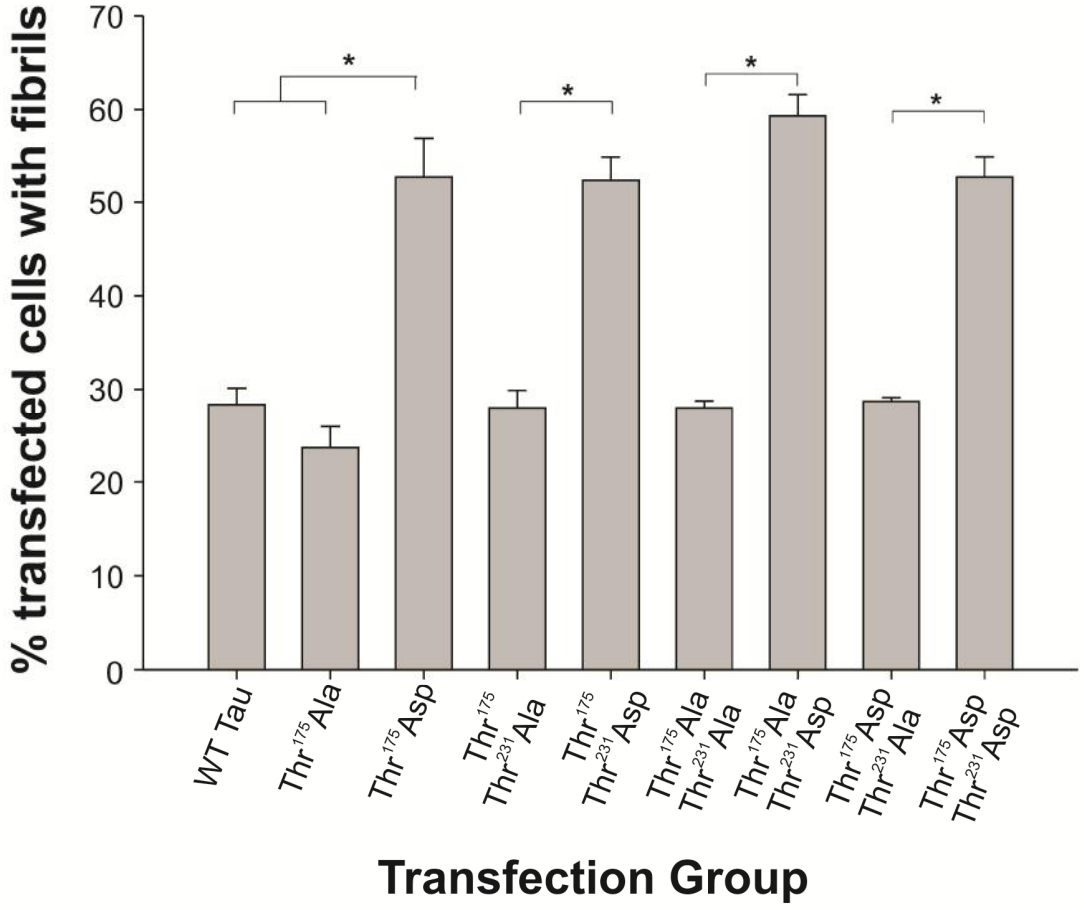


Figure 2.6: Thr^{231} phosphorylation is required for pathological fibril formation in Thr^{175} mutant tau. WT= wild type tau; Thr^{175}Ala= unphosphorylated mutant; Thr^{175}Asp= phosphomimic; Thr^{175} /Thr^{231}Ala= phosphorylation inhibited only at Thr^{231}; Thr^{175} /Thr^{231}Asp= phosphomimic only at Thr^{231}; Thr^{175}Ala /Thr^{231}Ala= phosphorylation prevented at Thr^{175} and Thr^{231}; Thr^{175}Ala /Thr^{231}Asp= phosphorylation prevented at Thr^{175} but phosphomimic at Thr^{231}; Thr^{175}Asp /Thr^{231}Ala= phosphomimic at Thr^{175} but phosphorylation prevented at Thr^{231}; Thr^{175}Asp /Thr^{231}Asp= phosphomimic at both Thr^{175} and Thr^{231}. * denotes $p<0.001$ post hoc after one way ANOVA. Values are representative of three independent experiments.

Supplemental Table 2.3: Tau fibril formation in Thr^{231} mutant containing constructs. WT Thr^{175}= wild type tau at Thr^{175}; Thr^{175}Ala= unphosphorylated mutant at Thr^{175}; Thr^{175}Asp= phosphomimic at Thr^{175}. WT Thr^{231}= wild type tau at Thr^{231}; Thr^{231}Ala= unphosphorylated mutant at Thr^{231}; Thr^{231}Asp= phosphomimic at Thr^{231}.Values represent mean±SEM % of GFP-tau expressing cells exhibiting fibrils. * denotes $p<0.05$ tukey's post-hoc test after one way ANOVA ($p<0.001$, $F=60.087$).

Group	**WT Thr^{231}**	**Thr^{231}Ala**	**Thr^{231}Asp**
WT Thr^{175}	28±1	28±1	52±2 *
Thr^{175}Ala	24±2	28±1	59±2 *
Thr^{175}Asp	53±3 *	29±0	53±2 *

2.4.7. Thr^{175}Asp mediated phosphorylation of Thr^{231} is not dependent on primed phosphorylation at Ser^{235}:

To test if Ser^{235} phosphorylation is necessary to prime phosphorylation of Thr^{231} in response to Thr^{175}Asp, site directed mutagenesis was used to create GFP-tagged tau protein with Ser^{235}Ala and Ser^{235}Asp mutations. A total of 6 mutants were made, each having a combination of Thr^{175} mutations as discussed previously. Ser^{235}Ala mutations did not prevent fibril formation in Thr^{175}Asp mutant transfected cells (increased relative to all others with $p<0.001$ Tukey's post-hoc test after one way ANOVA with $p<0.001$, and $F=70.537$), indicating that the mechanism of primed Thr^{231} phosphorylation is not necessary for the downstream pathology after Thr^{175} phosphorylation (Figure 2.7, Supplemental table 2.4). Ser^{235}Asp mutations did not increase fibril formation in the absence of Thr^{175} phosphomimic, indicating that its presence is not permissive to fibril formation either.

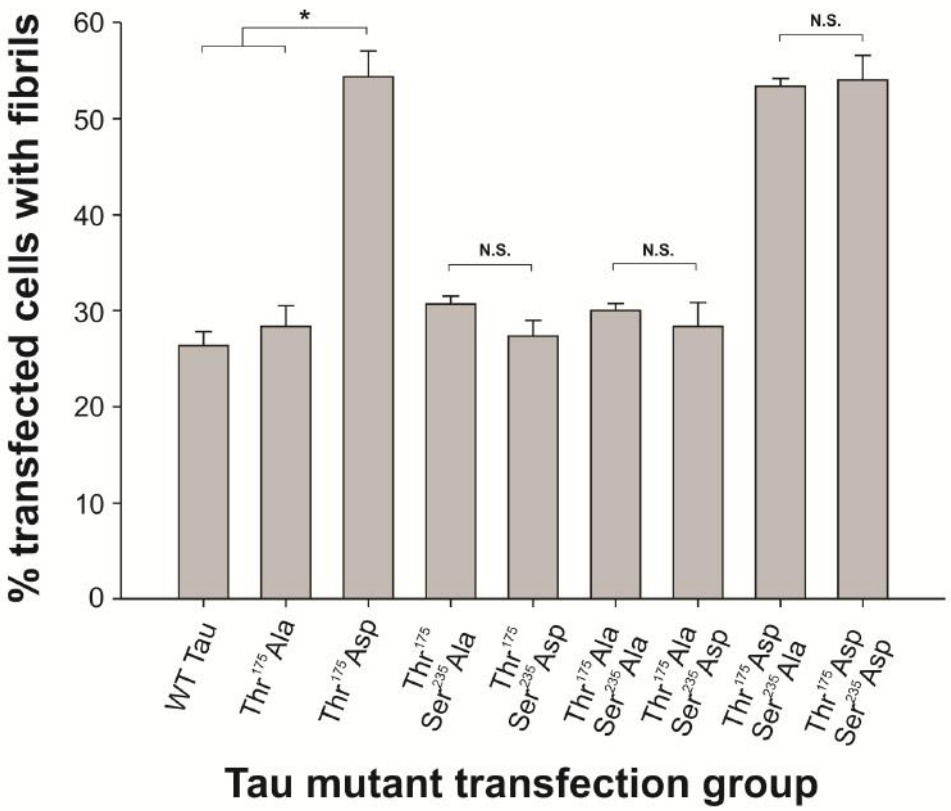


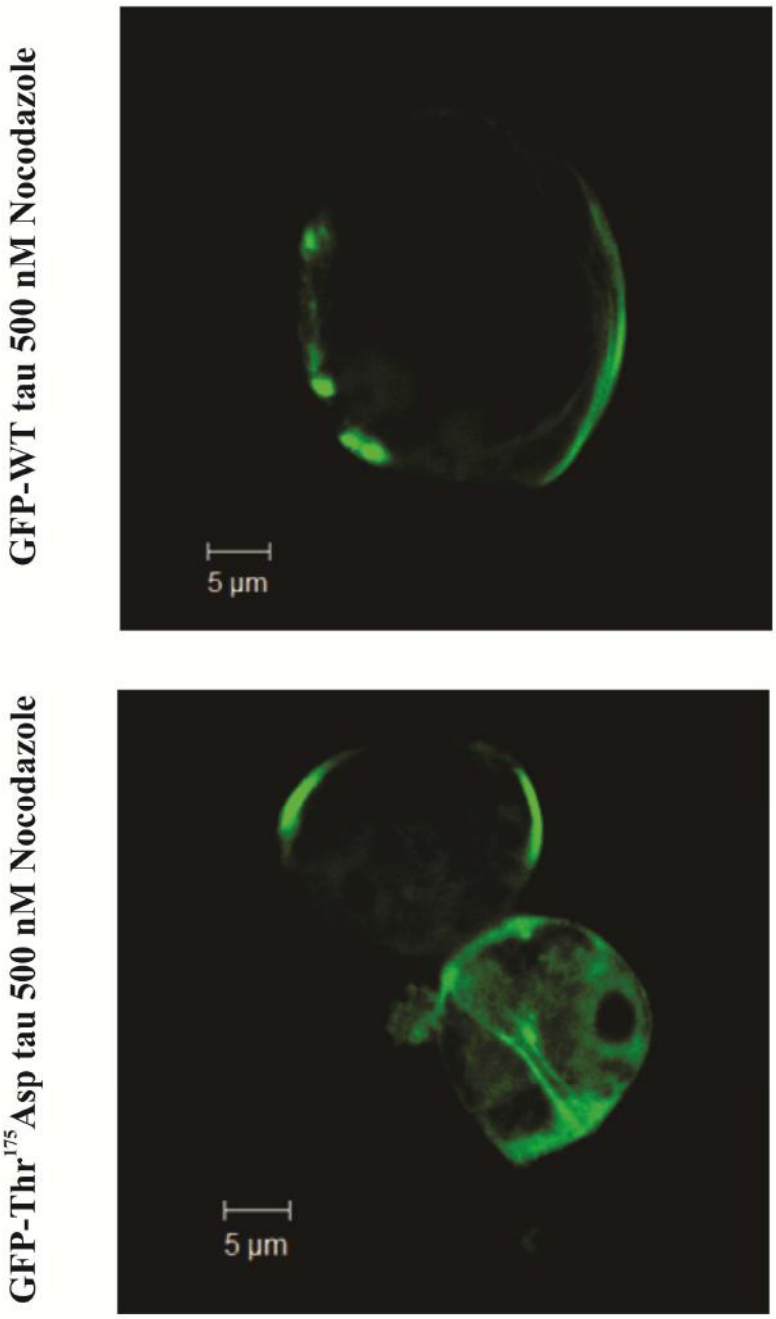
Figure 2.7: Ser^{235} phosphorylation is not required for Thr^{175} mediated fibril formation. . WT= wild type tau; Thr^{175}Ala= unphosphorylated mutant; Thr^{175}Asp= phosphomimic; Thr^{175} /Ser^{235}Ala= phosphorylation inhibited only at Ser^{235}; Thr^{175} Ser$^{235/}$Asp= phosphomimic only at Ser^{235}; Thr^{175}Ala /Ser^{235}Ala= phosphorylation prevented at Thr^{175} and Ser^{235}; Thr^{175}Ala /Ser^{235}Asp= phosphorylation prevented at Thr^{175} but phosphomimic at Ser^{235}; Thr^{175}Asp /Ser^{235}Ala= phosphomimic at Thr^{175} but phosphorylation prevented at Ser^{235}; Thr^{175}Asp /Ser^{235}Asp= phosphomimic at both Thr^{175} and Ser^{235}. * denotes $p<0.001$ post hoc after one way ANOVA. Values are representative of three independent experiments.

Supplemental Table 2.4: Tau fibril formation in Ser^{235} mutant containing constructs. WT Thr^{175}= wild type tau at Thr^{175}; Thr^{175}Ala= unphosphorylated mutant at Thr^{175}; Thr^{175}Asp= phosphomimic at Thr^{175}. WT Ser^{235}= wild type tau at Ser^{235}; Ser^{235}Ala= unphosphorylated mutant at Ser^{235}; Ser^{235}Asp= phosphomimic at Ser^{235}.Values represent mean±SEM % of GFP-tau expressing cells exhibiting fibrils. * denotes $p<0.001$ tukey's post-hoc test after one way ANOVA ($p<0.001$, $F=70.537$).

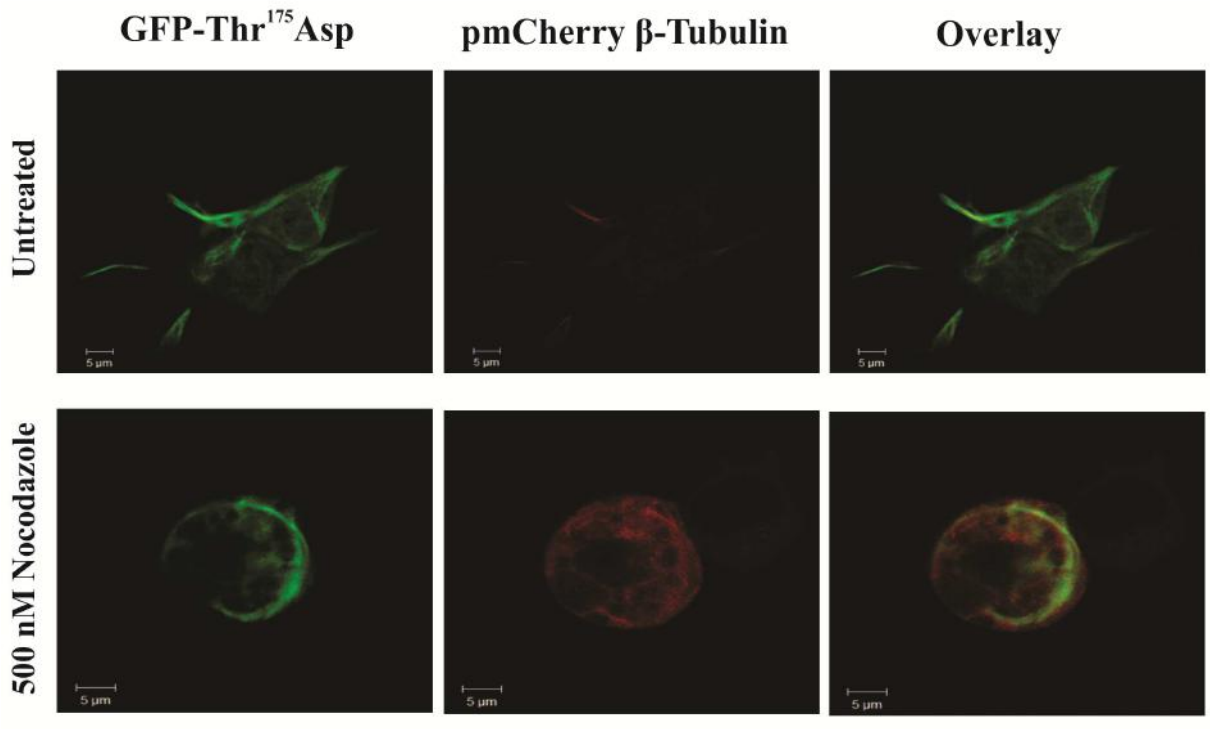
Group	**WT Ser^{235}**	**Ser^{235}Ala**	**Ser^{235}Asp**
WT Thr^{175}	26±1	31±1	27±1
Thr^{175}Ala	28±2	30±1	28±2
Thr^{175}Asp	54±2 *	53±1 *	54±2 *

2.4.8. Tau fibrils persist after nocodazole exposure

After 1 hour exposure to nocodazole, live cell confocal imaging showed that GFP-tau protein fibril structures were still present in cells transfected with both wild type GFP-tau protein and Thr^{175}Asp tau protein (Supplemental figure 2.3). 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 protein fibrils (Supplemental figure 2.4).



Supplemental figure 2.3: Thr^{175}Asp induced tau fibrils persist after 1 hour nocodazole treatment. Cells were treated with 500 nM nocodazole for 1 hour prior to live cell confocal imaging. WT= wild type tau, Thr^{175}Asp= phosphomimic. Images shown are live cells transfected with tau constructs. This experiment was performed once as a proof of concept. Images taken at 63x magnification.



Supplemental figure 2.4: β-tubulin co-localization with tau protein is lost in nocodazole treated cells. Untreated= double-transfected cells without nocodazole. 500 nM nocodazole= cells treated with 500 nM nocodazole for 1 hr. GFP-Thr^{175}Asp= Tau protein phosphomimic. pmCherry β-tubulin= β-tubulin. Overlay= both tau and tubulin channels. Images shown are live cells transfected with both tau and β-tubulin constructs. This experiment was performed once as a proof of concept. Images taken at 63x magnification.

2.4.9. Tau fibrils are not composed of β-tubulin

Co-localization of β-tubulin and tau protein 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 (Figure 2.8). This effect was especially clear when nocodazole treated cells showed the persistence of tau fibrils, but not the extending processes consisting of both proteins (Supplemental figure 2.4).

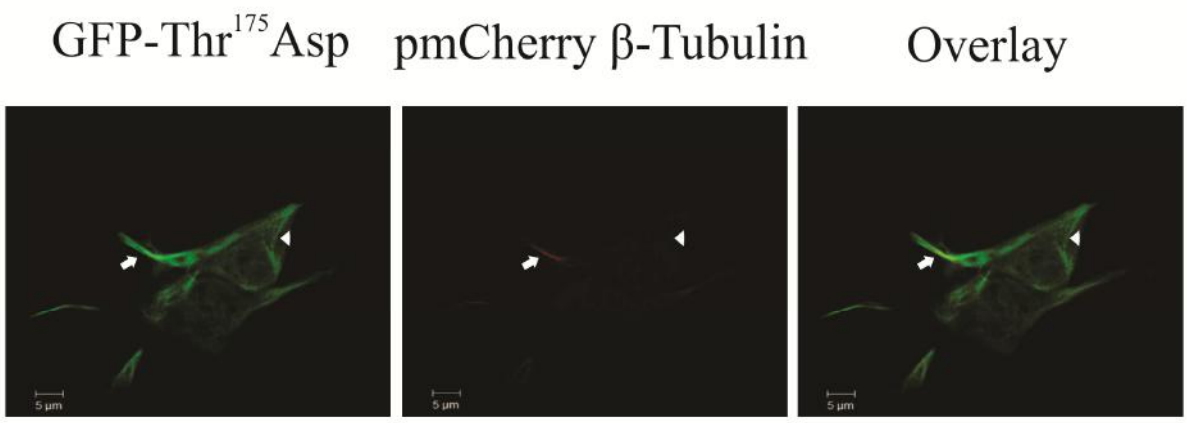


Figure 2.8: β-tubulin does not co-localize with tau protein in fibrils. Representative confocal micrograph depicting tau protein fibril in cell body (arrowhead) lacking tubulin co-localization. Co-localization occurs in the processes extending out from the cell (arrow). GFP-Thr^{175}Asp= Tau protein phosphomimic. pmCherry β-tubulin= β-tubulin. Overlay= both tau protein and tubulin channels. Images shown are live cells transfected with both tau protein and β-tubulin constructs. This experiment was performed once as a proof of concept. Images taken at 63x magnification.

2.5. Discussion:

We have demonstrated that phosphorylation of Thr^{175} induces pathological fibril formation by inducing GSK3β activation, which in turn leads to unprimed Thr^{231} phosphorylation. The latter step is both necessary and sufficient for the formation of pathological fibrils. Inhibition of this event using any of a panel of GSK3β inhibitors resulted in reduced fibril formation, and reduced 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 Thr^{231} in mediating a conformational change in tau protein has been previously described (Lin et al., 2007) and shown to have functional implications on tau protein's microtubule binding ability. Of note is its proximity to a bend in what has been proposed as tau protein's soluble global hairpin structure (Jeganathan et al., 2006). In this structure, the N-terminus of tau protein folds over the C-terminus, effectively sheltering 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 protein, conferring the ability to self-interact, effectively seeding insoluble aggregates with itself beginning with dimerization through cross linking of β pleated sheets located within the microtubule binding domains which are normally sheltered by the C- and N-termini (von Bergen M. et al., 2000).

Because GSK3β activity is enhanced by tau protein priming where an amino acid at the n+4 site has already been phosphorylated and because Ser^{235} is commonly found to be phosphorylated in conjunction with Thr^{231} (Cho and Johnson, 2004b), it was expected that Ser^{235} phosphorylation would enhance the observed pathology. However, we observed no impact of Ser^{235} phosphorylation on the extent of tau protein fibril formation, suggesting that GSK3β acts

on Thr^{231} in the absence of priming in response to $pThr^{175}$. This is not completely unexpected as GSK3β is known to phosphorylate tau protein 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 Thr^{231} 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 protein pathology is consistent with previous reports of GSK3β overexpression-induced neurodegeneration in transgenic mice (Lucas et al., 2001). Of specific importance to this study, GSK3β activity is also associated with phosphorylation at Thr^{231} (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 protein phosphorylation by GSK3α (Maurin et al., 2013), we have established that in our model there is no role for GSK3α in the pathological phosphorylation of tau protein downstream of Thr^{175} phosphorylation. This is in keeping with previous reports of the modulation of GSK3α and GSK3β activity. While GSK3β

can be highly upregulated, with increases in expression and activity, GSK3α is relatively consistently expressed across the lifespan (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 protein phosphorylation in normal development, as well as increased expression in the diseased state when tau protein phosphorylation is increased (Pei et al., 1997; Yang et al., 2008).

In this study, only the 2N4R isoform of tau protein was assessed. Of note, it has been shown that differential phosphorylation patterns have can have different, or even opposite effects on different tau protein 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 protein isoforms in the insoluble fraction from ALSci brains (Strong et al., 2006). The expression of all 6 isoforms in ALSci insoluble tau protein is consistent with the observation that Thr^{175}Asp tau protein induced aggregation in cells transfected with all 6 tau protein isoforms equally (Gohar et al., 2009). Therefore, the analysis of only the longest tau protein 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 Thr^{231} phosphorylation is necessary and sufficient for the induction of fibril formation after Thr^{175} phosphorylation does 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.

In order 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 overexpression (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 pathological tau protein modification in these experiments.

2.6. Conclusions:

These findings represent the first time a cascade-like sequence of phosphorylation events underpinning the induction of pathological tau protein aggregates in ALSci has been described. The focus of these studies was on the downstream effects of Thr^{175} phosphorylation. How this site is phosphorylated will be the subject of future investigations, and 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 Thr^{175}Asp expression are currently in progress.

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Chapter 3: Threonine^{175}, a novel pathological phosphorylation site on tau protein linked to multiple tauopathies.

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3.1 Abstract:

Microtubule associated protein tau (tau protein) deposition is associated with a spectrum of neurodegenerative diseases collectively termed tauopathies. We have previously shown that amyotrophic lateral sclerosis (ALS) with cognitive impairment (ALSci) is associated with tau protein phosphorylation at Thr^{175} and that this leads to activation of GSK3β which then induces phosphorylation of tau protein at Thr^{231}. This latter step leads to dissociation of tau protein from microtubules and pathological tau protein fibril formation. To determine the extent to which this pathway is unique to ALS, we have investigated the expression of $pThr^{175}$ tau protein and $pThr^{231}$ tau protein across a range of frontotemporal degenerations. Representative sections from the superior frontal cortex, anterior cingulate cortex (ACC), amygdala, hippocampal formation, basal ganglia, and substantia nigra were selected from neuropathologically confirmed cases of Alzheimer's disease (AD; n=3), vascular dementia (n=2), frontotemporal lobar degeneration (FTLD; n=4), ALS (n=5), ALSci (n=6), Parkinson's disease (PD; n=5), corticobasal degeneration (CBD; n=2), diffuse Lewy body dementia (DLBD; n=2), mixed DLBD (n=3), multisystem atrophy (MSA; n=6) and Pick's disease (n=1) and three neuropathologically-normal control groups aged 50-60 (n=6), 60-70 (n=6) and 70-80 (n=8). Sections were examined using a panel of phospho-tau protein antibodies ($pSer^{208,210}$, $pThr^{217}$, $pThr^{175}$, $pThr^{231}$, $pSer^{202}$ and T22 (oligomeric tau)). Across diseases, phospho-tau load was most prominent in layers II/III of the entorhinal cortex, amygdala and hippocampus. This is in contrast to the preferential deposition of phospho-tau protein in the ACC and frontal cortex in ALSci. Controls showed $pThr^{175}$ tau protein expression only in the 7^{th} decade of life and only in the presence of tau protein pathology and tau protein oligomers. With the exception of DLBD, we observed $pThr^{175}$ co-localizing with

pThr^{231} in the same cell populations as T22 positivity. This suggests that this pathway may be a common mechanism of toxicity across the tauopathies.

3.2 Introduction:

Microtubule associated protein tau (tau protein) is a cytoskeletal stabilizing protein involved in microtubule maintenance, fast axonal transport, and other physiological functions in neurons. Tau protein deposition is a characteristic of many neurodegenerative diseases that are collectively referred to as tauopathies. It has been shown that pathological species of tau protein are abnormally phosphorylated at multiple residues (Goedert et al., 1994) and that this is linked to a decrease in tau protein's ability to bind to and stabilize microtubules (Bramblett et al., 1993; Lin et al., 2007) with accompanying cytotoxicity (Bandyopadhyay et al., 2007). While the isoform composition of insoluble tau protein deposits and the structural formation of the protein aggregates differs, there are several phosphorylation sites that are thought to be universally important in induction of a tauopathy.

One potentially important phosphorylation site that has gone relatively unstudied is threonine^{175} (Thr^{175}). First identified in Alzheimer's disease as a phosphoepitope (Hanger et al., 1998), it was then determined that this site could be phosphorylated by multiple kinases linked to tau protein pathology including GSK3β, JNK, ERK2, and p38 (Reynolds et al., 2000). pThr^{175} tau protein was then identified in amyotrophic lateral sclerosis with cognitive impairment (ALSci) (Strong et al., 2009) and characterized in further detail in the context of this disease (Behrouzi et al., 2016; Strong et al., 2006; Yang et al., 2003; Yang et al., 2008; Yang and Strong, 2012). Importantly, pThr^{175} tau protein has been shown to induce tau protein fibril formation and cell death *in vitro* (Gohar et al., 2009). Unlike other widely accepted pathological

phosphorylation sites on tau protein, such as $pThr^{231}$ and $pSer^{202}$, $pThr^{175}$ has not been observed in the fetal brain where tau protein is hyperphosphorylated (Brion et al., 1993; Kenessey and Yen, 1993; Watanabe et al., 1993), suggesting that this site may be uniquely associated with pathological processes. $pThr^{175}$ tau protein has been shown to induce GSK3β activation in cell culture, and may therefore act as a destabilizing event resulting in enhanced phosphorylation of tau protein at other residues, resulting in dissociation from microtubules and neuronal toxicity (Moszczynski et al., 2015). In order to understand the extent to which this pathway of $pThr^{175}$ mediated tau protein aggregate formation underlies a broad range of tauopathies, we have used a panel of phospho-specific antibodies to characterize tau protein pathology with specific interest in the expression of $pThr^{175}$ tau protein across a broad range of tauopathies.

3.3 Methods:

Diseases studied included Alzheimer's (AD; 3 cases), vascular dementia (VD; 2 cases), ALS (5 cases), ALSci (6 cases), dementia with Lewy Bodies (DLBD; 2 cases), DLBD with mixed pathology (mDLBD; 3 cases including 2 with DLBD/VD and 1 with DLBD/AD)), frontotemporal lobar dementia (FTLD-TDP; 3 cases including one with a pathological C9orf72 hexanucleotide expansion with Type B pathology; a single case with Type A pathology and a single case with Type B pathology, FTLD-Tau; 1 case with familial history and no known mutations) (Mackenzie et al., 2011), multiple system atrophy (MSA; 6 cases), Parkinson's disease (PD; 5 cases), Pick's disease (1 case), and corticobasal degeneration (CBD; 2 cases) (Table 3.1). The institutional research ethics board approved the protocol and consent was given for use of all tissue used in this study. All neuropathological diagnosis were performed by a neuropathologist (RH, LCA). For all comparisons, we grouped the staining according to ALS (n=5), ALSci (n=6), or other tauopathy (n=22).

To assess the extent of pThr^{175} tau protein, pThr^{231} tau protein and tau protein oligomer pathological inclusions as a function of ageing, three groups of controls were studied, encompassing the 6th (n=6), 7th (n=6), and 8th (n=8) decades of life (Table 3.1). Hippocampal sections from each group were stained for pThr^{175} tau protein, pThr^{231} tau protein and oligomeric tau protein (T22). These cases have been previously characterized in a study examining age-dependant tau protein deposition in the frontal and entorhinal cortices and were shown to be free of neurodegenerative disease (Yang et al., 2005).

Table 3.1: Case demographics

Neuropathological diagnosis	Age	n (n Male)
AD	72±8	3 (2)
VD	78±11	2 (1)
ALS	56±16	5 (4)
ALSci	64±11	6 (5)
DLBD	68±1	2 (2)
mDLDB	83±6	3 (3)
FTLD	64±9	4 (1)
MSA	69±12	6 (3)
PD	77±2	5 (4)
Pick's	70±2	2 (2)
CBD	71±1	2 (1)
Control 1	55±2	4 (3)
Control 2	64±2	4 (3)
Control 3	75±3	4 (2)

AD: Alzheimer's disease, VD: Vascular dementia, ALS: amyotrophic lateral sclerosis, ALSci: ALS with cognitive impairment, DLBD: diffuse Lewy body dementia, mDLBD: Lewy body dementia with mixed pathology, FTLD: frontotemporal lobar dementia, MSA: multiple system atrophy, PD: Parkinson's disease, Pick's: Pick's disease, CBD: corticobasal degeneration. Control 1: 6^{th} decade control group, Control 2: 7^{th} decade control group, Control 3: 8^{th} decade control group.

Five to six micrometer paraffin-embedded sections from the superior frontal gyrus, anterior cingulate (ACC), hippocampus, entorhinal cortex, dentate gyrus, amygdala, basal ganglia and substantia nigra were used for all immunohistochemical analyses.

Cases were stained by haematoxylin and eosin (H&E) and Gallyas silver stain for routine histological analysis and overall pathology characterization. Immunohistochemistry was conducted using a series of antibodies (Table 3.2) previously characterized in ALSci (Yang and Strong, 2012), consisting of PHF tau protein (AT8; Thermo Fischer IL, Canada), $pThr^{175}$ tau protein, $pSer^{208,210}$ tau protein, $pThr^{217}$ tau protein (antibodies generated and designed in house (Yang and Strong, 2012), $pThr^{175}$ commercially available through 21st Century, MA, USA).

Table 3.2: Antibodies used

Antibody	**Clone**	**Titer**	**Antigen retrieval**	**Epitope**	**Company**
Tau pThr^{175}	Rabbit, polyclonal	1:1000	1	pThr^{175}	21st Century
Tau pThr^{217}	Rabbit, polyclonal	1:1000	1	pThr^{217}	21st Century
Tau pSer$^{208, 210}$	Rabbit, polyclonal	1:1000	1	pSer$^{208, 210}$	21st Century
PHF (AT8)	Mouse, monoclonal	2.5 ug/ml	No	pSer^{202}	Thermo Fischer
Tau pThr^{231}	Rabbit, polyclonal	1:1000	2	pThr^{231}	Thermo Fischer
T22	Rabbit, polyclonal	1:500	2	Tau oligomer	EMD Millipore
Alexa Fluor 488	Goat anti-rabbit	1:200	2	Secondary	Life Technologies

1) Boil in 10mM sodium citrate, 0.05% Tween 20 pH 6.0 for 2 min.

2) Pressure cooker (2100 Retriever; Aptum Biologics, UK) 10mM sodium citrate, 0.05% Tween 20 pH 6.0 for 15 min.

Antigen retrieval was conducted as necessary (Table 3.2). Endogenous peroxidase was quenched with 3% hydrogen peroxide (BDH Chemicals, VWR, On, Canada). Primary antibody incubation was performed at 4˚C overnight in blocking buffer (5% BSA, 0.3% Triton-X 100 in 1 X PBS). After washing, secondary antibody (1:200 biotinylated IgG) incubation was performed for 1 hour at room temperature in blocking buffer. Antigen:antibody complex was visualized with either horseradish peroxidase or alkaline phosphatase according to the manufacturer's instructions (Vectastain ABC kit, Vector Laboratories CA, USA), followed by substrate development with either DAB plus $NiCl_2$ or AP substrate kit III (Vector Laboratories). Counterstaining was performed using haematoxylin or nuclear fast red. The extent of pathology was described topographically and semi-quantitatively as previously reported (Yang and Strong, 2012). Representative images were captured with a 20x lens under light microscopy (Olympus BX45) and subsequently used for semi-quantitative analysis. The semi-quantitative scale was manually applied for each type of pathology by an evaluator blinded to the underlying diagnosis (WY) (neuronal, neuritic, or glial) separately as follows: '-' = none; '±' = less than 5 inclusions; '+' = less than 10 inclusions; '++' = more than 20 inclusions with scattered distribution; '+++' = more than 20 inclusions but with locally dense distribution; '++++' = more than 20 inclusions with a diffuse distribution. Additionally, the case positive ratio was defined for each antibody used and brain region investigated as the number of cases showing any pathology (± or more) compared to the total number of cases stained.

3.3.1 Oligomeric tau and $pThr^{231}$ staining:

Rabbit anti T22 (EMD Millipore CA, USA) and rabbit anti tau $pThr^{231}$ (Thermo Fischer) were used to probe tau protein inclusions for the presence of oligomeric tau protein (T22) and for phosphorylation at Thr^{231}. Tau protein oligomeric species are currently hypothesized to be more

toxic to neurons than the fibrillar inclusions themselves (Ward et al., 2012), and $pThr^{231}$ is thought to be a key site in the regulation of tau protein folding and ability to interact with microtubules (Lin et al., 2007; Schwalbe et al., 2015). Double labeling was performed on hippocampus from one case each from AD, ALSci, FTD, MSA, DLDB, and mDLDB. Tau protein was probed for $pThr^{175}$ using rabbit primary antibody (1:1000) overnight at 4˚C and Alexa Fluor goat anti-rabbit 488nm secondary (1:200, Thermo Fischer) for 1 hour at room temperature. Rabbit anti tau $pThr^{231}$ antibody was then labeled using a Zenon primary antibody labeling kit with Alexa Fluor 555nm dye (Thermo Fisher) and probed for 1 hour at room temperature. Slides were stored overnight at 4˚C and visualized within 24 hours of labeling by confocal imaging on a Zeiss LSM 510 Meta NLO multiphoton confocal microscope.

3.4 Results:

3.4.1 Tau antibody staining:

3.4.1.1 Neuronal tau:

ALS: Consistent with our earlier reports, we observed tau protein pathology in multiple brain regions in ALS, although to a lesser degree than either ALSci or the remaining tauopathies. Neuronal tau protein inclusions were most consistently observed in the entorhinal cortex, hippocampus, and amygdala. All antibodies were immunoreactive with neuronal tau protein inclusions in multiple brain regions (Table 3.3). Frontal and anterior cingulate pathology was limited in both load and case-positive incidence. In all regions studied, inclusions took the form of punctate cytosolic inclusions or tangles (Figure 3.1). Deposition was mainly restricted to the superficial cortical layers in the entorhinal cortex but restricted to deeper layers in the ACC and superior frontal cortex when present.

Table 3.3: ALS pathology

Stain	Frontal	Cingulate	Hippocampus	Dentate	Entorhinal	Amygdala	BG	SN
Neuronal								
pThr^{175}	± (1/5)	± (1/4)	± (1/5)	± (1/5)	± (2/4)	++ (1/2)	-(0/5)	- (0/5)
PHF	± (1/5)	± (1/4)	± (3/5)	± (2/5)	±-++ (5/5)	+++ (1/1)	± (1/4)	± (3/4)
pSer^{208,210}	- (0/5)	++ (1/4)	± (1/5)	- (0/4)	± (1/2)	++ (1/2)	- (0/5)	- (0/5)
pThr^{217}	+ (1/5)	± (1/4)	± (1/5)	- (0/4)	±-++ (3/4)	++ (1/2)	± (1/4)	± (2/5)
Glial								
pThr^{175}	++ (1/5)	- (0/5)	- (0/4)	- (0/5)	- (0/2)	± (1/2)	+ (1/5)	- (0/5)
PHF	± (1/5)	± (1/4)	± (1/5)	- (0/5)	+ (1/5)	++ (1/1)	- (0/4)	- (0/4)
pSer^{208,210}	- (0/5)	- (0/4)	- (0/5)	- (0/4)	± (1/2)	++ (1/2)	++ (2/5)	- (0/5)
pThr^{217}	+++ (1/5)	+++ (1/4)	+ (1/5)	- (0/4)	+++ (1/4)	++ (1/2)	++ (2/4)	- (0/5)
Neuritic								
pThr^{175}	- (0/5)	- (0/5)	± (1/4)	- (0/4)	± (2/5)	++ (1/2)	- (0/5)	± (1/5)
PHF	± (2/5)	± (1/4)	± (3/5)	- (0/5)	+-++ (5/5)	++ (1/1)	± (1/4)	± (3/4)
pSer^{208,210}	- (0/5)	± (1/4)	- (0/5)	- (0/4)	± (1/2)	- (0/2)	+ (1/5)	+ (2/5)
pThr^{217}	± (1/5)	- (0/4)	± (1/5)	- (0/4)	±-++ (3/4)	± (1/2)	+-++ (2/4)	+-++ (3/5)

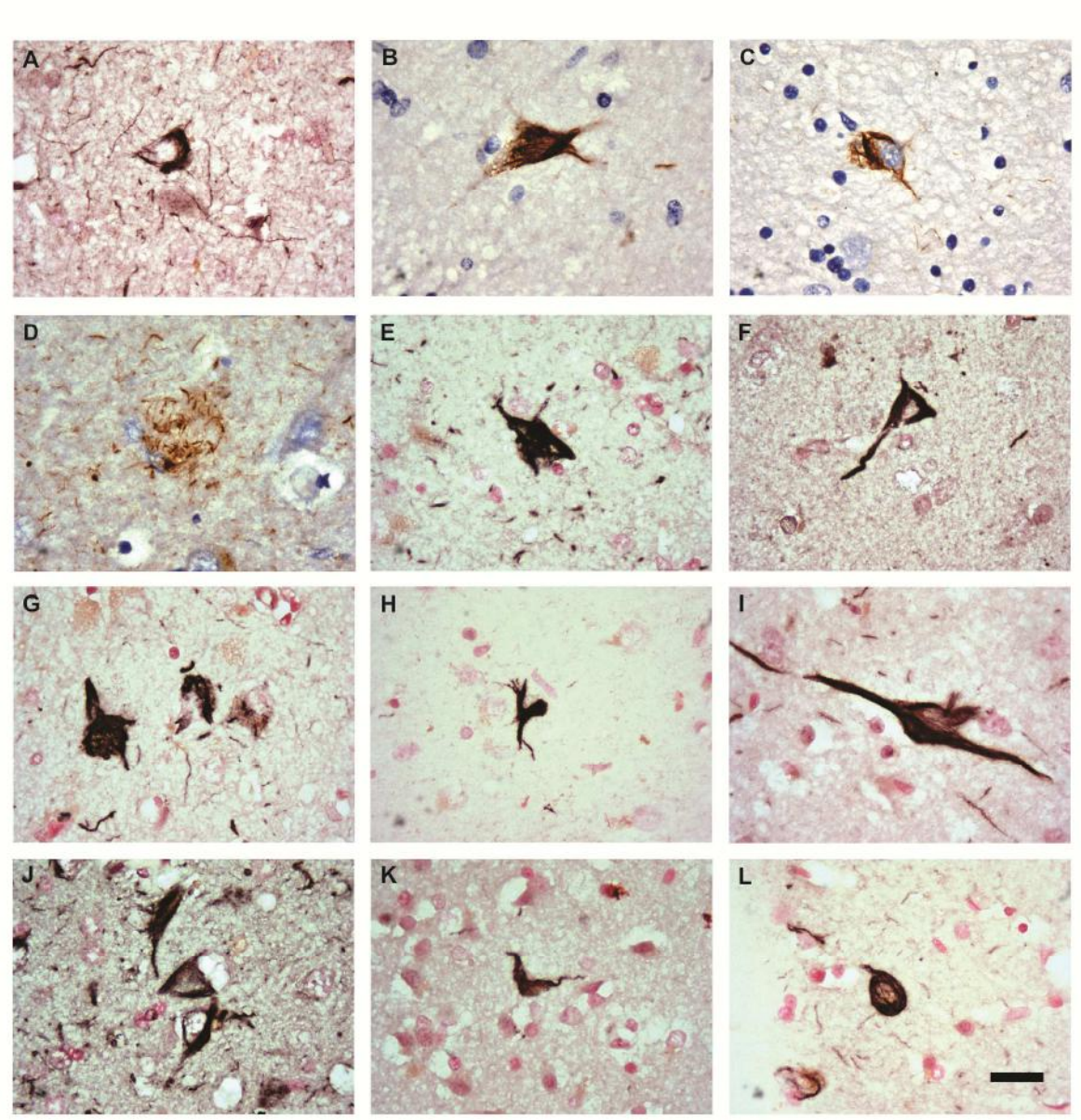
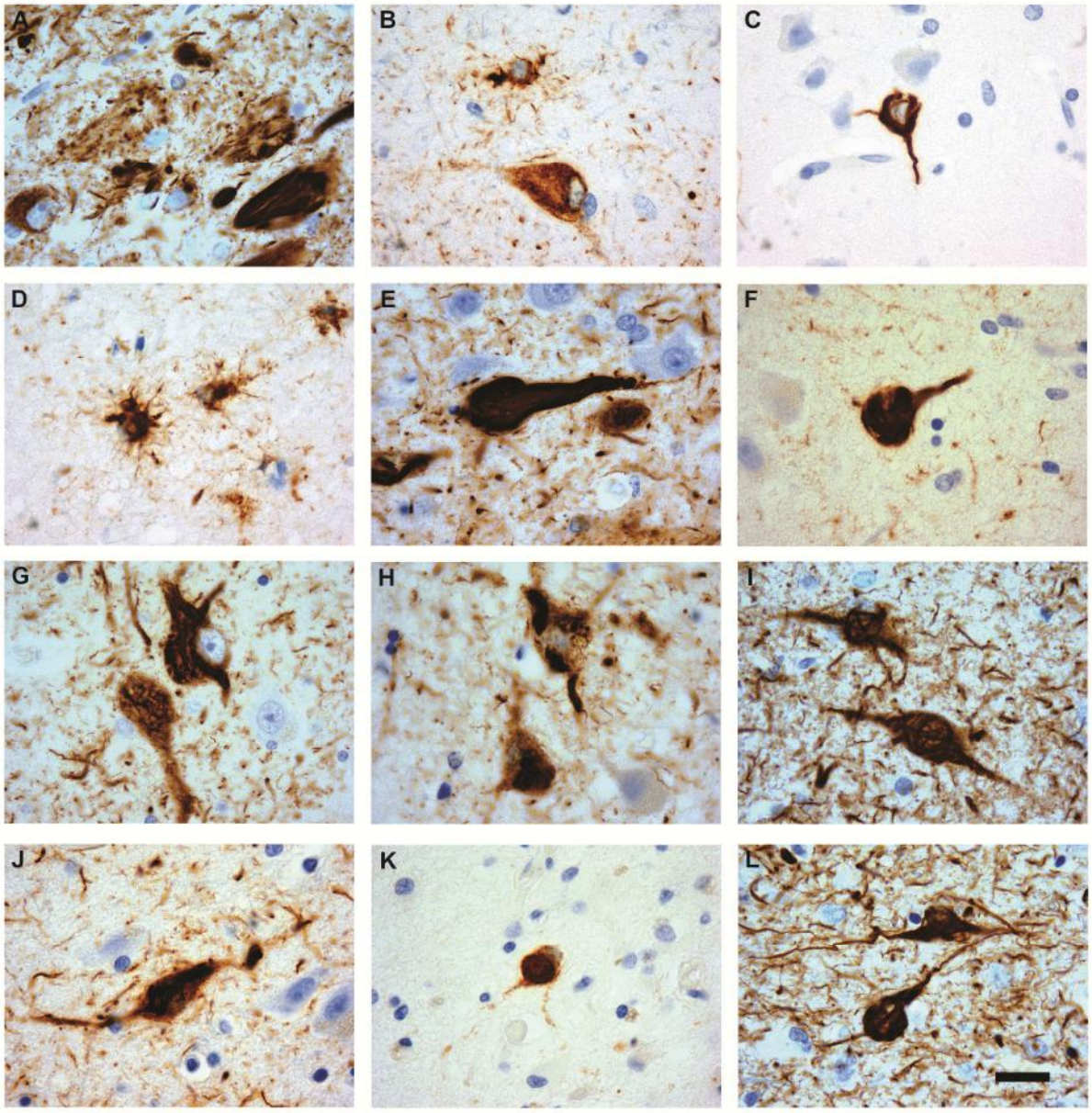
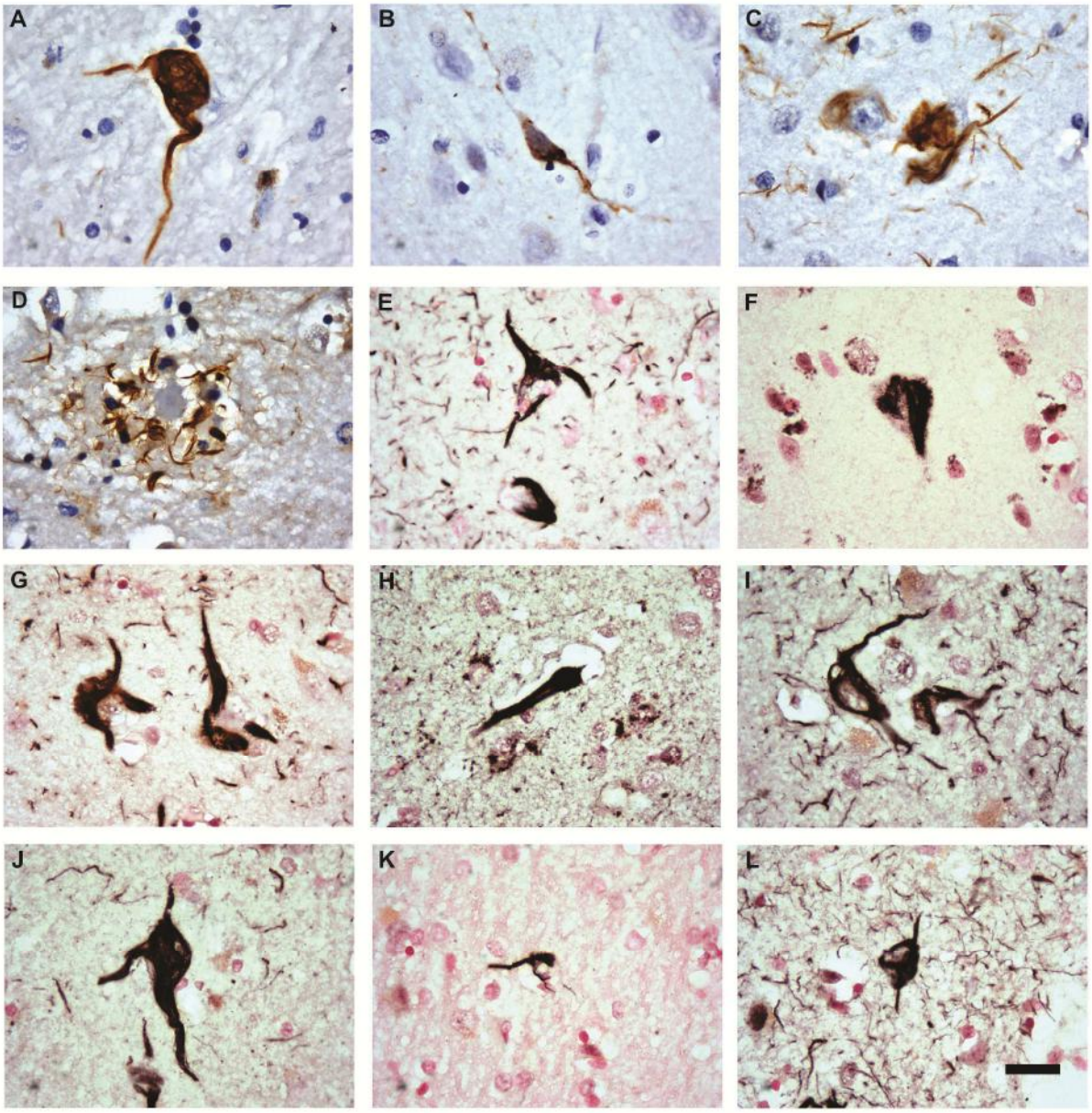


Figure 3.1: Representative pThr^{175} tau pathology in each neurodegenerative disease. A) AD Frontal cortex, B) ALS amygdala, C) ALSci amygdala, D) ALSci hippocampus neuritic plaque, E) CBD entorhinal cortex, F) DLBD amygdala, G) mDLBD entorhinal cortex, H) FTLD putamen, I) MSA amygdala, J) PD amygdala, K) Pick's entorhinal cortex, L) VD anterior cingulate cortex. Nuclear fast red or hematoxylin counterstain used. Original images taken at 100x. Scale bar= 20 μm.

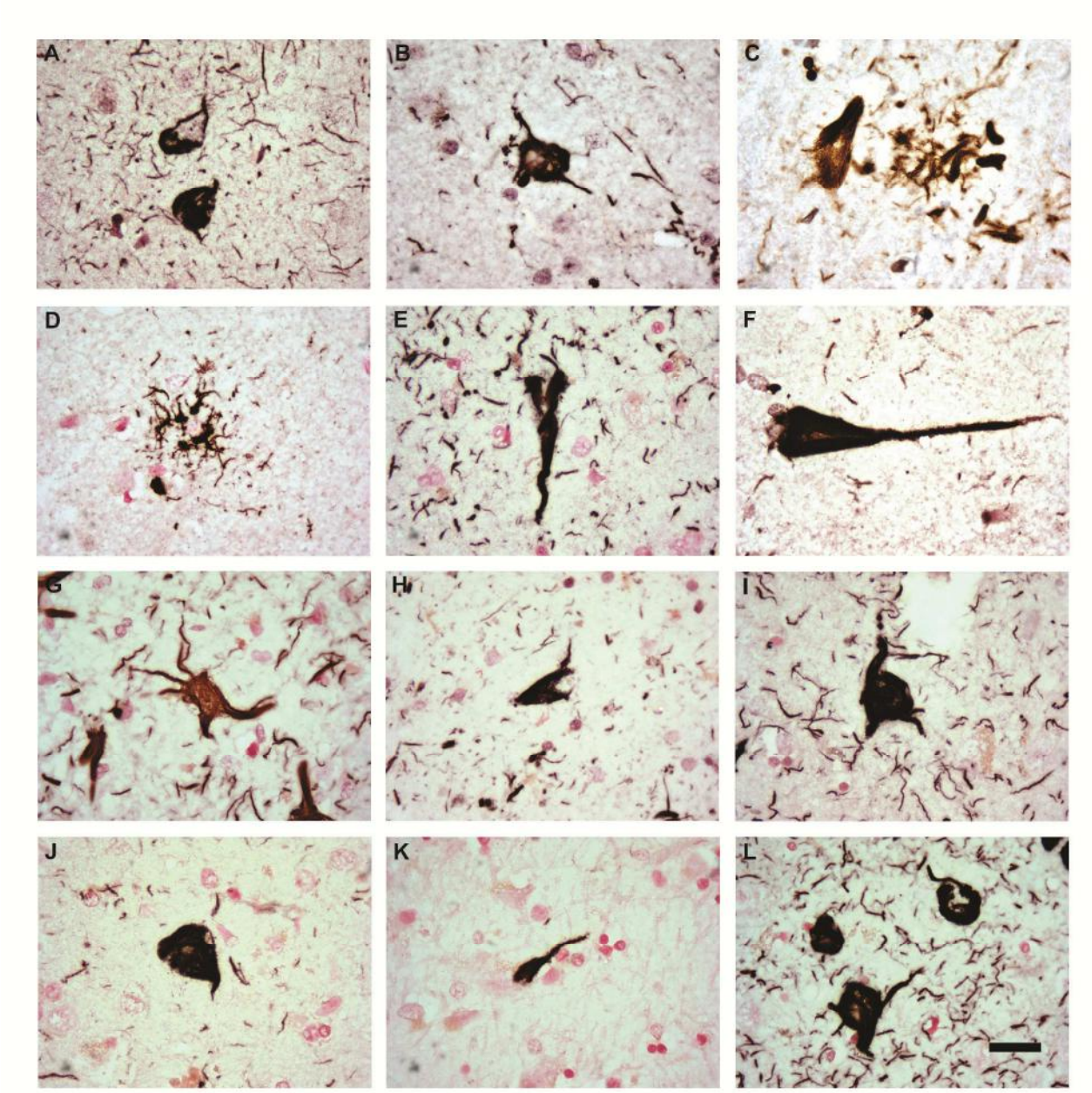
ALSci: Tau protein pathology (Figure 3.1, supplemental figures 3.1, 3.2, 3.3) in the form of tangles, skein-like inclusions, and punctate staining was observed to a greater extent in ALSci than ALS, especially in the ACC and superior frontal cortex. The load of pathology was increased in amount and distribution and the case positive ratio was higher than ALS in all brain regions studied (Table 3.4). As observed in ALS, pathological tau protein neuronal inclusions were observed predominantly in the superficial layers of the entorhinal cortex and within deeper cortical layers in the ACC and superior frontal cortex. However superficial layer involvement was noted in both the ACC and superior frontal cortex in ALSci, indicating a greater distribution across cortical layers in ACC and frontal cortex, further differentiating ALSci from ALS. Of note, Thr^{175} tau and PHF tau identified pathology to different extents in different brain regions. Notably, $pThr^{175}$ tau and $pThr^{217}$ tau identified a higher case positive ratio than PHF in the superior frontal cortex.



Supplemental Figure 3.1: Representative PHF tau pathology in each neurodegenerative disease. A) AD hippocampus, B) ALS amygdala, C) ALSci superior frontal cortex, D) ALSci superior frontal cortex astrocytic tau E) CBD entorhinal cortex, F) DLBD amygdala, G) mDLBD entorhinal cortex, H) FTLD entorhinal cortex, I) MSA amygdala, J) PD entorhinal cortex, K) Pick's amygdala, L) VD anterior cingulate cortex. Nuclear fast red or hematoxylin counterstain used. Original images taken at 100x. Scale bar= 20 μm.



Supplemental Figure 3.2: Representative pSer^{208,210} tau pathology in each neurodegenerative disease. A) AD substantia nigra, B) ALS amygdala, C) ALSci entorhinal cortax, D) ALSci ACC neuritic plaque, E) CBD entorhinal cortex, F) DLBD entorhinal cortex, G) mDLBD amygdala, H) FTLD superior frontal cortex, I) MSA amygdala, J) PD entorhinal cortex, K) Pick's entorhinal cortex, L) VD superior frontal cortex. Nuclear fast red or hematoxylin counterstain used. Original images taken at 100x. Scale bar= 20 μm.



Supplemental Figure 3.3: Representative pThr^{217} tau pathology in each neurodegenerative disease. A) AD anterior cingulate cortex, B) ALS entorhinal cortex, C) ALSci hippocampus, D) ALSci superior frontal cortex astrocytic plaque, E) CBD entorhinal cortex, F) DLBD hippocampus, G) mDLBD amygdala, H) FTLD amygdala, I) MSA amygdala, J) PD entorhinal cortex, K) Pick's entorhinal cortex, L) VD entorhinal cortex. Nuclear fast red or hematoxylin counterstain used. Original images taken at 100x. Scale bar= 20 μm.

Table 3.4: ALSci pathology

Stain	Frontal	Cingulate	Hippocampus	Dentate	Entorhinal	Amygdala	BG	SN
Neuronal								
pThr^{175}	± (4/5)	± (2/5)	± (5/5)	+ (1/5)	+ (4/4)	+ (1/1)	- (0/5)	- (0/3)
PHF	+ (2/4)	± (3/3)	±-+ (4/4)	±-+ (2/4)	±-++ (4/4)	±-++ (2/2)	- (0/5)	± (2/2)
pSer^{208,210}	± (2/5)	± (3/5)	±-++ (4/5)	± (1/5)	±-++ (3/4)	+ (1/1)	- (0/5)	- (0/3)
pThr^{217}	± (5/5)	±-+ (3/5)	±-++ (5/5)	±-++ (3/5)	±-++ (5/5)	+ (1/1)	- (0/5)	±-+ (2/2)
Glial								
pThr^{175}	±-++ (2/5)	±-++ (2/5)	- (0/5)	- (0/5)	± (1/4)	- (0/1)	±-+ (2/5)	- (0/3)
PHF	±-++ (4/4)	±-++ (2/3)	- (0/4)	- (0/4)	- (0/4)	+ (1/2)	± (3/5)	- (0/2)
pSer^{208,210}	++ (4/5)	++ (2/5)	- (0/5)	- (0/5)	± (1/4)	- (0/1)	±-+ (4/5)	± (1/3)
pThr^{217}	++-+++ (5/5)	+++ (2/5)	++ (2/5)	- (0/5)	± (1/5)	- (0/1)	+-++ (5/5)	- (0/2)
Neuritic								
pThr^{175}	±-+ (3/5)	±-+ (2/5)	±-+ (3/5)	±-++ (4/5)	±-++ (4/4)	- (0/1)	± (4/5)	± (1/3)
PHF	±-+ (2/4)	±-+ (3/3)	±-+ (3/4)	± (3/4)	±-++ (4/4)	±-++ (2/2)	± (2/5)	± (2/2)
pSer^{208,210}	±-+ (2/5)	± (3/5)	± (4/5)	± (2/5)	±-++ (4/5)	+ (1/1)	± (1/5)	± (1/3)
pThr^{217}	±-++ (5/5)	±-++ (2/5)	±-+ (4/5)	++ (2/5)	+-+++ (5/5)	+ (1/1)	± (3/5)	±-++ (2/2)

Tauopathies: Within the tauopathies and consistent with the literature, we observed tau protein neuronal pathology across multiple regions (Table 3.5). $pThr^{175}$ tau protein was present in all disease states where tau protein pathology was prominent. In Alzheimer's disease (AD), all tau antibodies showed robust neuronal pathology as neurofibrillary tangles and punctate cytoplasmic deposition in all brain regions studied. Across all cortical regions studied, neuronal pathology was present across all cortical layers but was most prominent in deeper layers (IV-VI). Amongst the tauopathies, the most prominent $pThr^{175}$ tau protein immunostaining was observed in AD. This included more prominent expression of $pThr^{175}$ tau protein than observed in ALSci.

Table 3.5: Tauopathies pathology

Stain	Frontal	Cingulate	Hippocampus	Dentate	Entorhinal	Amygdala	BG	SN
Neuronal								
pThr^{175}	±-++ (10/27)	±-++ (10/25)	±-+++ (20/26)	±-++ (8/28)	±-+++ (21/28)	±-+++ (19/23)	±-+ (10/29)	± (3/23)
PHF	±-++++ (13/27)	±-+++ (16/24)	±-++++ (28/29)	±-++++ (18/29)	±-++++ (28/29)	±-++++ (21/21)	±-++ (13/28)	±-++ (7/23)
pSer^{208,210}	±-+++ (12/27)	±-+++ (12/25)	±-+++ (25/29)	±-++ (10/28)	±-+++ (23/28)	±-+++ (23/28)	±-++ (9/28)	±-+ (5/23)
pThr^{217}	±-+++ (15/27)	±-+++ (14/24)	±-+++ (23/29)	±-++ (12/29)	±-+++ (24/29)	±-+++ (19/21)	±-+ (6/27)	± (7/23)
Glial								
pThr^{175}	±-+++ (6/27)	±-++ (6/25)	±-+ (4/29)	± (1/28)	± (2/28)	±-++ (3/24)	±-++ (16/29)	± (3/23)
PHF	±-++++ (8/27)	±-+++ (7/24)	±-+ (5/29)	- (0/29)	±-+++ (8/29)	±-+++ (12/21)	±-++++ (11/28)	- (0/23)
pSer^{208,210}	+-++ (2/28)	±-+++ (3/25)	++ (3/29)	++ (1/28)	±-++ (3/27)	±-+++ (3/22)	±-++ (10/28)	- (0/23)
pThr^{217}	±-+++ (7/26)	±-+++ (7/24)	±-+++ (3/29)	- (0/29)	++-+++ (2/29)	±-+++ (4/21)	±-+++ (15/27)	- (0/23)
Neuritic								
pThr^{175}	±-++ (10/27)	±-++ (9/25)	±-+++ (20/28)	±-++ (6/28)	±-+++ (21/28)	±-+++ (16/24)	±-+ (12/28)	±-++ (10/23)
PHF	±-++++ (14/27)	±-++++ (15/24)	±-++++ (24/29)	±-+ (11/29)	±-++++ (28/29)	±-++++ (17/21)	±-+++ (16/28)	±-++++ (13/23)
pSer^{208,210}	±-++++ (18/27)	±-+++ (9/25)	±-++ (18/29)	±-+ (6/28)	±-+++ (22/28)	±-+++ (18/23)	±-++ (11/28)	±-++ (11/23)
pThr^{217}	±-++++ (15/27)	±-++++ (14/25)	±-++ (21/29)	±-+ (11/29)	±-+++ (24/29)	±-++++ (18/23)	±-+++ (11/27)	±-++ (11/23)

As in AD, VD exhibited tau protein deposition as tangles and punctate cytoplasmic inclusions in all brain regions studied. This followed the same trend as AD with pathology being most prominent in deep cortical layers. In CBD, balloon neurons were observed and tau pathology was prominent in all brain regions as punctate inclusions and neurofibrillary tangles. Notably, PHF tau staining was more intense than $pThr^{175}$ tau in all regions, both in case positive ratio and in semiquantitative pathological load. In both DLBD and mDLBD, a similar degree of tau pathology was observed in the form of cytoplasmic punctate deposition and neurofibrillary tangles. Pathology within the dentate gyrus, basal ganglia and substantia nigra was present to a much greater extent in mDLBD than DLBD. In FTLD, tau pathology was observed as punctate inclusions and tangles in all brain regions investigated. In general, $pThr^{175}$ tau protein was less prominent than PHF tau protein, except in the frontal and cingulate cortex where it was more prominent on a case positive basis and the pathological load observed. In MSA, tau protein pathology in the form of tangles and punctate inclusions was present in all brain regions studied although frontal and ACC pathology was sparse. In Parkinson's disease, tau protein pathology was observed in all brain regions except the substantia nigra. Pathological tau protein expression was equivalent across all antibodies. In Pick's disease, all brain regions investigated exhibited tau protein pathology in the form of tangles, punctate inclusions and Pick bodies. Notably, PHF tau protein pathology was greater than the other antibodies including $pThr^{175}$ tau.

3.4.1.2 Neuritic tau

ALS: No neuritic plaques were observed in ALS. Neuritic pathology in the form of dystrophic neurites was observed to a limited extent in all brain regions studied and with a pattern of distribution mimicking that described above for neuronal pathology. Basal ganglia neuritic pathology was observed to a larger extent in the putamen than the globus pallidus by all tau

protein antibodies but pThr^{175} tau. Neuritic tau protein pathology within the substantia nigra was immunoreactive against all antibodies employed in the analysis.

ALSci: Neuritic tau protein pathology was observed predominantly as dystrophic neurites assuming a short curvilinear morphology. This was consistently observed in both cortical and subcortical tissues. Contrary to the superficial localization of frontal and ACC neuronal pathology, frontal neuritic pathology was observed mainly in deep layers as short curved neurites. Entorhinal neuritic pathology was observed mainly in the superficial layers in proximity to tau protein inclusion bearing neurons. Neuritic plaques were observed in the entorhinal cortex by all antibodies with the exception of the pThr^{175} tau antibody. Neuritic plaques within the amygdala were observed by PHF tau antibody labeling only. Neuritic plaques and tau protein positive neurites were observed in the hippocampus and were immunoreactive to all antibodies. Coiled bodies were observed throughout the basal ganglia.

Tauopathies: Neuritic pathology was prominent across all the tauopathies and typically mirrored the presence of tau protein immunoreactive neuronal pathology. Neuritic plaques were observed in AD as small atypical plaques and typical plaques in deeper layers (IV/V) more frequently than in superficial layers (II/III). No antibody identified neuritic plaques in basal ganglia or substantia nigra. Like neuronal tau ptotein, all cortical neuritic pathology was most prominent in deeper layers (IV-VI). All antibodies recognized neuritic pathology, most commonly in deeper cortical layers near tau protein positive neurons as short and curved, or long, straight neurites. Neuritic pathology was also observed to a lesser extent in white matter in frontal, ACC, and entorhinal cortices.

Similar to AD, neuritic pathology in VD was present as tau protein positive neurites and neuritic plaques in all brain regions studied except the substantia nigra. Also similar to AD, neuritic pathology followed a tendency to be most prominent in regions of prominent neuronal tau protein pathology and in particular in the deeper cortical layers. In CBD, neuritic plaques were only observed in the ACC and substantia nigra, and then, only using the PHF tau antibody. Neuritic pathology, as dystrophic neurites, was present in all brain regions investigated and mirrored the distribution of neuronal pathology. This was most evident in the entorhinal cortices where dystrophic neurites were most evident in superficial cortical layers. In contrast, dystophic neurites were most prominent in deep subcortical regions of the superior frontal cortex and ACC.

In DLBD, no frontal, ACC, dentate gyrus or basal ganglia neuritic pathology was observed. All other regions studied were positive for neuritic tau protein pathology, but no plaques were observed. Entorhinal neuritic pathology was most prominent in DLDB in layers II/III. In mDLBD, neuritic plaques were observed in all regions except for the substantia nigra. All regions studied exhibited neuritic pathology. In both FTLD and MSA, neuritic plaque pathology was not frequent and usually observed by PHF tau only. $pThr^{175}$ tau protein neuritic pathology was not as prominent as that observed using other tau protein antibodies. However, neuritic tau protein pathology was observed in all brain regions studied as with neuronal pathology. In PD, neuritic plaques were not observed, but tau protein positive neurites were observed in a similar distribution to neuronal pathology in all brain regions studied. In Pick's disease, neuritic pathology was identified mainly by PHF tau in all but the dentate gyrus.

3.4.1.3 Glial tau

ALS: Glial tau protein pathology was observed in all brain regions except the dentate gyrus, and substantia nigra (Table 3.3). Where present, glial pathology presented as astrocytic tangles and astrocytic plaques as previously described(Yang and Strong, 2012). The distribution was rare and followed that of neuronal and neuritic tau protein as described above and was similar in case positive ratio, although higher in semiquantitative load than neuronal and neuritic pathology in a single case.

ALSci: Glial pathology was present in ALSci (Figure 3.1, Supplemental Figures 3.1-3.3) to a similar degree as in ALS, but was more frequent in terms of regional distribution, case positive rate, and pathological load (Table 3.4). Frontal and ACC glial pathology was increased in both case positive incidence and pathological load.

Tauopathies: In the tauopathies, the extent of glial pathology was highly dependant on the underlying disease (Table 3.5). In AD, glial pathology was rare, and when present was usually observed only in the amygdala and basal ganglia. No glial pathology was observed in VD. Consistent with the literature, CBD contained astrocytic plaques throughout the grey and white matter across multiple brain regions. Additional astrocytic staining and tau protein positive microglia were observed. Glial pathology in CBD was identified to a far greater extent by PHF than the $pThr^{175}$ tau antibody. In DLBD, minimal glial pathology was observed in frontal cortex and basal ganglia, and when present, only as punctate astrocytic inclusions. In contrast to the limited glial pathology observed in DLBD, mDLBD showed much more frequent glial pathology across multiple brain regions, pathology that was less evident with the $pThr^{175}$ tau antibody. In FTLD, glial pathology was observed in all brain regions except the dentate gyrus and substantia

nigra. Notable in this disease, pThr^{175} tau identified glial pathology to a greater extent than PHF tau by both case positive incidence and increased load. In MSA, glial pathology was largely absent, being present only in the entorhinal cortex, amygdala and basal ganglia by multiple antibodies. Interestingly, the basal ganglia contained astrocytic inclusions in the putamen identified by all antibodies whereas in the globus pallidus they were only identified by pThr^{175} tau. In Parkinson's disease, glial pathology was present in all regions except the dentate gyrus and substantia nigra. Notably, the PHF tau antibody identified astrocytic plaques in the frontal cortex while tufted astrocytes were observed in the amygdala. In Pick's disease, glial pathology was observed in multiple brain regions using the PHF antibody mainly, although in the entorhinal cortex pThr^{175} tau was also positive for glial pathology.

3.4.2 pThr^{231} tau and T22 staining:

We examined the presence of oligomeric tau protein (recognized by the T22 antibody) and pathological tau protein phosphorylation at Thr^{231} using sections from the hippocampus of a single case each of AD, ALSci, FTD, MSA, DLBD, and mDLB. Cases were selected on the basis of the pathology described earlier. In each case, tau protein neuronal inclusions were recognized by both antibodies (Figure 3.2, Figure 3.3). Only DLBD showed notably reduced T22 pathology which, when present, was in the dystrophic neurites (Figure 3.3). Glial pathology was not observed with either antibody, regardless of diagnosis.

AD neuronal pathology was observed as fibrillar and punctate inclusions by pThr^{231} tau antibody but only as fibrillar/ tangles with T22. Neuritic pathology in the form of short and long torsional and dystrophic neurites was observed. pThr^{231} tau, but not T22, recognized plaques consisting of dystrophic neurites.

Similar to AD, ALSci neuronal pathology was observed by pThr^{231} tau and T22 as fibrillar and punctate staining. However additional solitary cytoplasmic inclusions were observed in neurons with the T22 antibody. Neuritic pathology was observed using both the pThr^{231} tau and T22 antibodies. Neuritic plaques were identified by both the pThr^{231} tau and T22 antibodies.

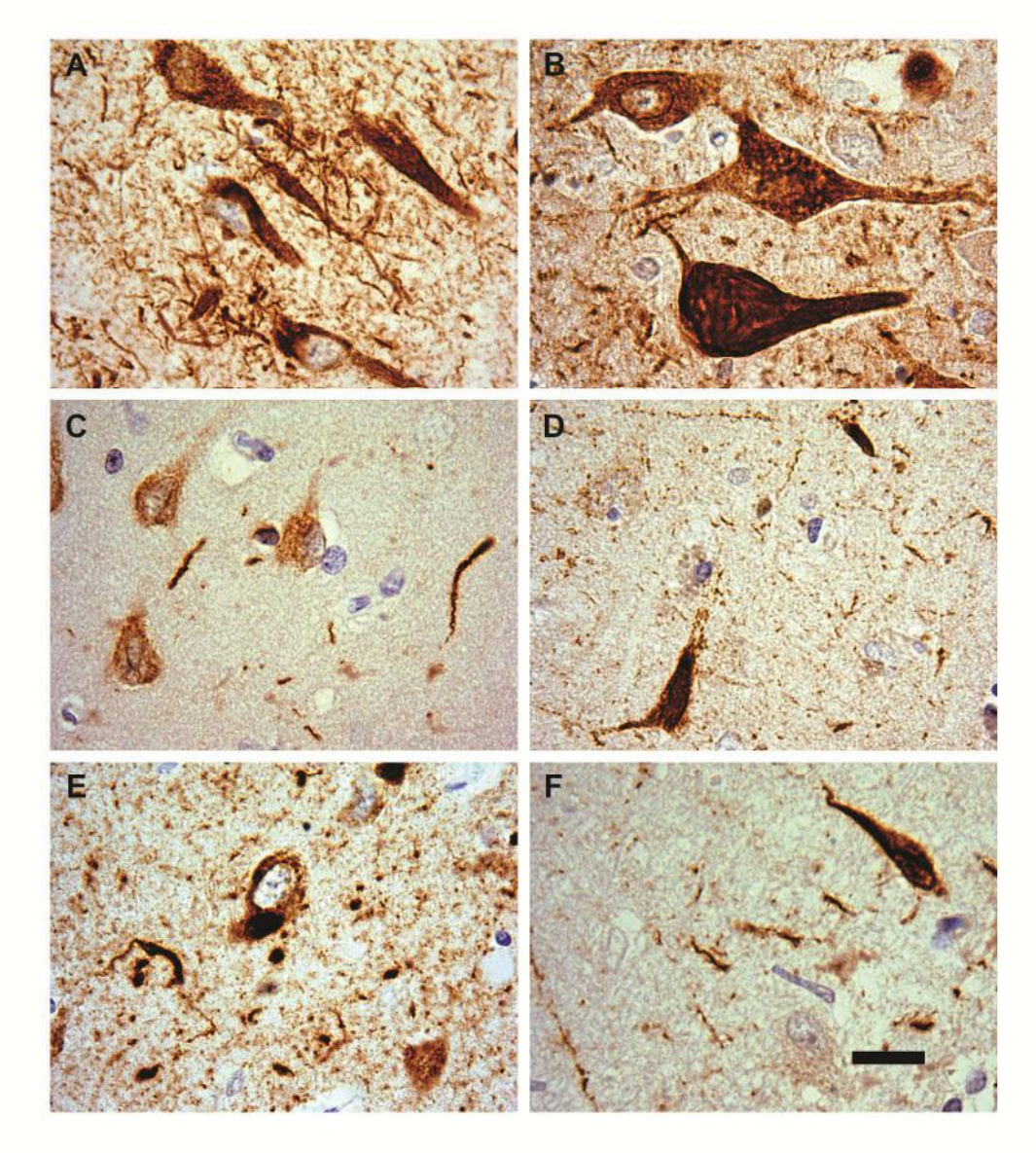


Figure 3.2: Representative hippocampal pThr^{231} tau pathology. A) AD, B) ALSci, C) DLBD, D) mDLBD, E) FTLD, F) MSA. Counterstained with hematoxylin. Original images taken at 100x. Scale bar= 20 μm.

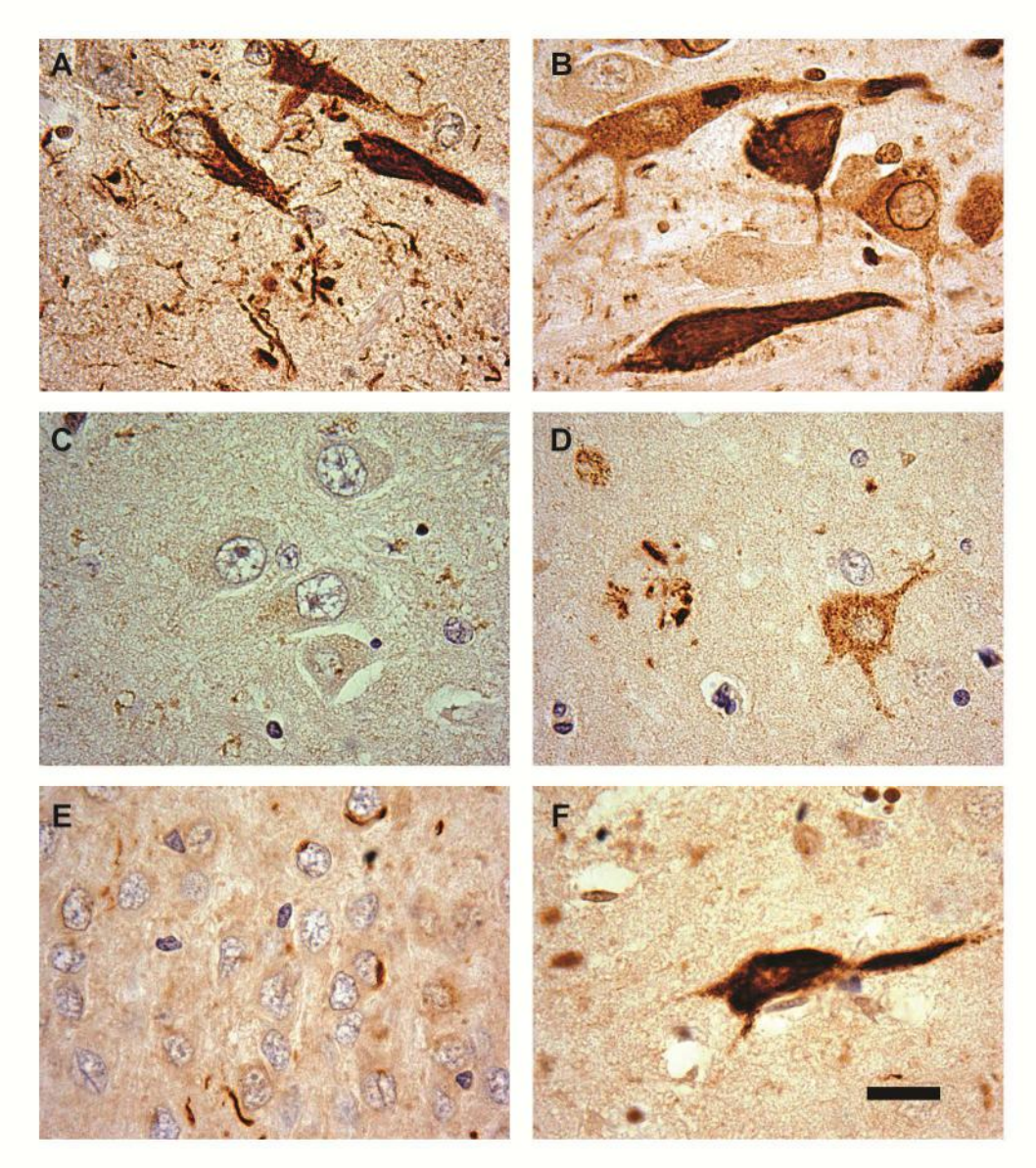


Figure 3.3: Representative hippocampal tau oligomer (T22) pathology. A) AD, B) ALSci, C) DLBD, D) mDLBD E) FTLD F) MSA. Counterstained with hematoxylin. Original images taken at 100x. Scale bar= 20 μm.

FTLD pathology was distinct from AD and ALSci in that pThr^{231} tau protein neuronal pathology was observed as a dense nuclear ring staining around abnormally folded nuclei, and solitary cytosolic inclusions on homogenously stained cytosol. While T22 neuronal cytoplasmic pathology was observed, neuritic pathology was observed as frequent short dystrophic neurites by both antibodies. No neuritic plaques were observed.

MSA pathology resembled AD and ALSci. Neuronal pathology was observed as tangles and punctate inclusions by both the pThr^{231} tau and T22 antibodies. In addition, pThr^{231} tau antibody diffuse cytoplasmic immunostaining was observed. Neuritic pathology was observed by both antibodies, although T22 immunoreactive neurites demonstrated a punctate staining pattern.

DLBD tau protein pathology was observed only faintly by pThr^{231} tau as punctate cytosolic deposition. While neuritic pathology was observed using the pThr^{231} tau antibody, no pathology was observed by T22 other than a few sporadic neurites. Conversely, mDLBD pathology was observed by both pThr^{231} tau and T22. pThr^{231} tau protein pathology was observed as tangles and punctate staining accompanied by dystrophic neurites and other neuritic pathology. No neuritic plaques were observed. T22 pathology however was observed as punctate cytosolic staining and dystrophic neurites.

Having confirmed that both T22 and pThr^{231} tau protein immunoreactive pathology was present, although as described to varying degrees, we next sought to confirm whether pThr^{175} tau protein and pThr^{231} tau protein co-localized using confocal imaging (Figure 3.4). Co-localization within neuronal tau protein inclusions was observed between pThr^{175} tau protein and pThr^{231} tau protein in each disease state except for DLBD. Co-localization was also observed in neuritic plaques in AD. No pThr^{175} tau protein immunoreactivity was observed in the absence of pThr^{231}.

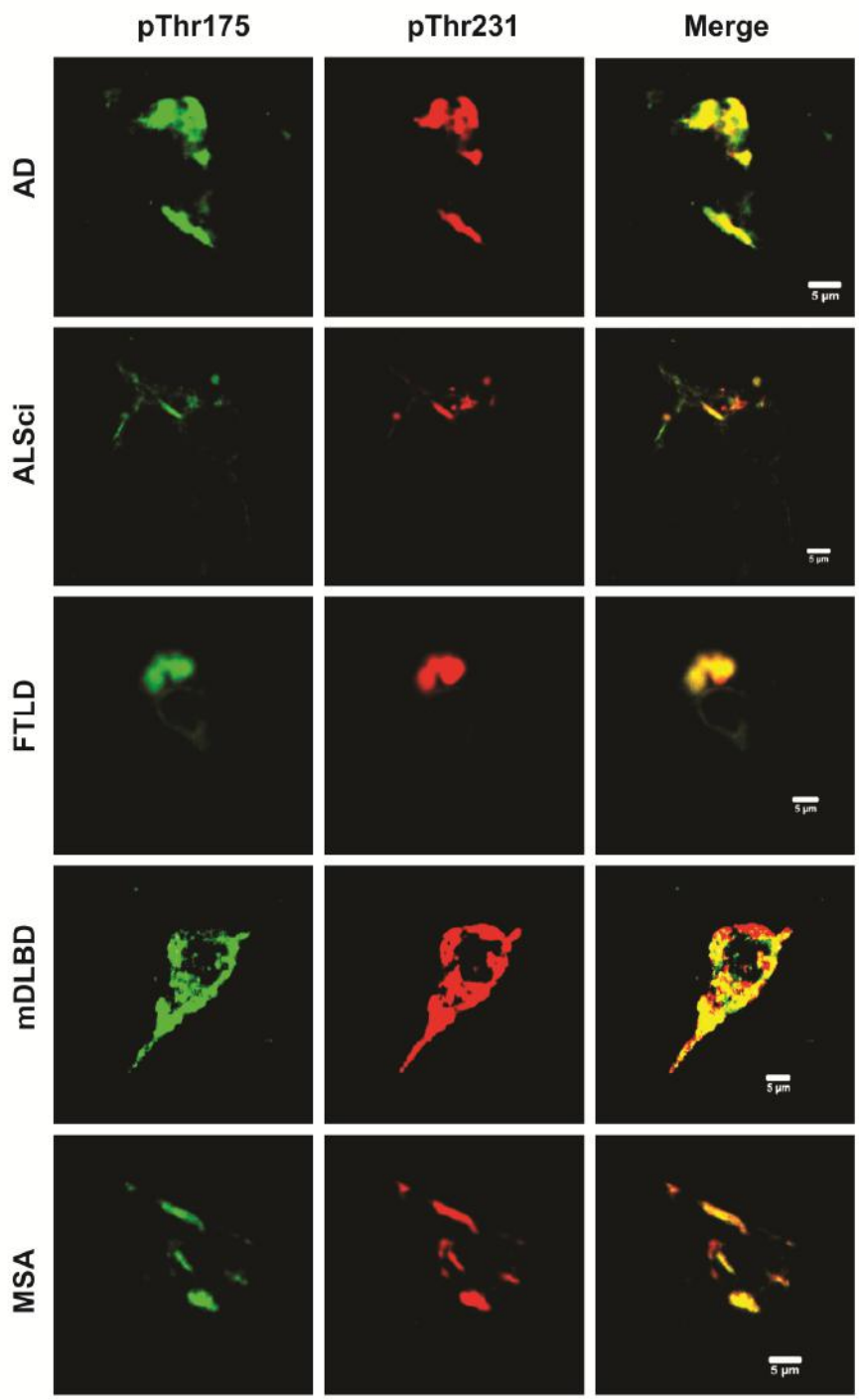


Figure 3.4: Co-localization of pThr^{175} and pThr^{231} tau in hippocampal neuronal inclusions. AD: Alzheimer's disease. ALSci: amyotrophic lateral sclerosis with cognitive impairment. FTLD: frontotemporal lobar dementia. mDLBD: mixed diffuse dementia with Lewy bodies. MSA: Multiple system atrophy. Co-localization stains were performed in one case per disease. Scale bar represents 5 µm.

3.4.3 Hippocampal pThr^{175}, pThr^{231} and oligomeric tau deposition as a function of aging:

Consistent with our previous report, we observed an increase in tau protein immunoreactive pathology beginning in the 7th decade of life (Yang et al., 2005). In contrast, no immunoreactive inclusions were observed to either the pThr^{175} tau protein or oligomeric tau protein (T22 immunoreactivity) in the 6th decade (Figure 3.5). pThr^{231} tau protein immunoreactivity was observed in each of the 6th, 7th and 8th decades within the hippocampus. In distinction to the pathological tau protein deposition observed in both ALSci and the tauopathies, neuronal Thr^{231} tau protein immunoreactivity was diffuse and localized to otherwise healthy appearing neurons and axonal processes. In the 7th decade, T22 immunoreactive neuronal cytoplasmic inclusions were observed minimally and when present were within the same regions in which we observed punctate pThr^{175} immunoreactivity. All but one case demonstrated pThr^{231} tau protein immunoreactivity, and importantly this case was negative for all three tau epitopes. In all cases, neuritic pathology was minimal or nonexistent, while neuronal positivity was mainly punctate tau protein expression.

In the 8th decade, we observed a marked increase in pThr^{175} tau, pThr^{231} tau and T22 immunoreactivity. In this decade, each antibody revealed tau protein immunoreactive punctate staining of neurons, neurofibrillary tangles, dystrophic neurites, and neuritic plaques. Across all three decades, T22 pathology was present in all cases and in regions where pThr^{231} tau protein and pThr^{175} tau protein was present, and was only positive in cases with prominent pThr^{175} tau protein positive cells and pathology.

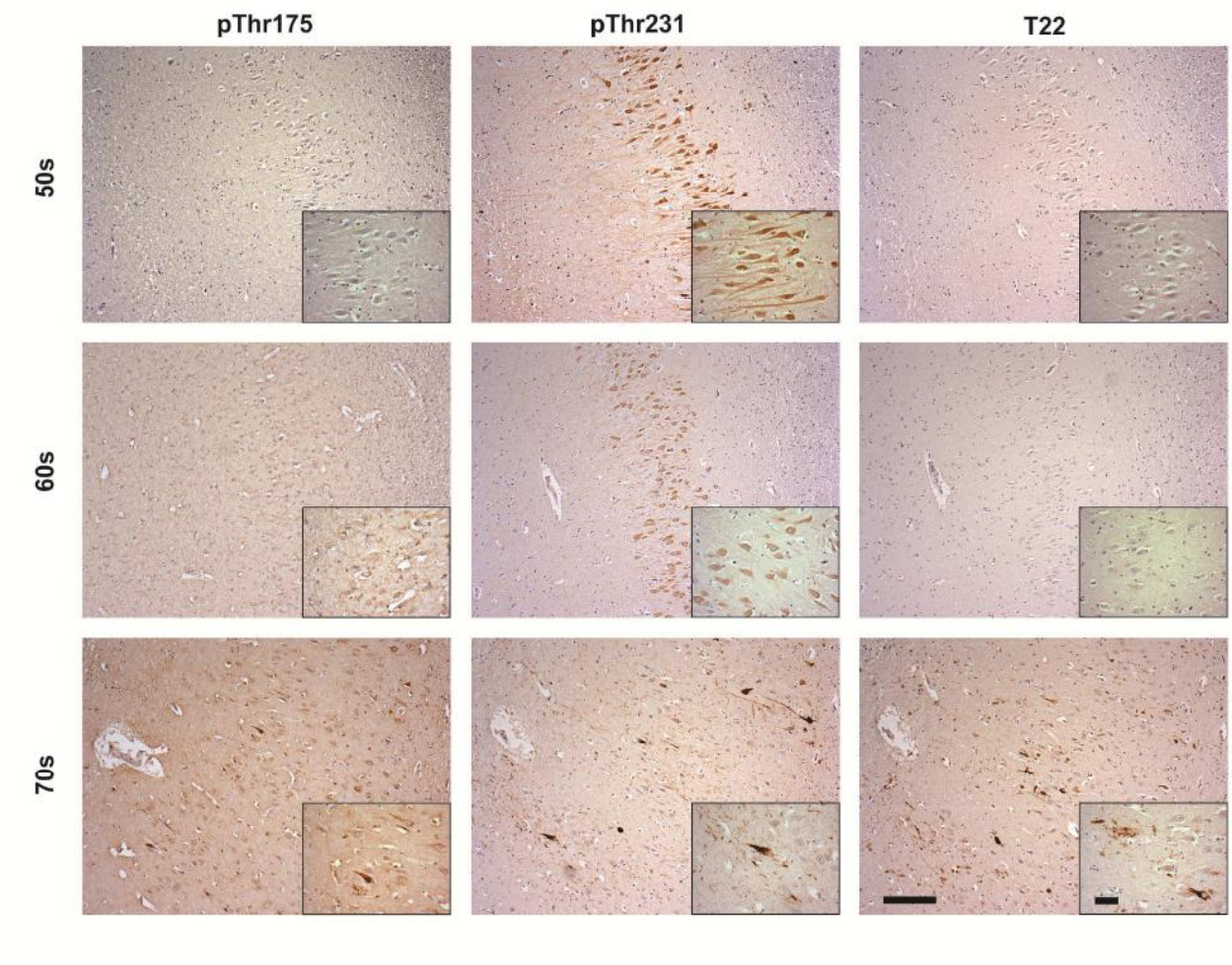


Figure 3.5: Age dependent tau pathology increases in the hippocampus of controls and is associated with pThr^{175} tau pathology in the 8th decade of life. Large images taken in the CA2 region of the hippocampus at 10x magnification, inset is same region at 40x magnification. Images are representative of 4 cases per age group. Scale bar large image= 100 μm, inset= 50 μm.

3.5 Discussion:

In undertaking these studies, we were specifically interested in determining whether the pathogenic phospho-tau protein species recognized by antibodies against $pThr^{175}$ tau protein and $pThr^{231}$ tau protein as well as oligomeric tau ptotein (T22) were expressed across a broad range of tauopathies. We were also interested in determining whether these pathological tau proteinspecies were co-localized in ALS and ALSci. It is known that the phosphorylation of tau protein at Thr^{231} is of both physiological and pathological significance in mediating the dissociation of tau protein from microtubules (Lin et al., 2007; Schwalbe et al., 2015; Sengupta et al., 1998). Thr^{231} is phosphorylated by activated GSK3β physiologically and in pathological states (Alonso et al., 2010; Cho and Johnson, 2004; Luna-Munoz et al., 2005; Sahara et al., 2008; Sengupta et al., 1998). We have previously shown that $pThr^{175}$ tau protein induces GSK3β phosphorylation and that this in turn leads to Thr^{231} tau protein phosphorylation resulting in tau protein fibril formation, and cell death *in vitro* (Moszczynski et al., 2015).

Although the number of cases studied here is limited, the intent was not to undertake a detailed topographic analysis of tau protein deposition across all tauopathies, but rather to determine whether the proposed pathway of $pThr^{175}$ tau protein mediated induction of $pThr^{231}$ tau protein with its attendant pathological tau fibril formation (as recognized by T22) was evident. It is noteworthy therefore that we observed that in each tauopathy studied, $pThr^{175}$ tau, $pThr^{231}$ tau and T22 immunoreactivity co-localized to the same inclusion-containing neuronal populations. In each case, neuronal $pThr^{175}$ tau protein co-localized with $pThr^{231}$ tau protein. This, paired with prior identification of $pThr^{175}$ tau protein in AD brain tissue but not controls (Hanger et al., 2007) and the lack of identified $pThr^{175}$ in fetal tau protein (Kenessey and Yen, 1993; Watanabe et al., 1993) suggests that $pThr^{175}$ is a key point in pathological tau protein

metabolism, as it is not a physiologically utilized site involved in the regulation of tau protein function during development or microtubule reorganization. This suggests that the downstream events triggered by pThr^{175} tau protein, including toxic oligomer formation, are common to each of these diseases.

To further assess the pathogenicity of pThr^{175} and pThr^{231}, we investigated each epitope in the hippocampus of control cases across three decades of life where tau protein pathology has been shown to increase with age (Yang et al., 2005). We observed no pThr^{175} tau protein pathology in the 6th decade with minimal immunoreactive neuronal inclusions in the 7th decade. pThr^{175} tau immunoreactivity was most prominent in the 8th decade. In each case in which we observed pThr^{175} tau immunostaining, we also observed T22 immunoreactivity. Similarly, we never observed T22 immunoreactivity in the absence of either pThr^{175} tau protein or pThr^{231} tau protein immunoreactivity. In contrast, pThr^{231} tau protein immunoreactivity was frequently observed in the absence of either pThr^{175} tau protein or T22 staining in younger individuals and when present, was within healthy appearing neurons and axonal processes. pThr^{175} and T22 did not show pathology in hippocampal regions spared from pThr^{231} pathology, and T22 was only positive in cases showing prominent pThr^{175} pathology.

Glial pathology was recognized to a greater degree by pThr^{217} tau and PHF tau than by the pThr^{175} tau antibody, suggesting that different pathological processes are at play in these cells. This is supported by the lack of identifiable glial pathology by pThr^{231} tau and T22. This paired with the low frequency of pThr^{175} tau protein glial pathology further strengthens the correlation between pThr^{175} and pThr^{231} in the induction of neuronal pathology and provides evidence that this pair of phosphorylation sites may be exerting specific neuronal toxicity in the disease process across multiple tauopathies.

Although limbic regions universally presented tau protein pathology, frontal and ACC tau protein pathology was present mainly in AD, VD, ALSci, FTLD, mDLBD and MSA. This paired with the deeper layer pathology in this region may indicate that tau protein pathology did not originate here but instead propagated from other regions. If tau originates in limbic structures, propagating along the Papez circuit, it is possible that it would arrive in ACC through thalamic projections to layer IV and V which could act as a hub for propagation to other brain regions such as frontal cortex through this well connected region. Regardless of the induction cause or place, tau protein toxicity is undeniable once initiated (Gomez-Ramos et al., 2006; Ward et al., 2012), and must be considered when attempting to understand the underlying biology of many neurodegenerative diseases. This hypothesis also implies that disease entities such as primary age-related tauopathies (PART) (Crary et al., 2014) may be in fact not age-related, but neuronal stress related, as increasing age would indicate longer time periods for normal mechanical stress on neurons to become pathological through stochastic processes (Kagias et al., 2012). Therefore, tau protein deposition should not be considered a simple function of normal ageing, but ageing should be considered a risk factor for tauopathy among a plethora of neuronal stresses. Of note as well is the frontal involvement in ALSci, which can be concluded is not likely a result of PART, which spares the neocortex by definition (Crary et al., 2014; Yang et al., 2005). We cannot conclude, however if the layer distribution of tau pathology resembles PART, as this was not described in the consensus report.

3.6 Conclusions:

These findings implicate a toxic axis of phosphorylation events beginning with Thr^{175} phosphorylation, dependent on further phosphorylation at Thr^{231}, which appears to be neuron specific and which may be common to the tauopathies. It may therefore be a contributor to

neuronal death in these diseases, and may be a point of intervention capable of slowing disease progression resulting from tau protein toxicity.

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Chapter 4: Chronic traumatic encephalopathy (CTE) and CTE with amyotrophic lateral sclerosis (CTE-ALS): evidence for trauma-induced pathological tau phosphorylation at Threonine[175]

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A version of this chapter is under review for publication in *Neurology*

4.1 Abstract:

Chronic traumatic encephalopathy (CTE) and chronic traumatic encephalopathy with amyotrophic lateral sclerosis (CTE-ALS) are neurodegenerative diseases associated with traumatic brain injury (TBI) characterized by pathological microtubule associated protein tau (tau protein) deposition. Common features of many tauopathies include: the pathological phosphorylation of tau protein at Thr^{175} ($pThr^{175}$ tau protein), GSK3β activation, the induction of tau protein phosphorylation at Thr^{231} ($pThr^{231}$ tau protein) and pathological tau protein oligomerization. To investigate whether CTE and CTE-ALS share these features, we characterized pGSK3β, $pThr^{175}$ tau protein and $pThr^{231}$ tau protein expression in human cases of CTE and CTE-ALS. To determine if GSK3β activation and pathological tau protein phosphorylation were a consequence of traumatic brain injury we analyzed these markers in a rat model of TBI. Tau protein isoform expression was assayed by western blot in 6 stage III CTE cases. We also used immunohistochemistry to analyze 5 cases each of CTE, CTE-ALS, and 5 controls for the expression of pGSK3β, $pThr^{175}$ tau protein, $pThr^{231}$ tau protein and oligomerized tau protein (T22) within spinal cord tissues and hippocampus. Using a rat model of moderate TBI, we assessed tau neuronal pathology and pGSK3β expression at 3 months post-injury. Both CTE and CTE-ALS are characterized by the presence of all 6 tau protein isoforms in both soluble and insoluble tau protein isolates. pGSK3β, $pThr^{175}$ tau protein, $pThr^{231}$ tau protein and oligomerized tau protein expression was observed in hippocampal neurons and in spinal cord. We also observed tau neuronal pathology (fibrillar inclusions and axonal damage) and increased levels of $pThr^{175}$ tau and activated GSK3β in moderate TBI rats. Pathological phosphorylation of tau at Thr^{175} and Thr^{231}, and activation of GSK3β are characteristic features of the tauopathy of CTE and CTE-ALS, a feature that can be replicated in an animal model of moderate TBI.

4.2 Introduction:

Chronic traumatic encephalopathy (CTE) and chronic traumatic encephalomyelopathy (CTE-ALS) are fatal neurodegenerative diseases that are closely associated with traumatic brain injury (TBI) (McKee et al., 2009). While typically associated with elite athletes, participants in recreational sport are experiencing increased rates of traumatic brain injury and the majority of TBI occurs as a result of non-sport related accidents, meaning the impact of traumatic brain injury are more widespread than just accidents and elite athletes (Coronado et al., 2015; Langlois et al., 2006) . Additionally, there is an increasingly evident relationship between TBI and neurodegenerative disease processes such as Alzheimer's disease (AD) Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Bazarian et al., 2009; Chen et al., 2007). Therefore, an understanding of the molecular changes and biochemistry associated with the neuropathology of protein aggregation and neuronal death in CTE and CTE-ALS may lead to a better understanding of pathophysiology of these disorders.

Both CTE and CTE-ALS share the pathological hallmark of neuronal and glial intracellular aggregates of microtubule associated protein tau (tau protein), placing these disorders amongst the tauopathies and thus potentially sharing a pathophysiology with AD, PD and ALS with cognitive impairment (ALSci) (McKee et al., 2009; Moszczynski et al., 2017; Tartaglia et al., 2014). Pathological tau protein phosphorylation at threonine 231 ($pThr^{231}$ tau protein) is considered to be a critical event leading to reduced interaction between microtubules and tau protein. The increased level of unbound tau protein promotes the formation of soluble pathogenic tau protein oligomers that further aggregate into insoluble pathological tau protein fibrils (Lasagna-Reeves et al., 2012; Lin et al., 2007; Nakamura et al., 2013; Ward et al., 2012). To date, there have been no detailed studies of the phosphorylation state of tau protein in either

CTE or CTE-ALS and thus it is unknown if the tauopathy of these disorders is homologous to that of related tauopathies.

We have recently described a sequence of phosphorylation events leading to tau protein toxicity in ALSci whereby tau protein phosphorylation at Thr^{175} ($pThr^{175}$ tau protein), that alone is insufficient to induce fibril formation, promotes further phosphorylation of tau protein at Thr^{231} by activated GSK3β (Moszczynski et al., 2015). We subsequently extended this observation to a broad range of tauopathies (Moszczynski et al., 2017). In this sequence, $pThr^{231}$ tau protein is the critical mediator of toxicity downstream of $pThr^{175}$ tau. Importantly, $pThr^{175}$ has only been observed in pathological states thus far while phosphorylation of tau protein at Thr^{231} appears to be a crucial physiological regulator of tau protein function (Lin et al., 2007; Schwalbe et al., 2015). Given the widespread applicability of this sequence in tauopathies, we have examined post mortem archival tissues from CTE and CTE-ALS patients to determine whether tau protein is pathologically phosphorylated at Thr^{175} and Thr^{231} and whether this is associated with increased levels of the active isoform of GSK3β (pGSK3β) and tau protein oligomerization. We have also examined whether this process is also triggered by moderate TBI in a rodent.

4.3 Methods:

4.3.1 CTE and CTE-ALS studies:

All studies were conducted in accordance with the institutional ethics board standards at University Hospital (London, ON, Canada) and Boston University (Boston MA, USA). Microscope slides with 6 μm thick hippocampal and spinal cord sections from fifteen cases (5 CTE, 5 CTE-ALS, and 5 Control; cases MSL1-MSL15) were used for immunohistochemical

studies. An additional 6 cases (cases 1-6) were obtained as frozen tissue from anterior cingulate and temporal pole. All tissue and slides were obtained from the Boston University CTE brain bank and were diagnosed as stage III CTE (McKee et al., 2013). Demographic data is summarized in supplemental table 4.1.

Supplemental Table 4.1: Case demographics

Case	Slide/ Tissue	CTE/CTE-ALS/Ctrl	Sex	Decade
1	Tissue	CTE	M	25
2	Tissue	CTE	M	58
3	Tissue	CTE	M	46
4	Tissue	CTE	M	71
5	Tissue	CTE	M	84
6	Tissue	CTE	M	69
7	Slides	CTE-ALS	M	49
8	Slides	CTE-ALS	M	31
9	Slides	CTE-ALS	M	41
10	Slides	CTE-ALS	M	67
11	Slides	CTE-ALS	M	62
12	Slides	CTE	M	82
13	Slides	CTE	M	73
14	Slides	CTE	M	56
15	Slides	CTE	M	75
17	Slides	CTE	M	50
18	Slides	Ctrl	M	83
19	Slides	Ctrl	M	61
20	Slides	Ctrl	M	61
21	Slides	Ctrl	M	55
22	Slides	Ctrl	M	81

4.3.1.1 Tau fractionation and Western blot:

Tau protein was isolated from the anterior cingulate gyrus and the temporal pole of 6 stage III CTE cases and a single Alzheimer's case as a control for the tau fractionation. Tau protein isolation, fractionation, and dephosphorylation was conducted as previously reported (Hanger et al., 1998; Strong et al., 2006). Tissue was homogenized in 1 ml of MES buffer (pH 6.5) and centrifuged at 27,000 x g for 60 minutes at 4˚C. The supernatant was then removed and centrifuged at 95,000 x g for 60 minutes at 4˚C. The supernatant containing the soluble tau protein fraction was saved and stored at -80˚C. The pellet containing the insoluble tau protein fraction was solubilized in 150 µl 4 M guanidine HCl for 1 hour at room temperature with a brief sonication and then dialyzed against 50 mM Tris-HCl, (pH 7.5, 1 mg/ml PMSF) overnight at 4^{o}C. The following day the dialysate was centrifuged at 15,000 x g for 60 minutes at 4^{o}C. The supernatant containing the insoluble tau protein was boiled at 100^{o}C for 10 minutes and then centrifuged at 15,000 x g for 30 minutes at 4^{o}C. Supernatant volume was then brought to approximately 3.0 ml in 50 mM Tris-HCl, 1.35 g ammonium sulphate added, and then cooled on ice for 15 minutes. Precipitated proteins were collected after centrifugation at 15,000 x g for 30 minutes at 4˚C and resuspended in 150 µl 50 mM Tris-HCl (pH 7.5). The suspension was dialyzed against 50 mM Tris-HCl (pH 7.5, 1 mg/ml PMSF) overnight at 4^{o}C and the dialysate clarified by centrifugation at 15,000 x g for 30 minutes at 4^{o}C.

Dephosphorylation of tau protein was conducted on aliquots of the soluble or insoluble tau protein in 50 mM Tris-HCl (pH 7.5) were incubated with lambda alkaline phosphatase (20 U/µl, Sigma, Oakville ON, Canada) for 6 hours at 30^{o}C (Strong et al., 2006). Reactions were stopped by the addition of 2x electrophoresis buffer (100 mM Tris-HCl, 4% SDS, 0.02% bromophenol blue, 20% glycerol, 200 mM DTT).

After fractionation and dephosphorylation with lambda alkaline phosphatase, equal aliquots of supernatant from dephosphorylated and non-dephosphorylated samples were run on 7.5% SDS-PAGE gels and electrophoretically transferred to a nitrocellulose membrane. Gels were probed for total tau protein with rabbit anti tau T14/T46 antibodies (1:1000 and 1:3000 titers, respectively; Thermo-Fischer, Burlington, Canada). After blocking with 10% bovine serum albumin (BSA) in tris-buffered saline with 0.2% tween (TBS-T) for 1 hour at room temperature, nitrocellulose membranes were probed with primary antibody overnight at 4˚C. Blots were then washed in TBS-T before probing with horseradish peroxidase tagged secondary antibody (Goat anti-Mouse IgG (1:5000 titer; Bio-Rad, Herculese, USA). Blots were visualized using enhanced chemiluminescence (Perkin Elmer, Waltham, USA).

4.3.1.2 Immunohistochemistry (IHC):

Six μm paraffin-embedded sections from the hippocampus and spinal cord were analyzed for all cases. Immunohistochemistry was conducted using a series of antibodies that recognized $pThr^{175}$ tau protein (1:1000 titer, antibody generated and designed in house, now commercially available through 21st Century, MA, USA), $pThr^{231}$ tau (1:1000 titer, Thermo Fischer) and oligomeric tau protein (1:500 titer, T22; EMD Millipore CA, USA) and activated GSK3β (1:50 titer, $pTyr^{216}$; BD Biosciences, Mississauga, Canada). Antigen retrieval (10 mM sodium citrate, 0.05% Tween 20 pH 6.0) was conducted for all antibodies using a pressure cooker (2100 Retriever; Aptum Biologics, UK, Supplemental table 4.2). Endogenous peroxidase was quenched with 3% hydrogen peroxide (VWR, Mississauga, Canada). Primary antibody incubation was performed at 4˚C overnight in blocking buffer (5% BSA, 0.3% Triton-X 100 in 1 X PBS). After washing, secondary antibody (1:200 biotinylated IgG) incubation was performed for 1 hour at

room temperature in blocking buffer. Antigen:antibody complex was visualized with horseradish peroxidase according to the manufacturer's instructions (Vectastain ABC kit, Vector Laboratories CA, USA), followed by substrate development with DAB. Counterstaining was performed using Harris haematoxylin.

Supplemental Table 4.2: Antibodies used

Antibody	**Clone**	**Titer**	**Epitope**	**Utility**	**Company**
Tau $pThr^{175}$	Rabbit, polyclonal	1:1000	$pThr^{175}$	WB, IHC	21^{st} Century
Tau $pThr^{231}$	Rabbit, polyclonal	1:1000	$pThr^{231}$	IHC	Thermo- Fischer
T22	Rabbit, polyclonal	1:500	Tau oligomer	IHC	EMD Millipore
T14	Mouse, monoclonal	1:3000	Tau aa's 141-178	WB, IP	Thermo-Fisher
T46	Mouse, monoclonal	1:1000	Tau aa's 404-431	WB	Thermo-Fisher
Total tau	Rabbit polyclonal	1:5000	Tau aa's 384-397	WB	Abcam
GSK3β	Mouse, monoclonal	1:10,000	GSK3β aa's 1-160	WB	BD Biosciences
GSK3β $pTyr^{216}$	Mouse, monoclonal	1:50 (IHC) 1:10,000 (WB)	$pTyr^{216}$	WB, IHC	BD Biosciences
Alexafluor 488	Goat anti-rabbit	1:200	Secondary	IHC	Life Technologies
Alexafluor 633	Goat anti-mouse	1:200	Secondary	IHC	Life Technologies

WB: western blot, IHC: immunohistochemistry, IP: immunoprecipitation

The extent of pathology was described semi-quantitatively as previously reported using visualization with a 20x objective under light microscopy (Olympus BX45) (Moszczynski et al., 2017; Yang and Strong, 2012). The semi-quantitative scale was applied as follows: '-' = none; '±' = less than 5; '+' = less than 10; '++' = more than 20 with scattered distribution; '+++' = more than 20 but with locally dense distribution; '++++' = more than 20 with a diffuse distribution. Additionally, the case positive ratio was defined for each antibody used as the number of cases showing any pathology (± or more) compared to the total number of cases stained. Spinal cord pathology was assessed by a binary scale due to the sparse nature of pathology where '+' = pathology present and '-' = pathology absent.

4.3.1.3 Co-localizations and fluorescence staining:

Double labeling was performed on sections from the hippocampus from one case per double label experiment. Tau protein was probed with $pThr^{175}$ or oligomeric tau (T22) rabbit primary antibody overnight at 4˚C and Alexafluor goat anti-rabbit 488 nm secondary (1:200, Thermo Fischer) for 1 hour at room temperature. Rabbit anti-tau $pThr^{231}$ antibody was then labeled using a Zenon primary antibody labeling kit with Alexafluor 555 nm dye (Thermo Fisher) and probed for 1 hour at room temperature. Slides were visualized within 24 hours of labeling by confocal imaging on a Zeiss LSM 510 Meta NLO multiphoton confocal microscope. For co-localizations with phospho-GSK3β (pGSK3β), staining was performed with mouse anti-$pTyr^{216}$ GSK3β antibody (1:50 titer, BD Biosciences) followed by secondary labeling with goat anti-mouse Alexafluor 633nm (Invitrogen).

4.3.2 *In vivo* studies

All protocols for these experiments were approved by the University of Western Ontario Animal Care Committee in accordance with the policies established in the guide to Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care. Twelve (12) adult female Sprague-Dawley rats were subjected to a left-sided 5 mm diameter craniotomy followed by a single moderate head trauma (3.5 m/s, 2 mm deep, with dwell time of 500 ms) using a cortical impactor (Precision systems model TBI 0310) (moderate TBI). After 3 months, all rats were euthanized by trans-cardiac perfusion with ice cold saline after intraperitoneal injection with a lethal dose of Euthanyl. Six (6) brains were drop-fixed in ice cold Bouin's fixative (Thermo-Fisher) for IHC analysis while 6 were frozen on dry ice for neurochemical analysis. Bouin's fixative was used to reduce artefactual tau pathology (Planel et al., 2004; Trojanowski et al., 1989). After 24 hours of fixation, tissue was embedded in paraffin.

4.3.2.1 Western blots:

Immunoblots were also performed using isolates from 6 moderate TBI rats and 4 age matched controls. Brain tissue was homogenized in RIPA buffer (1% NP40, 10% glycerol, 137 mM NaCl, 2mM EDTA) containing protease inhibitors (cOmplete; Roche Diagnostics, Indianapolis, USA) and phosphatase inhibitors (Phosstop, Roche Diagnostics) using a Brinkmann Polytron PT 3000 (Kinetamica, Bohemia, NY, USA). Protein concentration was determined by modified Bradford assay (BioRad).

Immunoprecipitations (IP) were performed on 1 mg of brain lysate protein using mouse anti-total tau (T46) in order to isolate all tau protein isoforms from the rat brain homogenates. The entire IP yield was then run on a 10% SDS-PAGE gel and probed with rabbit anti pThr[175]

tau (1:1000). Gels were stripped (2% SDS, 1M Tris, 7 μl/ml β-mercaproethanol) for 30 min at 50˚C and reprobed with rabbit anti-total tau (1:5000, Abcam ab24230). pGSK3β studies were performed on total brain lysate with mouse anti GSK3β $pTyr^{216}$ (1:10,000) followed by reprobing with mouse anti total GSK3β (1:10,000, BD Biosciences). Blots were visualized by enhanced chemiluminescence (Perkin Elmer) (BioRad Chemidoc MP imaging system and acquired with ImageLab 5.2.1 software). Densitometry was conducted in imageJ.

4.3.2.2 Immunohistochemistry:

6 moderate TBI and 3 age-matched control rat brains were cut to 5-6 μm thickness and stained for $pThr^{175}$ tau, $pThr^{231}$ tau protein and $pTyr^{216}$ GSK3β using the same antibodies and protocol used in human cases. GFAP staining was also conducted.

4.3.2.3 Statistical analysis:

Statistical analyses were conducted using SigmaPlot 10.0 software. A one way analysis of variance (ANOVA) was conducted following a Shapiro-Wilk test for normality. Post-hoc Tukey's test was conducted and a p value of 0.05 or lower was considered significant.

4.4 Results:

4.4.1 Western blot of human CTE:

Insoluble tau protein isolated from CTE cases contained all 6 isoforms. This was evident in both phosphorylated and dephosphorylated samples. This was in contrast a control Alzheimer's disease case in which the insoluble fraction consisted of mainly three isoforms constituting the paired helical filament motif as previously reported (Figure 4.1) (Strong et al., 2006).

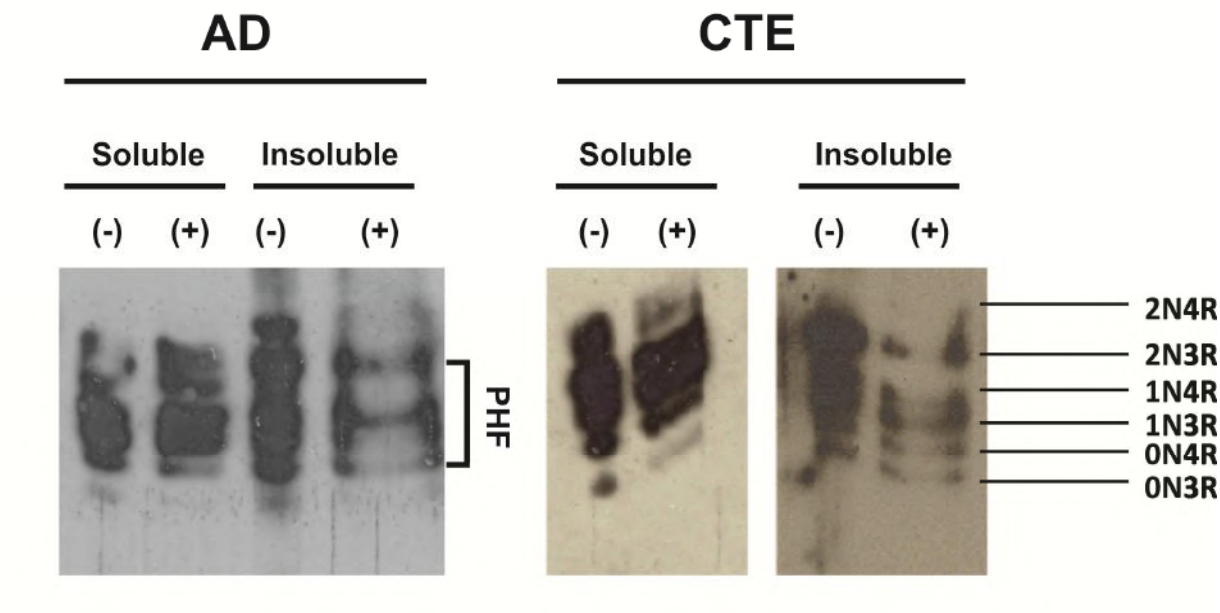


Figure 4.1: Representative Western blot of CTE-derived fractionated tau protein showing all 6 tau protein isoforms in the insoluble fraction in distinction to the 3 isoform motif which was observed in Alzheimer's disease. Images representative of 6 CTE cases and 1 Alzheimer's case. (Probed with mouse anti-T14/T46 total tau antibody; blots shown are from the same case on 2 separate blots).

4.4.2 Immunohistochemistry in CTE cases:

We observed immunoreactivity to $pThr^{175}$, $pThr^{231}$ and T22 within the hippocampal formation in all cases of CTE and CTE-ALS (Table 4.1, Figure 4.2a). This included tau protein immunoreactive tangles and dystrophic neurites throughout the CA1-4 regions and extending into the entorhinal cortex in all cases. Consistent with the limited $pThr^{175}$ tau protein non-neuronal pathology observed previously (Moszczynski et al., 2017), oligodendroglial tau protein immunoreactivity was observed to a limited extent in 5 cases only (Moszczynski et al., 2017). When present in control cases (3/5), tau protein immunoreactivity was observed only as faint immunoreactive punctate neuronal staining in the absence of tangles (Figure 4.2b). No neuritic pathology was observed in controls.

Table 4.1: Hippocampal and spinal cord pathology summary

Antibody	**CTE**	**CTE-ALS**	**Ctrl**
Hippocampus semi quantitation (case positive ratios)			
pThr^{175}	++++ (5/5)	+++-++++ (5/5)	+-++ (3/5)
pThr^{231}	++++ (5/5)	+++-++++ (5/5)	±-+ (3/5)
T22	++++ (5/5)	±-+++ (5/5)	±-+ (3/5)
Spinal cord pathology case positive ratios			
pThr^{175}	3/5	3/5	0/5
pThr^{231}	4/5	4/5	2/5
T22	4/5	1/5	0/5

Semi-quantitative scale was applied under 20x objective for neuronal pathology as follows: '-' = none; '±' = less than 5 inclusions; '+' = less than 10 inclusions; '++' = more than 20 inclusions with scattered distribution; '+++' = more than 20 inclusions but with locally dense distribution; '++++' = more than 20 inclusions with a diffuse distribution.

Figure 4.2 A

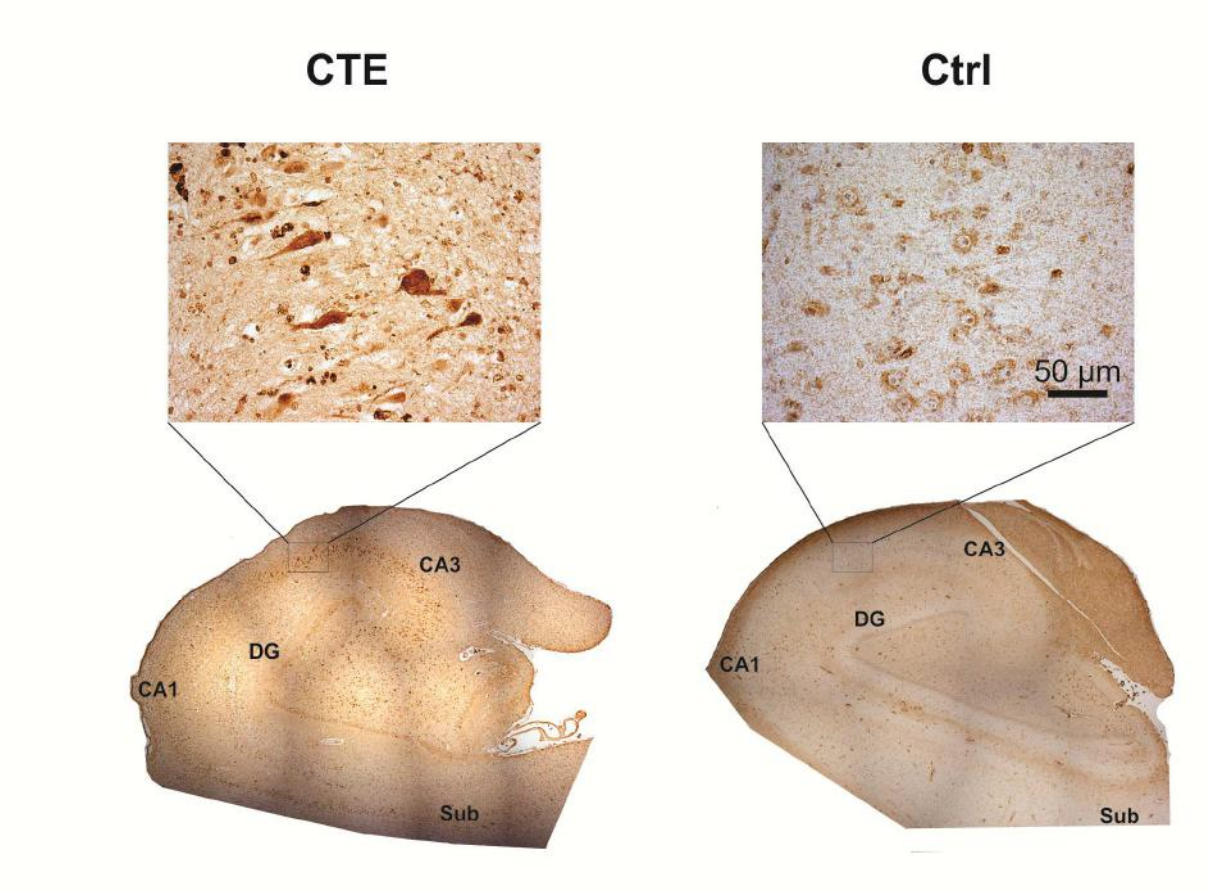


Figure 4.2 B

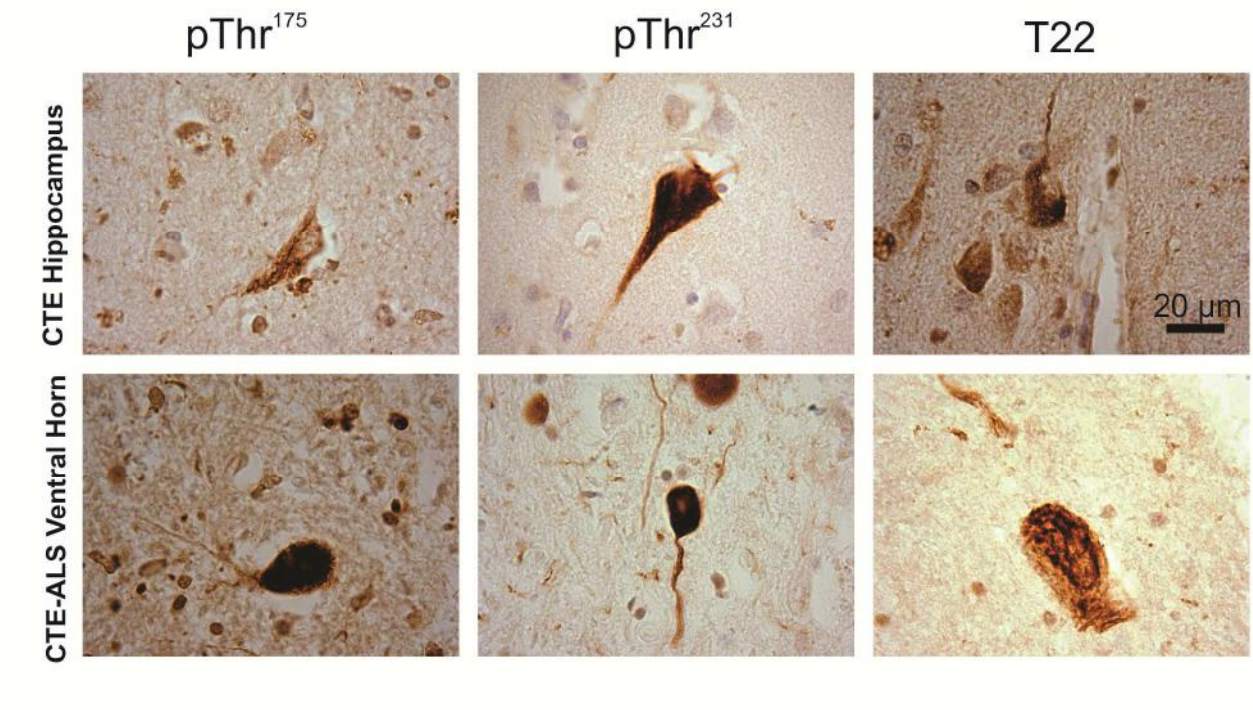
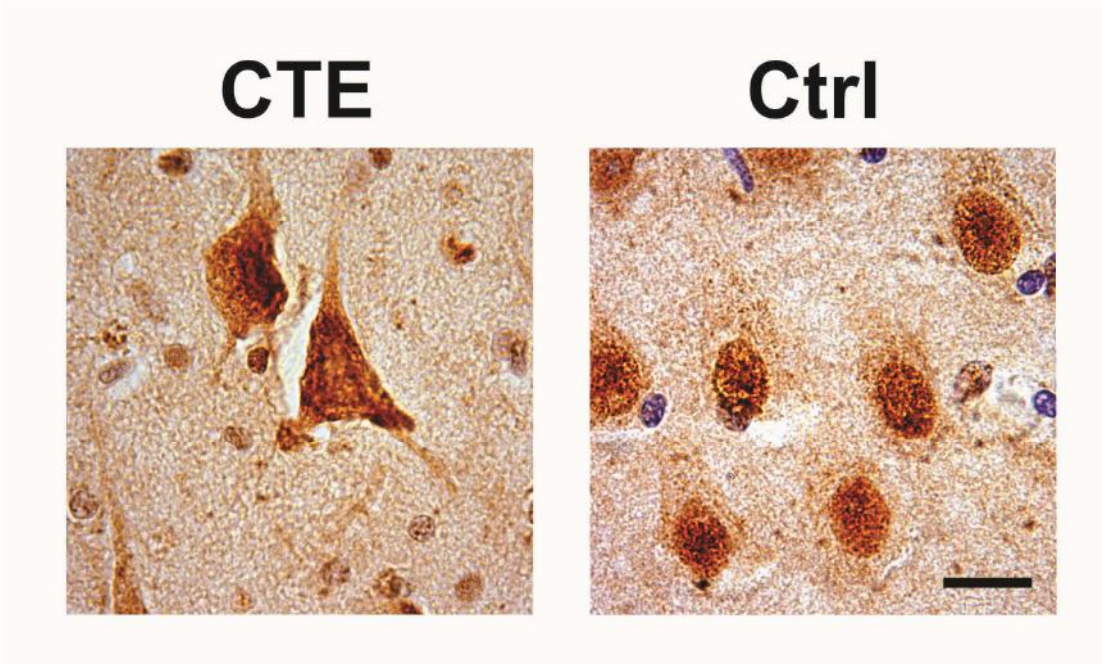


Figure 4.2: Tau pathology in hippocampus and ventral horn in CTE and CTE-ALS. A) Low magnification (4 x objective) composites of whole hippocampus stained for pThr^{175} tau protein in CTE and control (inlay is CA2 region taken with 40 x objective, scale bar = 50 µm). DG = Dentate gyrus, CA1 = CA1 region, CA3 = CA3 region, sub = subiculum (composite image based on images taken with a 4x objective). Note the presence of prominent tau protein immunoreactivity in CA1 through CA4, extending into the entorhinal cortex. B) Representative tau protein pathology observed in both the hippocampus (CTE case) and ventral horn of the spinal cord (CTE-ALS) when probed by rabbit anti-pThr^{175} tau, rabbit anti-pThr^{231} tau, or rabbit anti-T22 (tau oligomer) antibodies. Images taken with 100x objective (scale bar= 20 µm).

We also observed pathological tau protein deposition within the spinal cord regardless of the tau protein phosphoepitope studied (Figure 4.2b). The presence of tau protein pathology was independent of whether the underlying pathological diagnosis was CTE or CTE-ALS (Table 4.1) and consisted of sparsely distributed neurofibrillary tangles in motor neurons and dystrophic neurites. In all cases, pathological inclusions were minimal in number relative to a more diffuse immunoreactivity to $pThr^{231}$ tau and $pThr^{175}$ tau. No $pThr^{175}$ tau staining was observed in control motor neurons. $pThr^{231}$ tau protein was observed but only as diffuse perikaryal staining of motor neurons when present. Lipofuscin staining was also observed in some controls.

We also noted a significant alteration in the immunoreactivity towards pGSK3β from a primarily nuclear to a diffusely cytosolic pattern of immunoreactivity (Supplemental Figure 4.1). This pattern was observed in both hippocampal CA2 and spinal cord motor neurons in all CTE cases while only being present in occasional isolated cells in controls.



Supplemental Figure 4.1: Activated GSK3β (pGSK3β) localization in hippocampus of CTE and control. A redistribution of pGSK3β is observed in CTE and CTE-ALS cases in which immunoreactivity is prominent in both the nuclear and cytosolic compartments whereas in control cases, immunoreactivity is restricted to the nuclear compartment. Tissue stained with mouse anti-GSK3β pTyr^{216}. Images taken from hippocampal CA4 region with 100x objective. Scale bar= 20 μm.

Double-immunolabeling of the hippocampus demonstrated that $pThr^{175}$ tau and $pThr^{231}$ tau co-localize consistently such that $pThr^{175}$ always co-occurred with $pThr^{231}$ immunoreactivity. However, as observed previously (Moszczynski et al., 2017), $pThr^{231}$ tau immunoreactivity could occur independently of $pThr^{175}$ tau suggesting that a subset of $pThr^{231}$ tau pathology is $pThr^{175}$ positive (Moszczynski et al., 2017). Additionally, we observed co-localization of $pThr^{231}$ tau and T22, suggesting that oligomeric tau was a component of $pThr^{231}$ tau pathology. Due to the nature of the antibodies, it was not possible to test for co-localization of $pThr^{175}$ with T22 (oligomerized tau protein) as they were raised in the same species, not purified and not compatible with the primary antibody labeling system available to us. We can therefore only infer that $pThr^{175}$ tau protein co-localizes with oligomeric tau protein as well. We did however observe $pThr^{175}$ co-localization with active GSK3β (Figure 4.3).

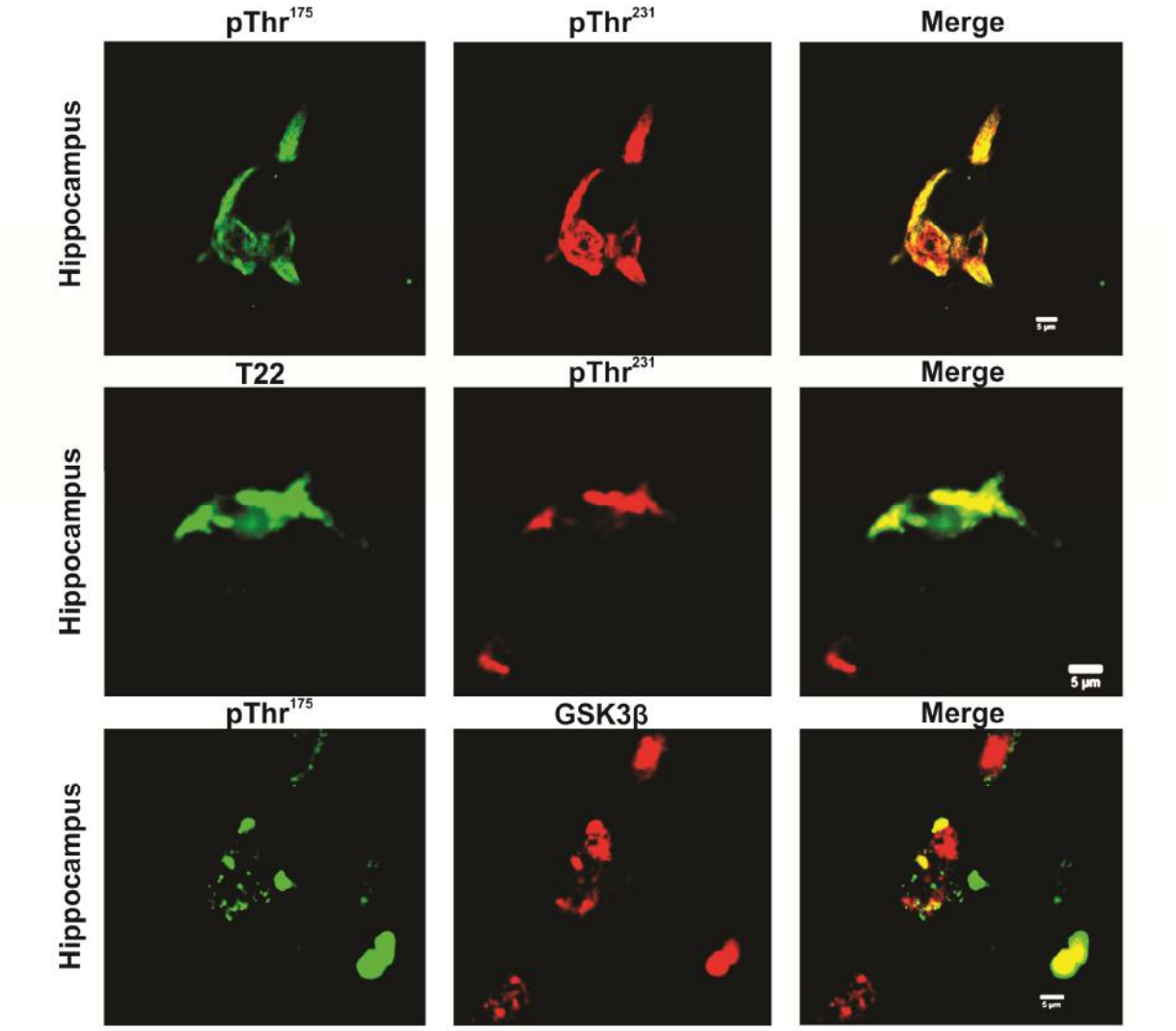
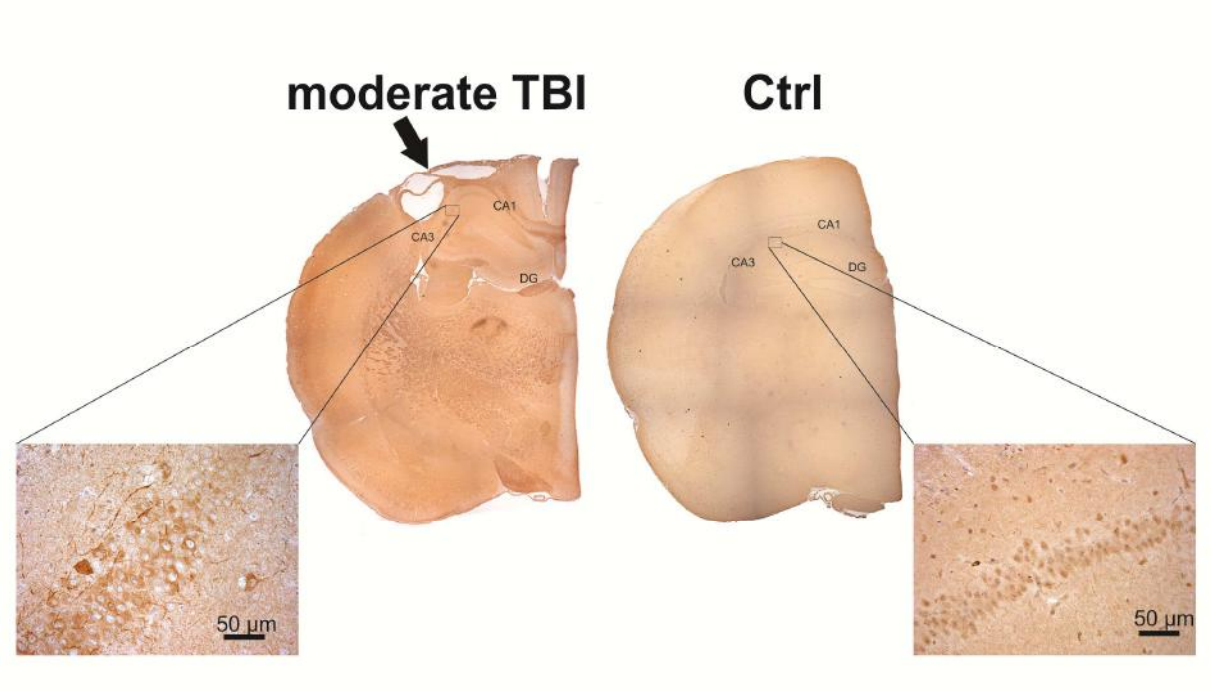


Figure 4.3: Co-localization of pThr^{175} tau and pThr^{231} tau was observed in hippocampal neurons of CTE (upper panels). The presence of pathological tau protein oligomers (T22 immunoreactivity) was co-localized to pThr^{231} tau protein immunoreactive neurons in CTE (middle panels). Consistent with a role in activation of GSK3β in inducing pathological tau protein deposition, we observed the co-localization of pThr^{175}tau protein with the active pGSK3β immunoreactivity (lower panel). Tissues were immunolabelled with rabbit anti-pThr^{175} tau protein, rabbit anti-pThr^{231} tau, rabbit anti-T22 antibody and mouse anti GSK3β pTyr^{216} antibody. For double-labeled tissue, red channel antibodies were labeled directly with Alexafluor 555. Co-localization stains were performed in one case per antibody combination. Scale bar = 5 µm.

4.4.3 pThr^{175} and pThr^{231} expression in moderate TBI:

Both pThr^{175} and pThr^{231} tau neuronal immunoreactivity was observed in moderate TBI rat brains (Figure 4.4). Of note, pThr^{175} positive neuronal staining was observed in regions distant to the injury site mainly as axonal staining; however, no fibrillar inclusion type pathology was observed in regions distant from the injury site. While pThr^{175} tau protein was significantly elevated within the hemisphere ipsilateral to the injury, the contralateral hemisphere also showed a trend to increased pThr^{175} tau protein expression when normalized against total tau protein ($p=0.05$ and 0.065 respectively after one way ANOVA with $p=0.008$ and $F= 5.757$). Diffuse pThr^{231} staining was observed in healthy neurons. Pathology-bearing pThr^{231} tau protein was only observed near the site of injury. GFAP staining did not show inclusions at site of injury indicating that pathology was a protein specific phenomenon. Additionally previous studies in our lab have shown the specificity of the pThr^{175} antibody to tau protein using peptide blockers (Yang et al 2012).

Figure 4.4 A



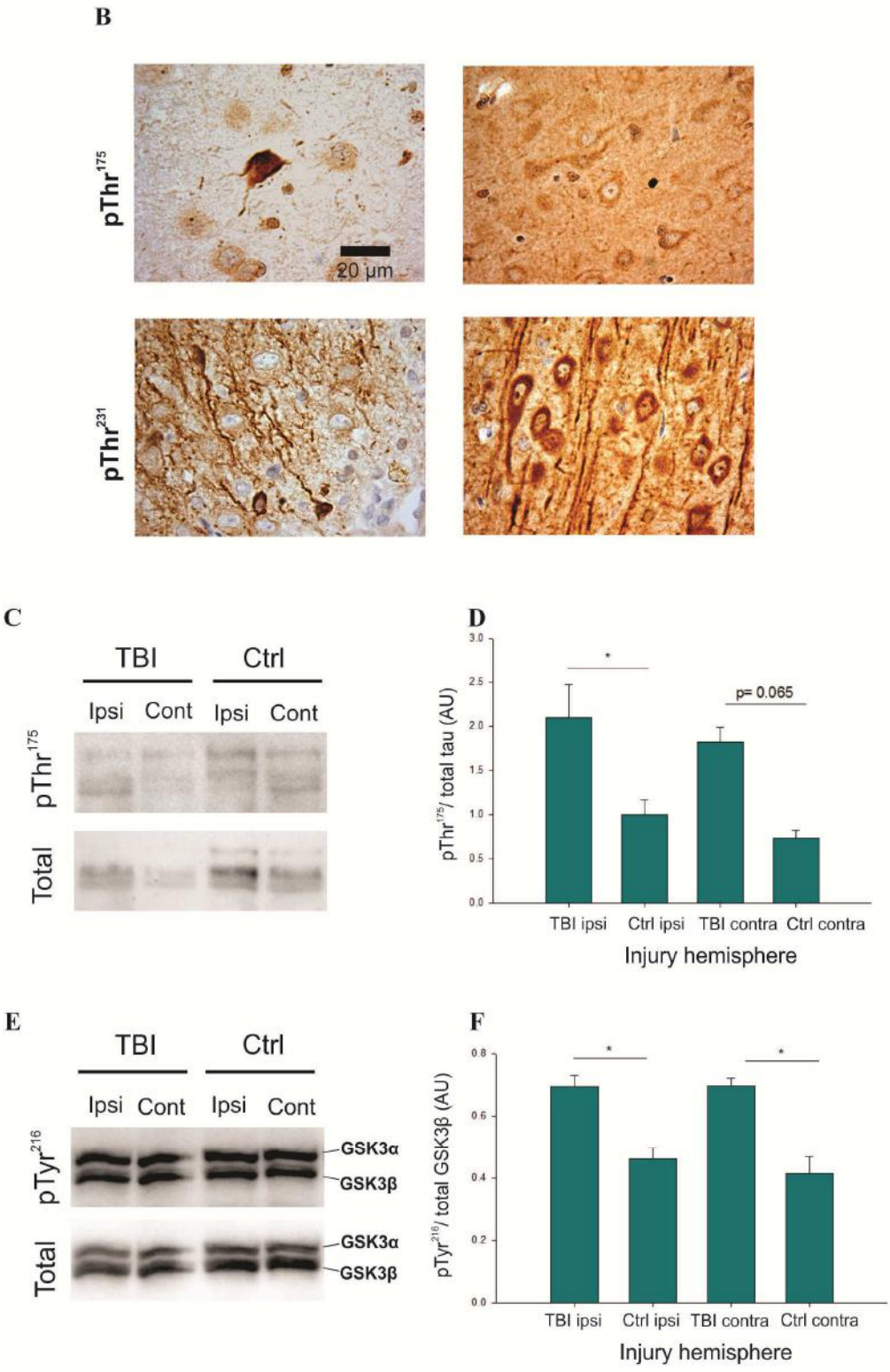
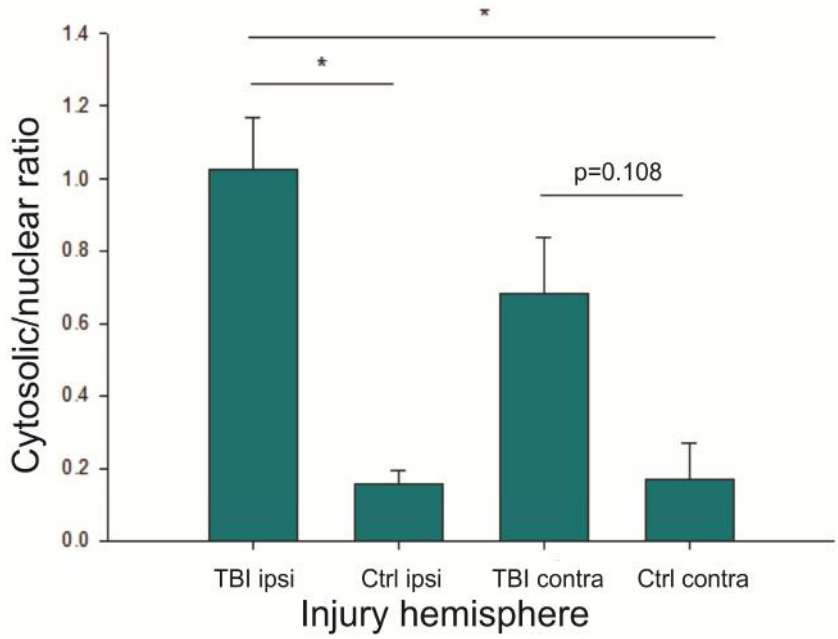

B
pThr175
20 µm
pThr231
C
TBI
Ctrl
Ipsi
Cont
Ipsi
Cont
pThr175
Total
D
pThr175/ total tau (AU)
*
p= 0.065
TBI ipsi
Ctrl ipsi
TBI contra
Ctrl contra
Injury hemisphere
E
TBI
Ctrl
Ipsi
Cont
Ipsi
Cont
pTyr216
GSK3α
GSK3β
Total
GSK3α
GSK3β
F
pTyr216/ total GSK3β (AU)
*
*
TBI ipsi
Ctrl ipsi
TBI contra
Ctrl contra
Injury hemisphere

Figure 4.4: pThr^{175} tau pathology is recapitulated in an *in vivo* model of moderate TBI at 3 months post injury. A) Composite images of whole brain sections stained for pThr^{175} tau protein. Images were taken with a 4 x objective. Inlay image taken with 40 x objective. Arrow denotes site of injury. DG= Dentate gyrus, CA1= CA1 region, CA3= CA3 region. Scale bar= 50 μm. B) High magnification images with moderate TBI showing neuronal and neuritic pathology. Images were taken with 100 x objective. Scale bar = 20 μm. C) Western blots of pThr^{175} tau protein and total tau protein in ipsilateral and contralateral brain injuries. D) densitometry of western blots probed for pThr^{175} tau protein and total tau protein (pThr^{175}/total tau). E) pTyr^{216} GSK3β and total GSK3β (pTyr^{216}/ total GSK3β) in ipsilateral and contralateral brain injuries. F) densitometry of western blots probed for pTyr^{216} GSK3β and total GSK3β. * represents $p < 0.05$. Ipsi: ipsilateral injury hemisphere, contra: contralateral injury hemisphere. Data are representative of 6 rats (TBI; IHC and western blot), or 4 rats (control; western blot), or 3 rats (control; IHC).

We investigated tau protein phosphorylation and GSK3β activation by western blot (Figure 4.4c). $pTyr^{216}$ GSK3β was also significantly elevated in ipsilateral and contralateral hemispheres relative to uninjured controls ($p=0.005$ and 0.001 respectively after one way ANOVA with $p<0.001$ and $F=13.928$) when normalized against total GSK3β (Figure 4.4). Finally, we observed the same change in localization of pGSK3β in moderate TBI rat brains as in CTE cases. This was quantified by blinded counts in which we observed a significant increase in diffuse expression of $pTyr^{216}$ GSK3β in the injured hemisphere compared to uninjured control rats ($p= 0.01$ by Tukey post hoc test after one way ANOVA $p= 0.004$ and $F= 7.559$; Supplemental figure 4.2).



Supplemental Figure 4.2: Quantification of cytosolic active GSK3β in both injured and uninjured rats. Bar graph showing ratio of cells with widespread pTyr^{216} GSK3β compared to cells showing predominantly nuclear pTyr^{216} GSK3β. Values represent counts from 3 rats (control) or 6 rats (TBI). * represents $p < 0.05$.

4.5 Discussion:

We have observed that both CTE and CTE-ALS are tauopathies in which pathological tau protein aggregates contain aberrantly phosphorylated tau protein with immunoreactivity to both $pThr^{175}$ tau and $pThr^{231}$ tau. The presence of T22 immunoreactivity (recognizing oligomeric tau protein) is consistent with a pivotal role for phosphorylation at Thr^{175} in the pathogenesis of CTE and CTE-ALS. The inference can be made on the basis of our previous study which showed that $pThr^{175}$ tau protein pathology only occurs in pathological conditions and that the expression of $pThr^{175}$ tau protein coincides with oligomerized tau protein in the same neuronal populations (Moszczynski et al., 2017). This was observed at the same time as $pThr^{231}$ tau protein was observed in pathological inclusions. This continues therefore to be consistent with the postulated role of pathological tau protein phosphorylation across a broad range of tauopathies (Moszczynski et al., 2017). Uniquely, we have also observed that this pathological process of tau protein phosphorylation can be triggered experimentally in an *in vivo* model of moderate TBI. The finding that the tauopathy of CTE, CTE-ALS consists of the expression of all 6 tau protein isoforms in both the soluble and insoluble tau protein isolates further suggests that this process is biochemically distinct from the tauopathy of AD.

Consistent with our previous reports, $pThr^{175}$ tau staining was only observed when other pathological tau protein markers were also present (Moszczynski et al., 2017) and as such, $pThr^{175}$ tau positive staining in controls was restricted to mainly those individuals with advanced age where some tau protein pathology is expected in the hippocampal formation (Yang et al., 2005). Therefore beyond its role in toxicity itself, $pThr^{175}$ also appears to also be an indicator of toxicity and neuronal damage. Because of this, $pThr^{175}$ tau protein may be useful as a biomarker of tauopathy, being more specific than other tau protein phosphoepitopes that have been

investigated previously as CSF biomarkers of disease (Mattsson et al., 2009). If this proves true for general tauopathies, it may be useful to investigate the ratios of $pThr^{175}$ tau protein and other proteins that have previously been investigated such as14-3-3 (Foote and Zhou, 2012), and neurofilament proteins (Li et al., 2016) in CSF or blood of CTE and CTE-ALS patients.

The tau protein isoform composition profile observed in CTE in this study and previously in ALSci (Strong et al., 2006) in which all 6 tau protein isoforms are observed in the soluble and insoluble tau protein isolates is in distinction to that observed in a number of tauopathies, including AD (in which the pathogenic tau protein marker is the PHF triplet), CBD and PSP (4R tauopathies) and Pick's disease (3R tauopathy) (Buee, V et al., 1996; Delacourte et al., 1996; Delacourte et al., 1998). This could be interpreted as indicative that the tauopathy of CTE, CTE-ALS and ALSci is a "secondary event" which is triggered in response to a primary neuronal injury. In both CTE and CTE-ALS, this can be postulated to be directly due to the traumatic brain injury itself, a hypothesis that is strongly supported by the *in vivo* moderate TBI experimental paradigm. While it is less clear what the 'trigger' for the tauopathy of ALS may be, it is clear that once initiated, the induction of phosphorylation at Thr^{175} leads to a cascade of events that culminates in neuronal death *in vitro* (Gohar et al., 2009; Moszczynski et al., 2015). At this time, it is unknown whether or not $pThr^{175}$ tau protein pathology is capable of transneuronal propagation as has been posited with regards to other neurodenegeration related proteins. It may be that a longer time than the three months in this study is required for this to occur from a state of no disease to sufficient changes and pathological load necessary for propagation.

In relationship to our understanding of the pathophysiology of ALS, upwards of 10% of CTE cases also develop motor symptoms consistent with ALS (CTE-ALS), a rate much higher

than the incidence of ALS (2-3 per 100,000) observed in the general population (McKee et al., 2010). Unique to the motor neuron degeneration associated with CTE-ALS is tau protein pathology in the spinal cord. The only other instance of a disseminated tauopathy in association with motor neuron degeneration is that which was observed in the previously hyper-endemic Western Pacific variant of ALS; a variant of ALS increasingly recognized to be at the intersection of an environmental insult in an at risk population (Garruto, 1991; Hirano et al., 1961). While we have observed pathological tau protein phosphorylation in the spinal cords of both CTE and CTE-ALS patients in this study, the variability observed warrants investigation on a larger cohort of cases with regional stratification. The resources available to us in this study did not allow for regional stratification due to the random nature of tissue selection (cervical, thoracic, or lumbar for different cases, but not all) and thus we were not able to discern whether there was a correlation between motor symptom progression and regional tau protein deposition. However, the failure to observe tau protein deposition in the spinal cords of individuals afflicted with sporadic ALS suggests that the spinal motor neuron tauopathy of CTE-ALS is not an incidental finding or secondary to the primary neuronal injury of ALS. It is possible that tau protein phosphorylation and pathology begins early in the neurodegenerative process in CTE or CTE-ALS, in which case spinal cord pathology would be expected to precede symptom onset in patients that would otherwise develop motor impairment.

To conclude, we report the presence of all 6 tau protein isoforms in the insoluble fraction of tau protein isolated from CTE and CTE-ALS, and have observed $pThr^{175}$ tau, in conjunction with $pThr^{231}$ tau, oligomerized tau, and changes in active GSK3β localization are consistent with a pathological tauopathy driven by the aberrant phosphorylation of Thr^{175} tau protein. The observation that this pathway can be triggered following a single moderate TBI suggests that

brain trauma itself can drive this process directly. Understanding how moderate TBI drives phosphorylation at Thr^{175} is the topic of current studies. However, given our previous findings that this pathological cascade of tau protein phosphorylation can be fully inhibited, and that this inhibition abolishes $pThr^{175}$ tau protein induced neuronal death suggests that both CTE and CTE-ALS may be amenable to pharmacological inhibition of GSK3β activation.

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Chapter 5: Somatic gene transfer using a recombinant adenoviral vector (rAAV9) encoding pseudophosphorylated human Thr^{175} tau in adult rat hippocampus induces a pathological tauopathy

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5.1 Abstract

Aberrant phosphorylation of the microtubule associated protein tau (tau protein) is associated with multiple neurodegenerative diseases (collectively known as tauopathies) where it is a critical contributor to toxicity in neurons. An individual phosphorylation site at Thr^{175} ($pThr^{175}$) has been observed in multiple tauopathies and has been shown to exert toxicity when expressed as a pseudophosphorylated tau construct ($Thr^{175}Asp$) *in vitro*. This toxicity is exerted through activation of GSK3β and further phosphorylation of tau protein at Thr^{231}. In this study, we used a recombinant adenoviral expression vector (rAAV9) to express a GFP-tagged $Thr^{175}Asp$ tau protein construct in adult female Sprague-Dawley rat hippocampus to assess the ability of $pThr^{175}$ tau protein to exert toxicity *in vivo*. 10 rats per group were injected with rAAV9 vectors encoding either GFP, wild type GFP-tagged tau protein, $Thr^{175}Ala$ tau protein or $Thr^{175}Asp$ tau protein. 12 months post-injection all rats were euthanized and investigated by immunohistochemistry for GFP (extent of vector expression), $pThr^{231}$ tau protein, activated GSK3β, and caspase-3 cleavage. $Thr^{175}Asp$ tau protein inoculated neurons showed tau protein pathology in the form of axonal beading, fibrils, and neurofibrillary tangles. Caspase-3 cleavage was observed in the $Thr^{175}Asp$ tau protein group but not others. These results indicate that $pThr^{175}$ tau protein is capable of exerting toxicity *in vivo* and that this may be a therapeutic intervention in neurodegenerative diseases exhibiting this pathological phosphoepitope.

5.2 Introduction

Over 50% of ALS cases present with, or develop, cognitive impairment in the form of a frontotemporal dysfunction (Strong et al., 2009; Strong et al., 2017). These neuropsychological deficits are also associated with shorter patient survival and an increased caregiver burden (Chio et al., 2010; Govaarts et al., 2016). Previous studies have demonstrated the presence of pathological intracellular inclusions of the microtubule associated protein tau (tau protein) in the subgroup of cognitively impaired ALS patients known as ALS with cognitive impairment (ALSci). Tau protein in ALSci is associated with neuronal and glial tau protein inclusions and is phosphorylated at Thr^{175} ($pThr^{175}$ tau) (Strong et al., 2006; Yang et al., 2003; Yang and Strong, 2012). Further study revealed that the presence of neuronal cytoplasmic inclusions of tau protein in ALSci is associated with activated GSK3β, a kinase that has been clearly demonstrated to phosphorylate tau protein in other tauopathies (Yang et al., 2008). *In vitro* studies using pseudophosphorylated tau protein constructs to mimic $pThr^{175}$ (Thr^{175}Asp) showed that this phosphoepitope induces tau protein aggregate formation and increased cell death *in vitro* (Gohar et al., 2009). This was then shown to be reliant on increased GSK3β activity and further phosphorylation of tau protein at Thr^{231} (Moszczynski et al., 2015). In order to determine whether this pathway leading to pathological tau phosphorylation could be applied to the tauopathies, we evaluated a larger cohort of ALS cases (in collaboration with the Mann group) (Behrouzi et al., 2016) and a broad range of neuropathologically confirmed tauopathies (Moszczynski et al., 2017b). Critically, in the latter study, we observed that the presence of $pThr^{175}$ tau protein inclusions was also associated with GSK3β activation and the induction of $pThr^{231}$ tau protein with associated pathological tau protein oligomerization (recognized by the antibody T22) (Lasagna-Reeves et al., 2012). In our most recent studies, we have shown that this $pThr^{175}$ tau protein is present within tau neuronal cytoplasmic inclusions in both hippocampal

neurons and motor neurons in chronic traumatic encephalopathy (CTE) with or without an associated motor neuron degeneration (CTE-ALS) (Moszczynski et al., 2017a). Of importance to future mechanistic studies, a remarkably similar pathology inclusive of both $pThr^{175}$ tau protein and $pThr^{231}$ tau protein can be induced by traumatic brain injury (TBI) in a rodent model of moderate TBI (Moszczynski et al., 2017a).

The role of $pThr^{175}$ tau protein in the induction of a tauopathy seems therefore to have wide applicability to a range of neurodegenerative processes, including ALSci, CTE and CTE-ALS. However, the effects of $pThr^{175}$ tau protein have not been investigated *in vivo*. The purpose of this study was to investigate the effects of expressing pseudophosphorylated tau protein (Thr^{175}Asp tau protein) in a rat model to characterize its capacity to induce tau protein fibrillization and toxicity as it does *in vitro*.

5.3 Methods:

All experimental protocols were approved by the University of Western Ontario Animal Care Committee (AUP #2013-008) in accordance with the policies established in the guide to Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care.

5.3.1 Somatic gene transfer:

A previously described (Mustroph et al., 2012) somatic gene transfer technique was used to express recombinant adeno-associated virus (rAAV9) vectors: rAAV9 tau (wt-human tau), rAAV9 Thr^{175}Alanine-tau (phosphorylation inhibition), rAAV9 Thr^{175}Asp tau protein (phosphorylation mimic), rAAV9 EGFP (control) into both hippocampus of adult, wild-type female Sprague Dawley rats.

5.3.2 Surgical procedures:

Two-month old wild type female Sprague-Dawley rats (Charles River Canada) were housed in pairs prior to surgery and randomly assigned to an experimental or control group. Food and water were provided *ad libitum*. Ten rats were used per inoculum; thus a total of 40 rats underwent surgery. Each rat weighed approximately 250 g at the time of surgery.

All surgical procedures were conducted at level 2+, in a biosafety cabinet. Rats were weighed, anesthetized under an induction dose of 5% isofluorane and 2 L/min oxygen, and shaved at the top of their head. With a stereotaxic apparatus, each skull was immobilized using ear bars and a mouth-piece. A plastic mask covered the nose and delivered a maintenance dose of 2-3% isoflurane anesthetic with 1% oxygen. Eye lubricant was applied to the eyes to prevent eye damage during the procedure. The shaved area of the head was disinfected using a three-stage preparation: rub with soap, then isopropyl alcohol, and finally iodine. Body temperature was maintained by placing a 37˚C isothermal pad beneath the rat for the duration of the procedure. A rostrocaudal incision was made along the midline of the scalp, and the subcutaneous tissue and periosteum were elevated to expose the underlying bone. The head was leveled using Bregma and Lambda as reference points. Four small holes (1mm) were bored into the skull using an electric drill. Injections of the rAAV9 vectors were conducted using stereotactic coordinates, with Bregma as the reference point. Four inoculations per animal were conducted at two sites per side (3 μL per site), all within the hippocampus at the following coordinates: A/P: -5.5 mm, M/L: ±4.6 mm with D/V: -3.2 mm, and M/L: ±6.0 with D/V: -6.0 mm. Using a Hamilton syringe, a total volume of 12 μL (3 μL per site) was injected over the course of 20 minutes (5 minutes per site) for a total of 1.32×10^{10} vector genomes (2 injections each hippocampus). After injection, the syringe tip remained in place for 2 minutes, before

withdrawal. The vector volume was adopted from previously reported studies utilizing adenoviral vectors to express tau protein in hippocampus (Mustroph et al., 2012).

Rats were then sutured and administered 0.1 mL/100 g Baytril, and 0.1 mL/100 g Meloxicam subcutaneously. After surgery, rats were placed in a recovery cage under a heat lamp until fully recovered from the anesthesia. Rats were then singly housed for 1 week to allow for the incision to close, and then were housed in pairs.

5.3.3 Behavioural and imaging analysis:

All rats underwent Morris water maze testing, startle box testing, as well as open field testing as part of a behavioural battery, and neuroimaging on a 9.4 T magnetic resonance imaging (MRI) scanner for structural and diffusion tensor imaging analysis. These were conducted as the focus of separate graduate theses. Hence, the details of these methods and results will not be considered in detail for this chapter, given that my thesis focused on the pathological analysis.

5.3.4 Immunohistochemistry:

At 12 months post injection, 4 rats per group were sacrificed by trans-cardiac perfusion with heparinized saline (10 units heparin/ mL, 9% NaCl) followed by perfusion fixation with 4% formaldehyde (pH 7.4) after intraperitoneal injection with a lethal dose of Euthanyl. Brains were removed and stored in 4% formaldehyde for 24 hours before tissue processing and embedding in paraffin wax. Tissue was then serially sectioned at 4-6 μm thick sections and mounted to microscope slides.

Immunohistochemistry was conducted using a series of antibodies that included rabbit anti-GFP (1:750 titer, Life Technologies, OR, USA) and rabbit anti-pThr^{231} tau (1:7500 titer,

Thermo Fischer). Antigen retrieval (10 mM sodium citrate, 0.05% Tween 20 pH 6.0) was conducted for all antibodies using a pressure cooker (2100 Retriever; Aptum Biologics, UK). Endogenous peroxidase was quenched with 3% hydrogen peroxide (VWR, Mississauga, Canada). Primary antibody incubation was performed at 4˚C overnight in blocking buffer (5% BSA, 0.3% Triton-X 100 in 1 X PBS). After washing, secondary antibody (1:200 biotinylated IgG) incubation was performed for 1 hour at room temperature in blocking buffer. Antigen:antibody complex was visualized with horseradish peroxidase according to the manufacturer's instructions (Vectastain ABC kit, Vector Laboratories CA, USA), followed by substrate development with DAB. Counterstaining was performed using Harris haematoxylin.

5.3.5 GFP expression and pathology mapping:

Every tenth slide from the serially sectioned brain was stained with rabbit anti-GFP and analyzed for expression. For analysis, the hippocampus was divided into dentate gyrus, CA4, CA3, CA2, CA1, subiculum and fimbria. Expression in each region was graded as present (positive) or absent (negative) for each animal, regardless of how many positive cells were present or expressing pathology. The percentage of animals expressing the GFP construct or pathological tau inclusions was documented. Thalamus, cerebellum and brainstem were used as negative controls.

For each GFP expressing region, the extent of tau protein deposition and pathology was mapped by probing for GFP and $pThr^{231}$ tau protein separately, and then expressed according to the same hippocampal breakdown and positive or negative criteria in each animal for all GFP-expressing regions. One slide per animal was selected for a detailed analysis based on presence of GFP construct expression in the region of interest. The evaluator (AM) remained blinded to the grouping of the rodents until all aspects of the analysis had been completed.

5.3.5 Co-localization and fluorescence staining:

Tau protein was probed with chicken anti-GFP (1:500, Abcam, MA, USA) in conjunction with rabbit anti-pThr^{231} tau protein (1:1000, Thermo Fischer), rabbit anti-cleaved (Asp^{175}) caspase-3 (1:200 titer, Cell signaling, MA, USA) or mouse anti-pTyr^{216} GSK3β (1:50 titer, pTyr^{216}; BD Biosciences, Mississauga, Canada) primary antibodies overnight at 4˚C and Alexafluor goat anti-chicken 488 nm and goat anti-rabbit 555nm or donkey anti-mouse 565 nm secondary (all used at 1:200, Thermo Fischer) for 1 hour at room temperature. Rabbit anti-tau protein pThr^{231} antibody was then labeled using a Zenon primary antibody labeling kit with Alexafluor 555 nm dye (Thermo Fisher) and probed for 1 hour at room temperature. Slides were visualized within 24 hours of labeling by confocal imaging on a Zeiss LSM 510 Meta multiphoton confocal microscope. For co-localizations with phospho-GSK3β (pGSK3β), staining was performed with mouse anti-pTyr^{216} GSK3β antibody (1:50 titer, BD Biosciences) followed by secondary labeling with goat anti-mouse Alexafluor 633nm (Invitrogen).

5.3.6 Quantification and Statistical analysis:

Randomly selected fields within the GFP-expressing CA2 hippocampal pyramidal layer were photographed by confocal microscopy with a 25x objective in GFP-expressing CA2 regions for each animal. The total number of GFP-expressing cells, and the number of cleaved Caspase-3 positive, GFP-expressing cells were counted and expressed as Caspase-3/total cells. Statistical analyses were conducted using SigmaPlot 10.0 software. A one way analysis of variance (ANOVA) was conducted following a Shapiro-Wilk test for normality. Post-hoc Tukey's test was conducted and a p value of 0.05 or lower was considered significant.

5.4 Results:

5.4.1 Behavioural and imaging:

Although the results of the behavioural and neuroimaging studies are the topic of independent thesis projects, it was noteworthy that no overt differences were observed between groups by any method of behavioural analysis or imaging at any time in this study. Fifteen rats developed mammary (Wild type human tau protein n= 2 rats, Thr^{175}Asp tau protein n= 4 rats, Thr^{175}Ala tau protein n= 3 rats, GFP n= 2 rats) or pituitary tumours (Thr^{175}Asp tau protein n= 2 rats, Thr^{175}Ala n= 2 rats) and were sacrificed or died prior to the 12 month timepoint. This extent of benign tumour development was unexpected but upon literature review, it has been documented that the development of tumours can occur spontaneously in up to 50% of rats in the Sprague-Dawley line after the age of 6 months (Giknis and Clifford, 2013).To increase the number of rats for statistical power for imaging and behavioural studies, fifteen additional surgeries were performed to replace the missing rats from each group. None of these rats were used in histological analyses given that I was specifically interested in pathology at the 12 month time interval.

5.4.2 GFP construct expression in the hippocampus:

Hippocampal GFP expression was observed in all groups throughout the anterioposterior axis of the hippocampus (Figure 5.1). The CA2 subregion revealed GFP positive staining in pyramidal neurons as well as in projections within the radiatum layer and cell bodies within the oriens layer in all animals regardless of group, suggesting consistent long term expression of the adenoviral vector across all inoculums. In regions outside of the CA2, we observed variability in the extent of GFP expression within each construct-expressing group. Staining in non-CA2 regions was never as intense as the positive staining in the CA2 region. Cortical GFP neuronal

expression was also observed in the subcortical white matter just superficial to the hippocampus. Extrahippocampal staining was observed in the cortex superficial to the hippocampus only, and was not observed in control regions studied including the thalamus, cerebellum and brainstem.

Figure 5.1 A

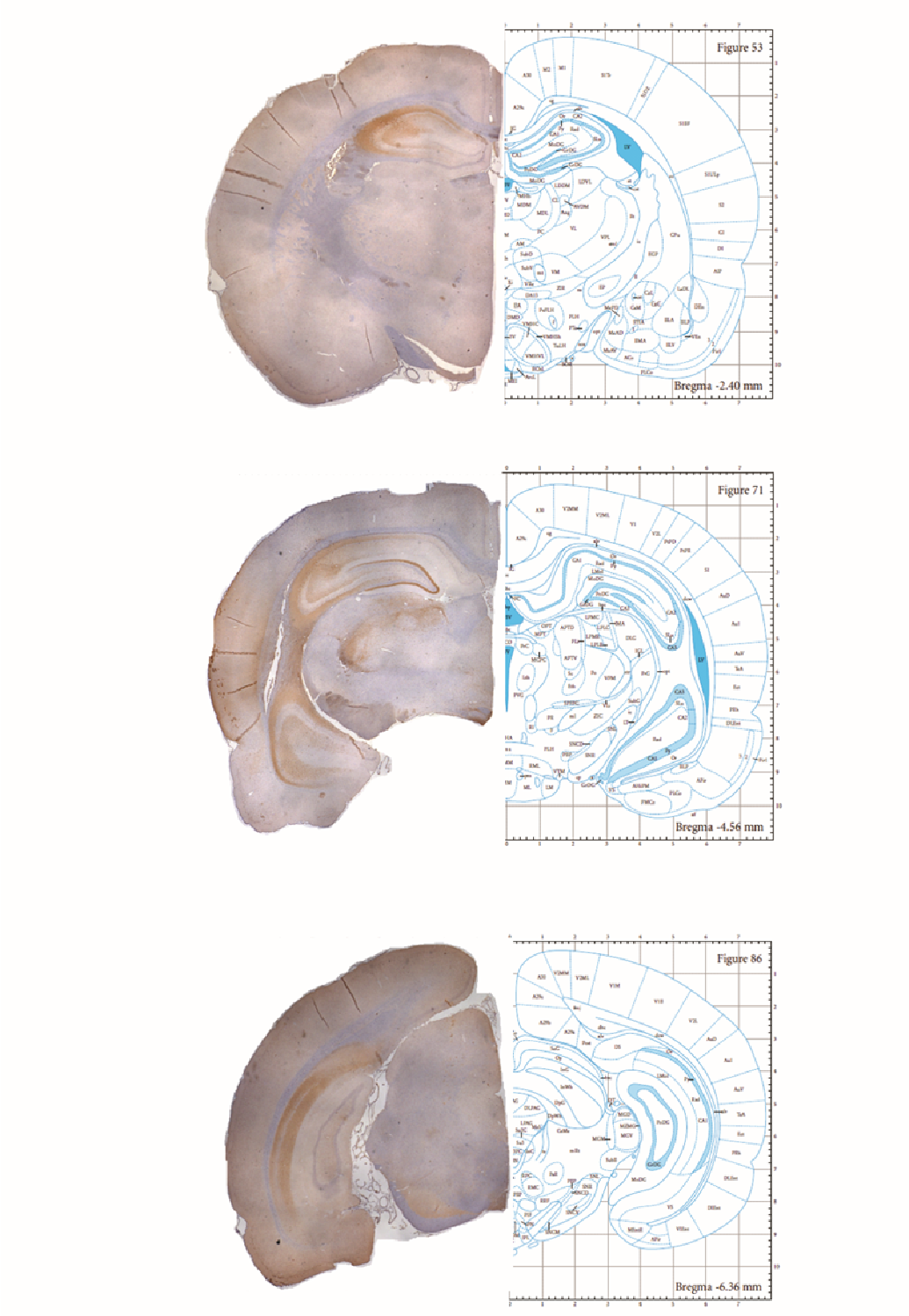
Figure 53
Bregma -2.40 mm
Figure 71
Bregma -4.56 mm
Figure 86
Bregma -6.36 mm

Figure 5.1 B

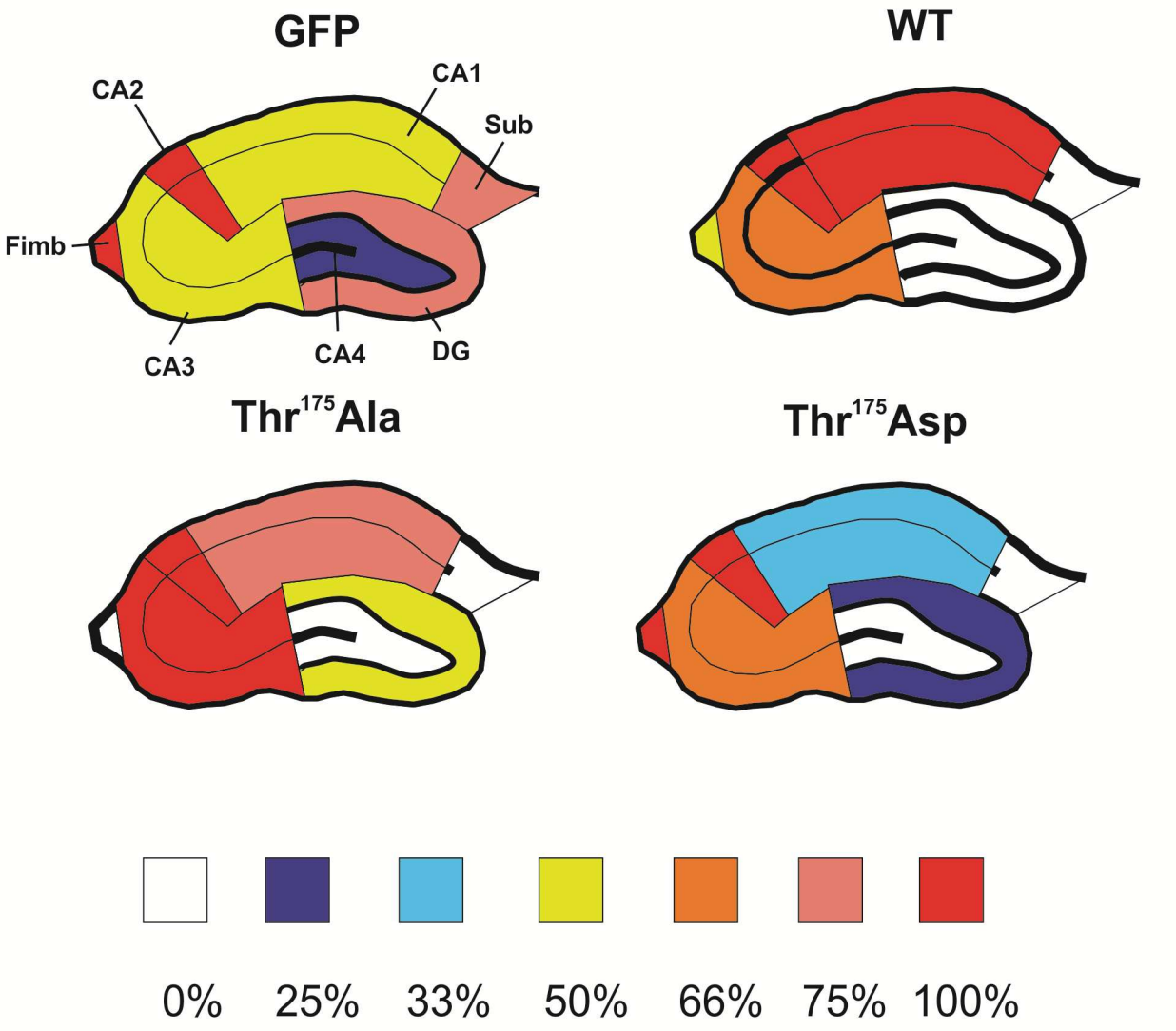

Figure 5.1: AAV9-mediated GFP construct expression. A) We observed adenoviral construct expression (GFP immunoreactivity) throughout the rostrocaudal axis of the hippocampus. B) The most consistent expression was observed within the CA2 region (red) of all animals regardless of the rAAV9 construct with varying degrees of expression throughout the remaining aspects of the hippocampus. % score represents percentage of animals with GFP-positive immunoreactivity in each region. DG=dentate gyrus, SUB= subiculum. Images are composites of photographs taken with a 10 x objective. Data are representative of 3-4 rats per group.

5.4.3 Activated GSK3β expression:

In the CA2 pyramidal neurons of all animals, activated GSK3β was observed in both the nucleus and cytosol. No difference in the GSK3β localization or apparent expression could be detected between groups, although we were unable to reduce the degree of nonspecific background immunoreactivity (Figure 5.2).

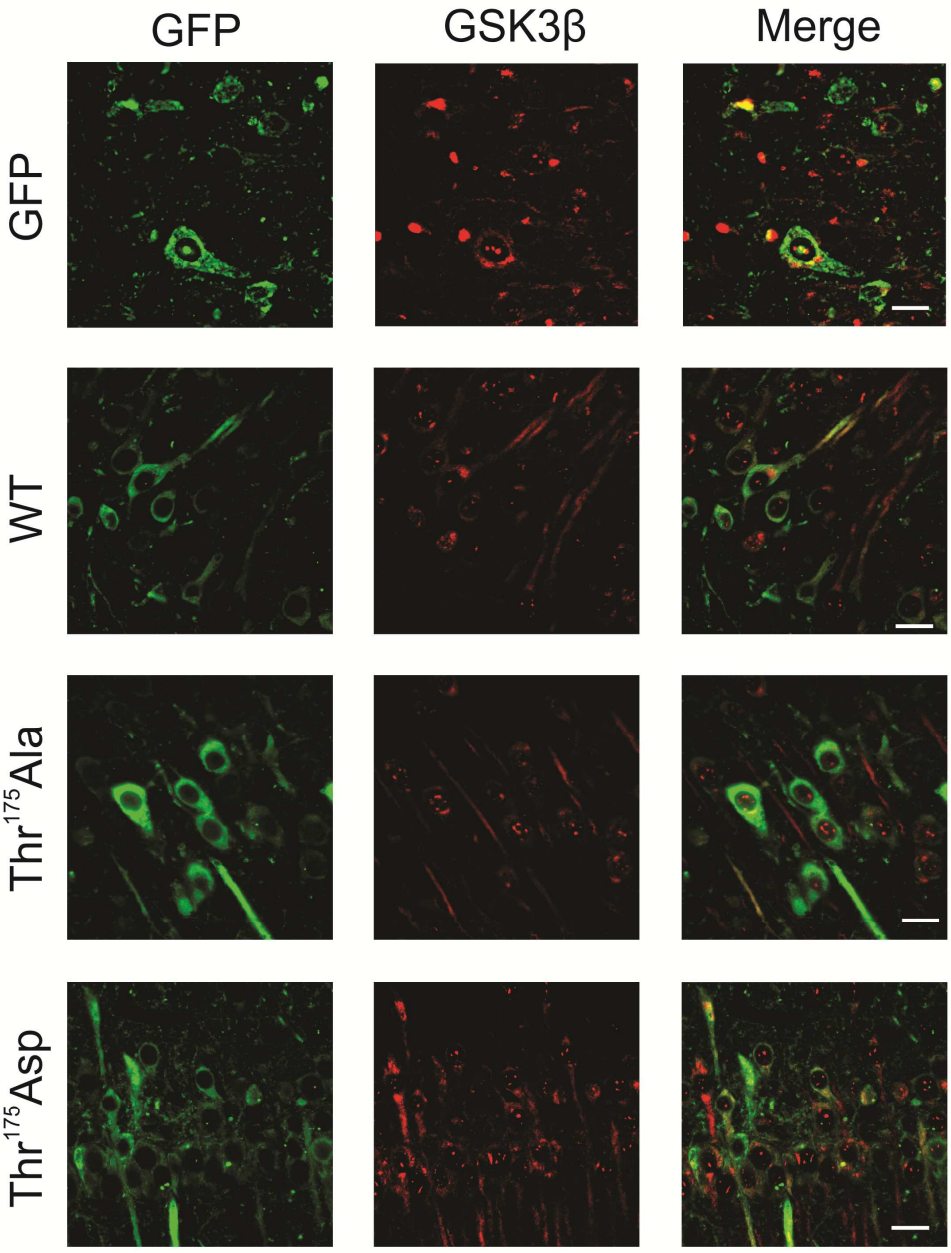

Figure 5.2: Activated GSK3β is expressed in all groups. Nuclear and cytosolic GSK3β expression was observed in GFP-expressing hippocampal neurons in all construct-bearing groups. No inter-group differences were observed. Scale bar = 20 μm. Data are representative of 3 rats per group.

5.4.4 Tau protein pathology expression *in vivo*:

Tau immunoreactive neuronal cytoplasmic inclusions were observed exclusively in the Thr^{175} Asp tau protein expressing rats in all hippocampal regions expressing GFP constructs. This was evident using either the rabbit anti-GFP (recognizing tau constructs, Figure 5.3) or the $pThr^{231}$ tau antibody (Figure 5.4) and could be characterized as axonal beading, fibrils, and tangles (see Figure 5.3 for examples as observed using anti-GFP immunohistochemistry). In addition to this, $pThr^{231}$ staining revealed fibrillar neuronal inclusions and glial positive cells in a phenotype resembling coiled bodies (Figure 5.4).

We observed some degree of axonal beading in all groups within GFP-immunoreactive processes, suggesting that this was a nonspecific feature of the inoculation itself. However, no other pathology was observed outside of that found in Thr^{175} Asp tau protein expressing rats.

Figure 5.3 A

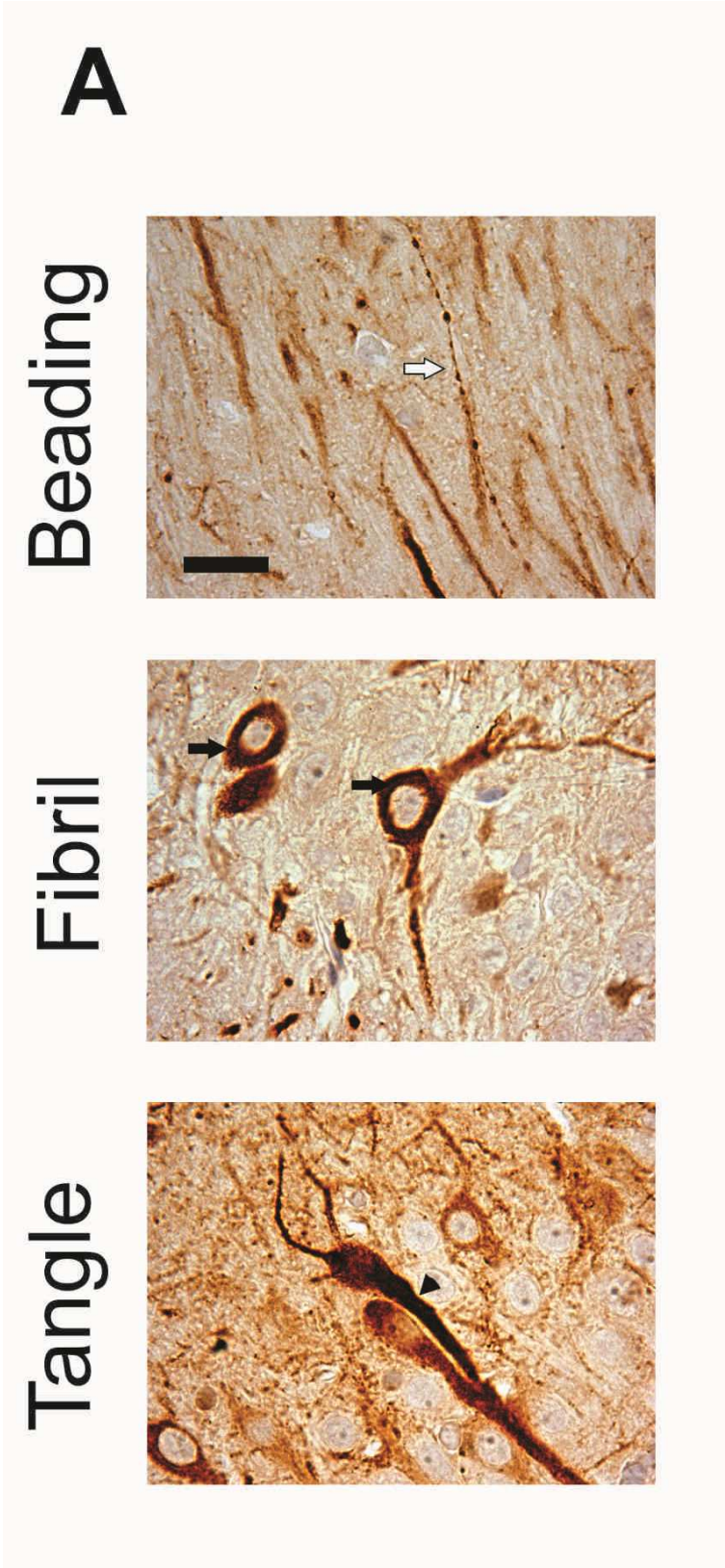

Figure 5.3 B

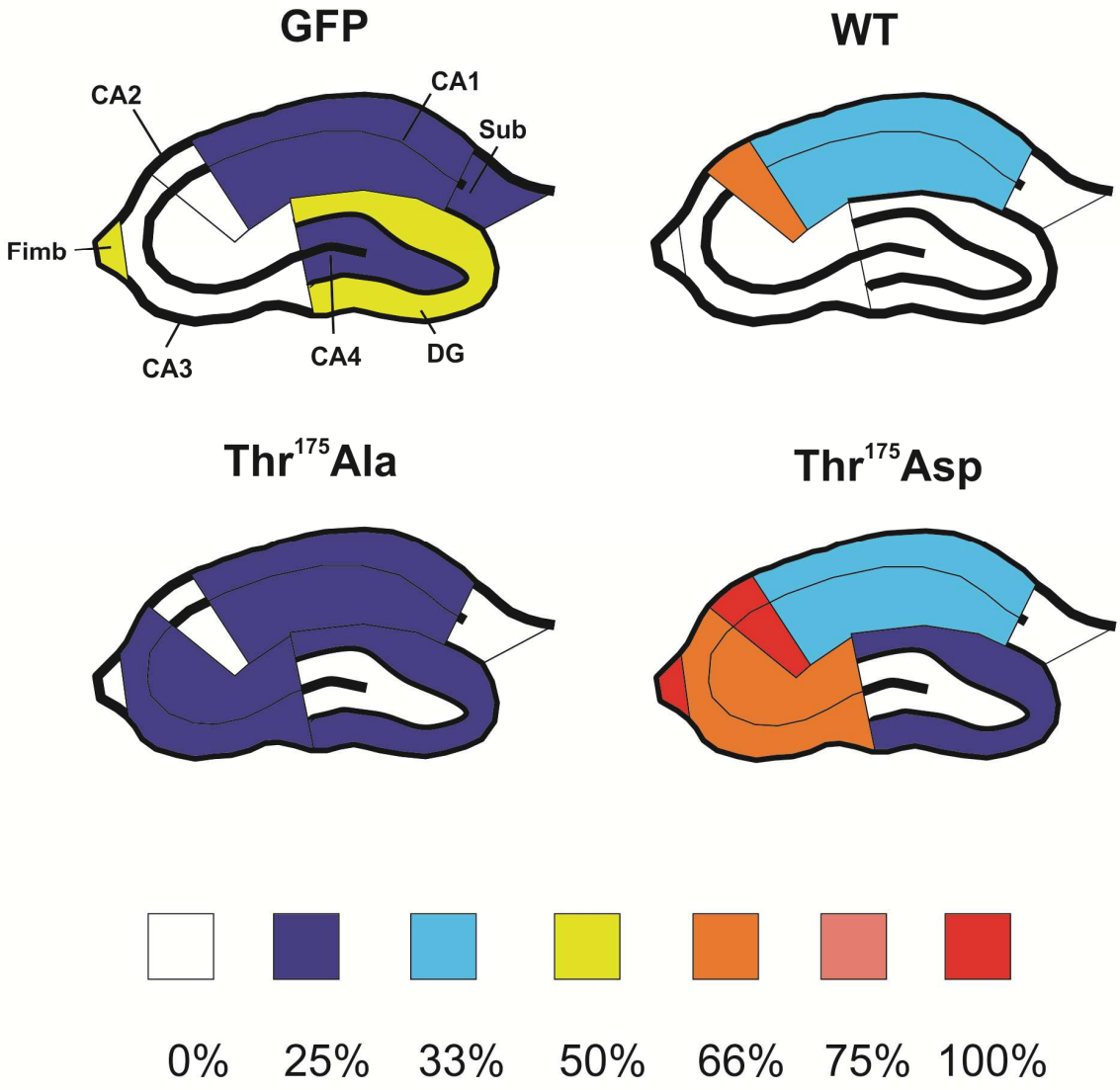

Figure 5.3: Thr^{175} Asp tau protein pathology. A) GFP probing by immunohistochemistry reveals GFP-tau protein pathology expression in the form of axonal beading (white arrow), fibrils (black arrow) and tangles (black arrowhead). B) Pathology was expressed in Thr^{175} Asp tau protein expressing rats to a greater extent than all other groups. % score represents percentage of pathology-positive animals in each region. DG=dentate gyrus, SUB= subiculum, Fimb= fimbria. Images taken with a 100x oil immersion objective. Data are representative of 3-4 rats per group. Scale bar= 20 μm.

Figure 5.4 A

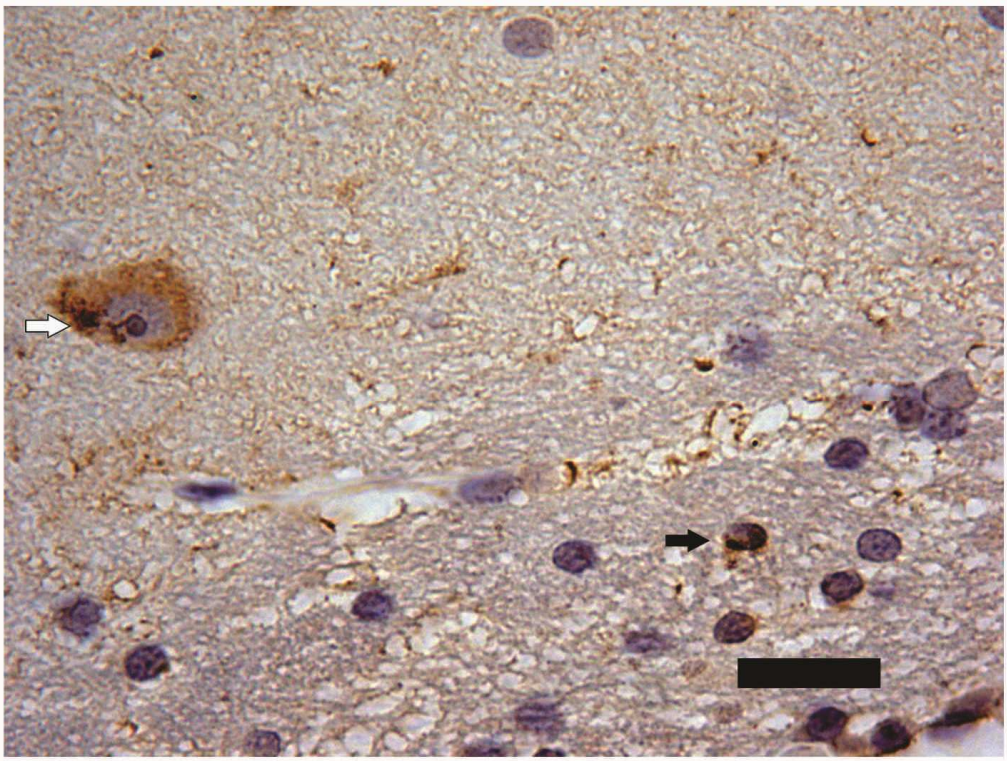

Figure 5.4 B

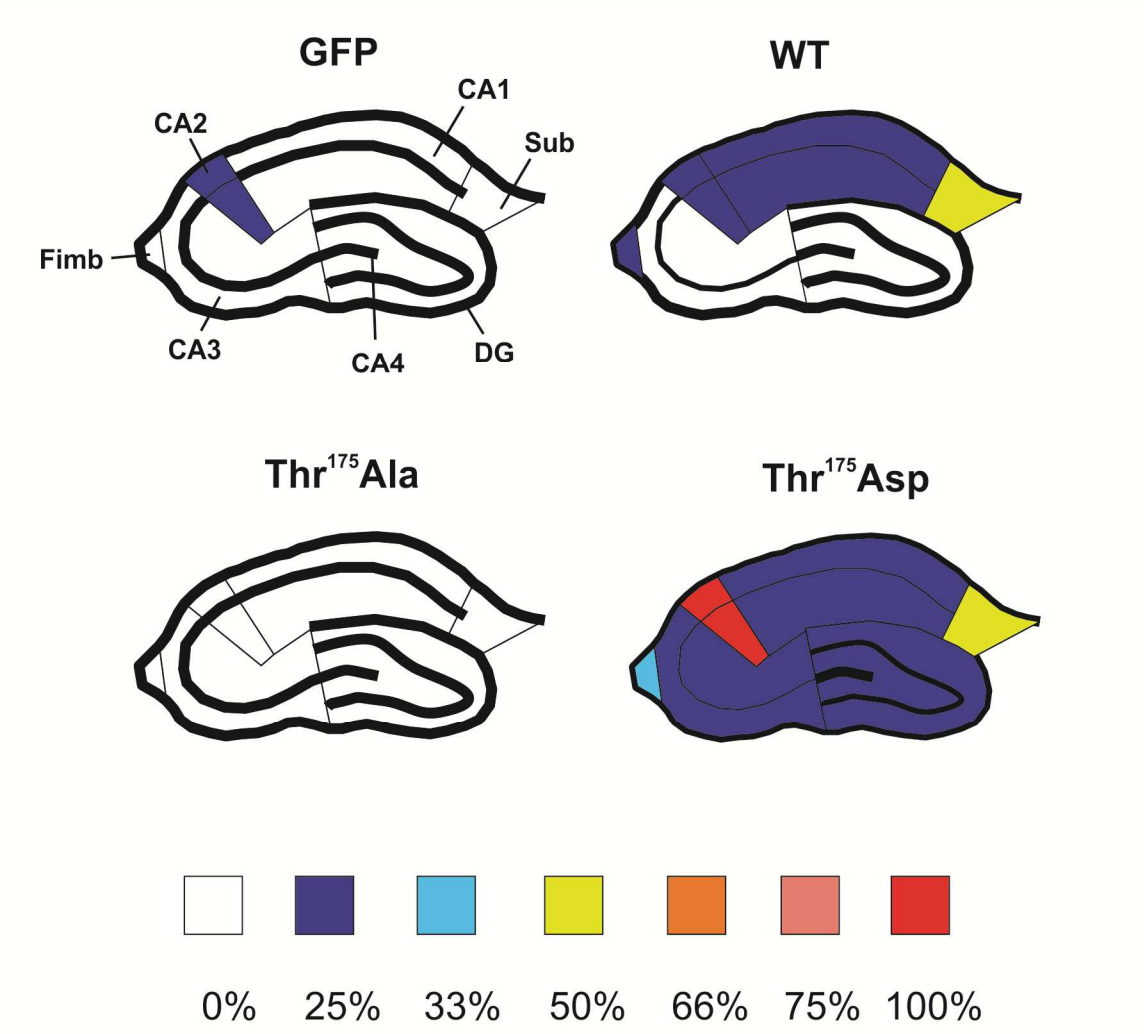

Figure 5.4: pThr^{231} tau protein pathology. A) pThr^{231} tau protein probing by immunohistochemistry reveals GFP-tau protein pathology expression in the form of neuronal fibrils (white arrow) and glial coiled body-like inclusions (black arrow). B) Pathology was expressed in Thr^{175} Asp expressing rats to a greater extent than all other groups. % score represents percentage of pathoplogy-positive animals in each region. DG=dentate gyrus, SUB= subiculum, Fimb= fimbria. Image taken with a 100x oil immersion objective. Data are representative of 3-4 rats per group. Scale bar= 20 μm.

5.4.5 pThr^{231} tau protein co-localizes with tau protein fibrils *in vivo*:

Consistent with the known physiological role of pThr^{231} in modulating microtubule/tau protein interactions, I observed similar degrees of pThr^{231} immunoreactivity across all animal groups, regardless of construct expressed. Uniquely within Thr^{175} Asp tau protein expressing neurons in which tau protein fibrils were observed, pThr^{231} tau protein immunoreactivity co-localized in fibrils (Figure 5.5). In Thr^{175} Asp tau protein expressing neurons where GFP-positive fibrils were present, pThr^{231} positive staining was always present. Conversely, no pThr^{231} positive fibrils occurred in the absence of GFP suggesting that the presence of pThr^{231} immunoreactive fibrils was dependant on the expression of the pathological human tau construct.

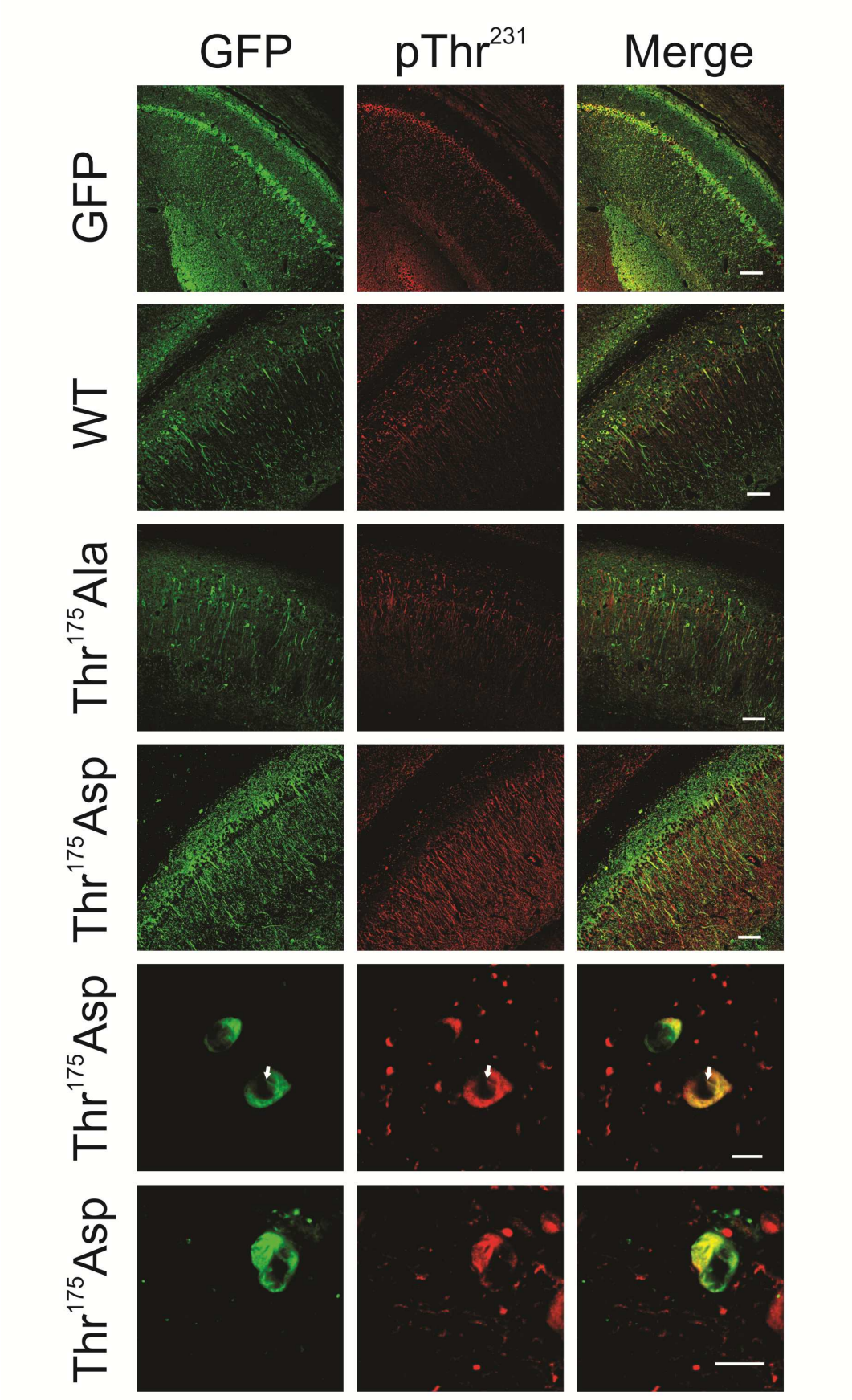
GFP
pThr231
Merge
GFP
WT
Thr175Ala
Thr175Asp
Thr175Asp
Thr175Asp

Figure 5.5: pThr^{231} tau protein is expressed in hippocampal neurons and co-localizes with GFP-tau protein pathology. No difference in pThr^{231} tau protein immunoreactivity was detected between groups. pThr^{231} tau protein co-localized to pathological inclusions Thr^{175} Asp tau protein expressing neurons (white arrows). Scale bar = 100 μm for low magnification images and 10 μm high magnification images. Images are representative of 4 rats per group.

5.4.6 Thr^{175}Asp tau protein expressing cells are positive for cleaved caspase-3:

The number of GFP-positive cells expressing cleaved caspase-3 was calculated as a proportion of the total number of GFP-positive cells in the field of randomly photographed GFP-expressing CA2 region taken by confocal microscopy with a 25x objective. Representative high magnification GFP-expressing neurons are shown in Figure 5.6A. While all groups exhibited some level of caspase-3 positive staining (WT tau protein: 4.9 ± 3.5%, Thr^{175}Asp tau protein: 38.5 ± 9.7%, Thr^{175}Ala tau protein: 6.3 ± 1.7%, GFP: 3.8 ± 1.7%) showed that Thr^{175}Asp tau protein positive neurons expressed a higher proportion of cleaved caspase-3 expressing cells than all other groups ($p=0.004$ vs GFP, $p= 0.003$ vs WT tau protein and $p= 0.005$ vs Thr^{175}Ala tau protein after one way ANOVA with $p= 0.002$ and $F= 10.518$; Figure 5.6B).

Figure 5.6 A

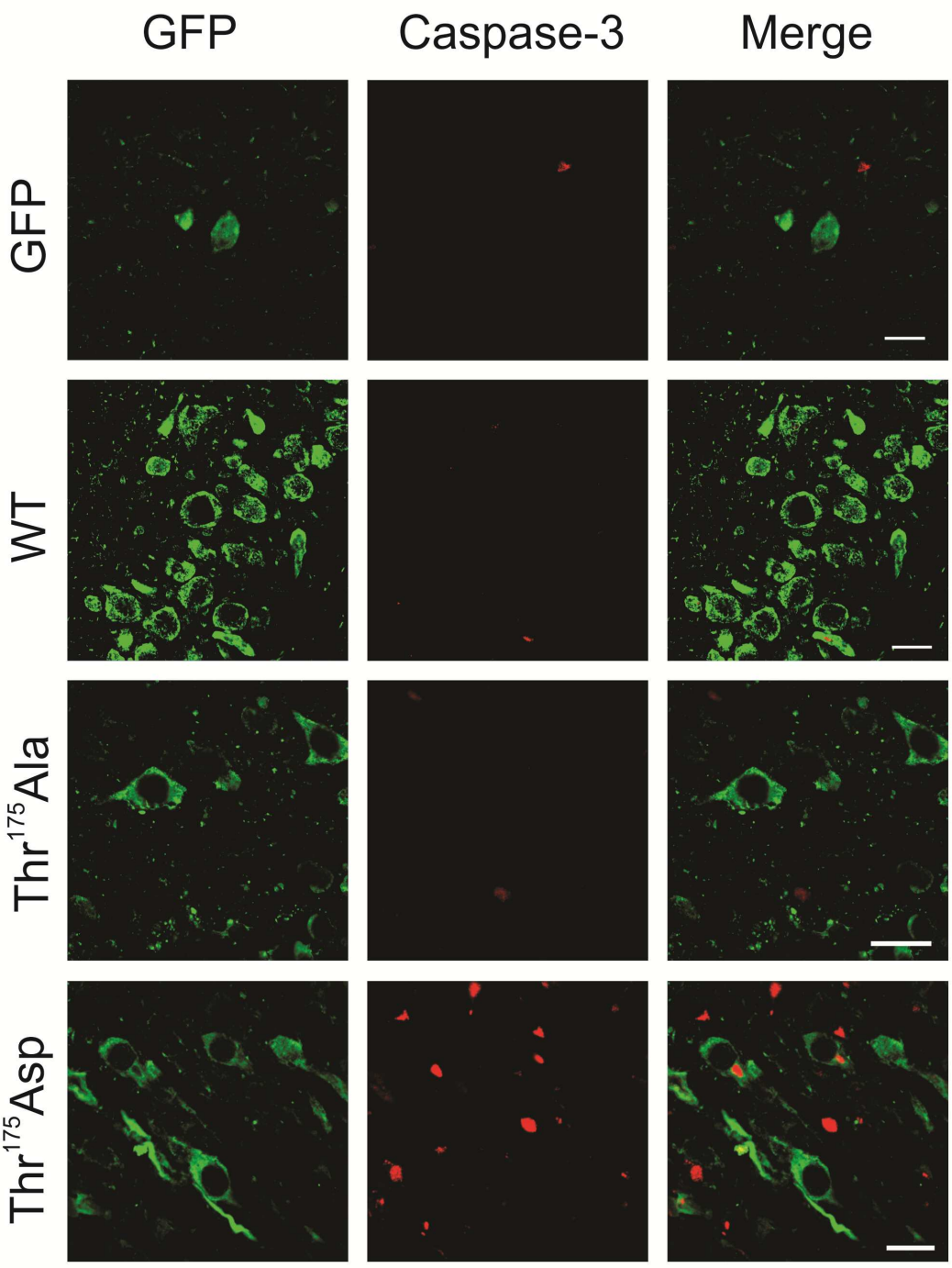

Figure 5.6 B

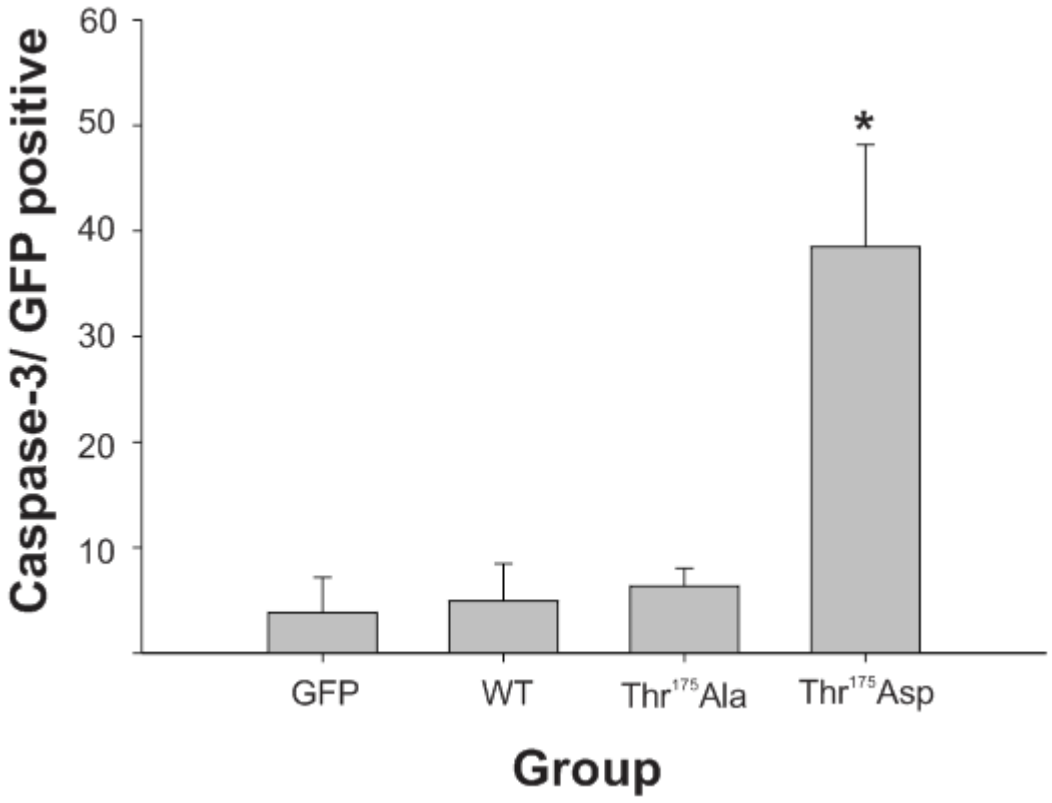

Figure 5.6: Caspase-3 cleavage occurs in Thr^{175}Asp tau protein-expressing hippocampal neurons. A) Thr^{175}Asp tau protein inoculated brains exhibited caspase-3 positive GFP-expressing neurons to a greater extent than all other groups. Scale bar = 20 μm. B) Blinded counts revealed that Thr^{175}Asp tau protein-expressing neurons express cleaved caspase-3 significantly more than all other groups. *denotes $p<0.05$ by Tukey's post hoc test after significant one-way ANOVA. Data are representative of 4 rats per group.

5.5 Discussion:

We have shown that the expression of Thr^{175}Asp tau protein, a pseudophosphorylated construct mimicking $pThr^{175}$ tau protein, in the adult rodent hippocampus is capable of inducing fibrillar tau protein pathology. Furthermore, this is associated with induction of $pThr^{231}$ immunoreactive neuronal cytoplasmic inclusions, which may be occurring through activated GSK3β which is ubiquitously expressed through the cytosol in GFP-expressing neurons. Thr^{175}Asp tau protein expression was associated with increased markers of apoptosis.

That $pThr^{175}$ toxicity is evident in this animal model builds on our previous *in vitro* observation of $pThr^{175}$ tau mediated neurotoxicity and confirms that this phosphoepitope on tau protein is directly responsible to setting off a cascade of phosphorylation events that are toxic in an intact brain (Moszczynski et al., 2015). Additionally, it shows that onset of expression in the adult brain will lead to pathological fibril formation as well as apoptotic cell death. Therefore the $pThr^{175}$ pathology observed in those neurodegenerative diseases that we have thus far investigated, including ALSci, CTE, and CTE-ALS suggests a common mechanism of tau mediated neurotoxicity across a broad range of tauopathies (Moszczynski et al., 2017a; Moszczynski et al., 2017b).

Although the animal model we utilized in this study did express pathology, it was localized to the discrete region bearing the GFP-tau construct. Most specifically, tau pathology in this experimental model was limited to the hippocampal region CA2, a region for which the behavioural testing paradigm was insensitive. Moreover, it is also quite likely that the load of tau deposition and the extent of neuronal death or dysfunction was insufficient for a phenotype to be expressed. A similar argument can be made for the failure to observe pathology using high

resolution MRI. To address this, future behavioural studies will employ testing paradigms that are more sensitive to deficits in CA2 (such as social memory) in evaluating Thr^{175}Asp tau protein-mediated behavioural changes (Hitti and Siegelbaum, 2014). With respect to the neuroimaging studies, it is known that *ex vivo* imaging methods have a much higher resolution than the *in vivo* imaging used in these studies (Dyrby et al., 2011). As such, future studies using this model will require more sensitive measures of behaviour and imaging.

Alternatively, using a genetic model with more widespread expression of Thr^{175}Asp tau protein may be a useful tool now that the pathogenicity of this tau protein construct *in vivo* has been confirmed. This may be the more useful tool for assessing therapeutic potential due to the possibility of a more aggressive pathology and behavioural phenotypes that will be more easily measured over time.

To varying degrees, we observed axonal damage in all 4 groups, likely a result of the invasiveness of surgery. That Thr^{175}Asp tau protein expressing brains had more axonal pathology and was the only group showing fibrillar tau protein inclusions with a widespread nature in every animal indicates that the results are due to the expressed construct. This is consistent with our previous *in vivo* studies of Thr^{175}Asp tau protein constructs which also demonstrated increased fibril formation relative to other groups (Gohar et al., 2009; Moszczynski et al., 2015). Finally, the induction of caspase-3 cleavage indicates apoptotic cell death which was only observed in this group. This is consistent with our previous reports that Thr^{175}Asp tau protein expression *in vitro* leads to caspase-3 cleavage (Gohar et al., 2009).

Given the evidence of toxicity and the known mechanism by which this is exerted downstream of Thr^{175} tau phosphorylation, the inhibition of GK3β may be a useful therapeutic

strategy in neurodegenerative diseases bearing $pThr^{175}$ tau protein pathology. The potential efficacy of GSK3β inhibition will be the focus of future studies in animal models of $pThr^{175}$ toxicity. There is already evidence of GSK3β inhibition reducing tau protein based pathological processes *in vitro* (Hong et al., 1997; Moszczynski et al., 2015) and *in vivo* (Noble et al., 2005). Additionally, evidence from bipolar disorder patients on Lithium treatment suggests that chronic Lithium administration is associated with reduced rates of Alzheimer's disease, indicating therapeutic potential as a strategy of preventing tau protein phosphorylation-based pathological processes (Kessing et al., 2010; Nunes et al., 2007).

5.6 Conclusions:

Taken together, these data suggest that $pThr^{175}$ tau protein is capable of inducing fibril formation, tau protein-based pathology, neurotoxicity and death *in vivo*. Further investigation using GSK3β inhibitors will determine therapeutic potential to inhibit $pThr^{175}$ based tau protein toxicity in a complete physiological system and whether this may be effective in human tauopathies.

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Chapter 6: Discussion

6.1 Summary of results

Thr^{175} tau which has been implicated in the pathogenesis of cognitive impairment in ALS is toxic and capable inducing tau aggregates both *in vitro* and *in vivo*. This toxicity is mediated through increased activation of GSK3β and is dependent on phosphorylation at Thr^{231} in a process which can be fully prevented *in vitro* by inhibition of GSK3β pharmacologically or through molecular knock-down.

In this thesis, I have expanded the relevance of Thr^{175} phospho-tau and the cascade triggered by $pThr^{175}$ tau protein in the induction of pathogenic tau fibrils from ALSci and Alzheimer's disease to other tauopathies. I have proposed that this pathway of pathological tau protein fibril formation may represent a common mechanism of toxicity in these disease processes. Most critically, Thr^{175} tau is phosphorylated only in diseased states and appears to be a specific indicator of pathological tau, unlike other phospho-tau variants. The $pThr^{175}$ containing pathology is associated primarily with neuronal cells and not glial cells. Notably in all of these diseases, Thr^{175} tau phosphorylation is always associated with Thr^{231} tau phosphorylation and tau oligomerization, further emphasizing the importance of this pathway.

Although the initiating mechanism is unknown, I have shown that Thr^{175} tau phosphorylation and pathology can be induced by traumatic brain injury in a rat model of mild traumatic brain injury. This pathology is observed in human CTE and CTEM in hippocampal and motor neuron populations, and is associated with activated GSK3β as well as tau oligomerization. In this model, $pThr^{175}$ tau protein and pathology can be induced by a single traumatic brain injury in wild type rats and persists 3 months after injury. In traumatic brain

injury and CTE, GSK3β activity appears to shift from a primarily nuclear to an even distribution through nucleus and cytosol.

6.2 Implications

6.2.1 Mechanism of toxicity

The toxicity observed downstream of Thr^{175} phosphorylation relies on increased activation of GSK3β beyond baseline levels of activity. There are three potential mechanisms by which this may be induced by tau protein, all of which rely on tau functional changes, likely promoted by a conformational shift from its global hairpin structure. Firstly, opening of the hairpin may simply provide increased substrate for GSK3β by exposing more Ser and Thr residues on tau protein for phosphorylation by GSK3β. It has been shown that increasing substrate availability can increase the activity of kinases including GSK3β (Frame et al., 2001). Secondly, opening of the hairpin may expose the proline-rich domain, allowing for tau interactions with other signaling molecules. These occur through SH3 homology domains which have been shown to interact with Fyn kinase (Klein et al., 2002). This in turn has been demonstrated to phosphorylate GSK3β at Tyr^{216}, enhancing its activity, leading to increased tau phosphorylation *in vitro* (Lesort et al., 1999). Finally, opening of the tau protein hairpin conformation may expose the N-terminal phosphatase activating domain (PAD), consisting of amino acids 2-18. The PAD domain has been shown to directly activate GSK3β related pathways (Kanaan et al., 2011). While each of these possibilities is dependent on opening of the hairpin loop structure, it is unknown whether the effect of Thr^{175} phosphorylation can directly cause an opening of the global hairpin conformation or if this is mediated by a downstream effect. However the location of this phosphoepitope on the Pro-rich domain/ hinge region of the hairpin

suggests that this is possible based on previous reports of phosphorylation in the proline rich domain (Jeganathan et al., 2006; Jeganathan et al., 2008).

The trigger for Thr^{175} phosphorylation remains unknown. However, the upstream cascade of kinase activation that would lead to the phosphorylation of Thr^{175} tau was not the focus of this thesis; rather it was to determine whether $pThr^{175}$ tau was a pathogenic post-translational modification, and if so, how. In future studies, it will be key therefore to use the rat model of mild traumatic brain injury to understand this process further (discussed in future directions section). It is interesting to note however that Thr^{175} tau protein has been shown to be phosphorylated *in situ* by several kinases including MAPK, JNK/SAPK, GSK3β, and LRRK2; hence, there are several candidates (Atzori et al., 2001; Hanger et al., 1998; Hanger, 2017; Reynolds et al., 2000). Of note, the MAPK family of kinases, specifically JNK has exhibited increased activity after traumatic brain injury (Tran et al., 2012), in which case it is a strong candidate. In fact, JNK inhibition has been previously reported to reduce tau pathology after traumatic brain injury in a rodent model (Tran et al., 2012).

One possible means of tau phosphorylation by activated JNK is spatial proximity (Zeke et al., 2016). Neuronal injury requires microtubule remodeling (Kleele et al., 2014; Tang and Chisholm, 2016; Tang-Schomer et al., 2010). This would be a circumstance in which tau protein must disengage the microtubule to enable microtubule disassembly before re-polymerization. While unbound, large proportions of tau could be re-localized to the soma. Here tau may be situated in proximity to stress-activated JNK, which could phosphorylate tau by this stochastic process. If this happens to occur at Thr^{175}, the downstream pathogenic process of GSK3β activation and Thr^{231} phosphorylation would lead to an inability of tau to return to the microtubule, and fibril formation. It is also possible that phosphorylation of Thr^{175} can occur

through different pathways. For example LRRK2-induced phosphorylation in Parkinson's disease linked to mutations in LRRK2 (Shanley et al., 2015) or already elevated GSK3β activity in Alzheimer's disease (Guerreiro et al., 2016). Given this, it is reasonable to propose that there may be multiple pathways of Thr^{175} tau phosphorylation, with varying degrees of generalizability to cellular injury response or disease specificity. This may explain the heterogeneity of diseases associated with $pThr^{175}$ tau.

6.2.2 Pathology vs. physiology

An important observation is that $pThr^{175}$ tau was only observed in pathological or injured states. As mentioned above, the likely pathways to Thr^{175} induction are all abnormal and may occur in "primed cells". It is possible then that $pThr^{175}$ represents a tipping point in pathological tau metabolism and further study of the regulation of $pThr^{175}$ dephosphorylation is required to establish whether it is a reversible event. The pathological state specificity of $pThr^{175}$ implies that beyond its direct role in toxicity itself, $pThr^{175}$ tau appears to also be an indicator of toxicity and neuronal damage. Because of this, $pThr^{175}$ tau may be useful as a biomarker of tauopathy, being more specific than other tau phosphoepitopes that have been investigated previously as CSF biomarkers of disease such as $pThr^{181}$ (Mattsson et al., 2009). If this proves true for general tauopathies, it may be useful to investigate the ratios of $pThr^{175}$ tau and other proteins that have previously been investigated such as14-3-3 (Foote and Zhou, 2012), and neurofilament proteins (Li et al., 2016) in CSF or blood. These ratios may provide utility in distinguishing between different neurodegenerative diseases or stratifying patients with the same clinical diagnosis as candidates based on molecular pathology for specific interventions or combinations of interventions. The utility of combination therapy for treatment of neurodegenerative disease is discussed further in the Future Directions section.

The pathological specificity of $pThr^{175}$ was in contrast to $pThr^{231}$ tau which may be of both pathological as well as physiological significance. As mentioned above, it has been shown that microtubule dynamics are more plastic after axonal damage (Kleele et al., 2014), in which case tau protein would need to disengage the microtubule. This physiological response may be regulated by Thr^{231} phosphorylation (Cho and Johnson, 2004; Lin et al., 2007; Schwalbe et al., 2015; Sengupta et al., 1998). As such, Thr^{231} phosphorylation must be carefully regulated by the cell as anything leading to dysregulation, such as incorrect isomerisation about the phosphate group (causing inability of dephosphorylation) can cause cell toxicity (Nakamura et al., 2013). If kinase activity is enhanced and Thr^{231} phosphorylation is driven by an external factor, it may be that it is phosphorylated at a faster rate than it can be dephosphorylated, leading to an inability to return to the microtubules, further tau phosphorylation, and tau-mediated toxicity culminating in apoptotic cell death. This dysregulation may be mediated by $pThr^{175}$ making the pathogenic state of Thr^{231} phosphorylation dependent on Thr^{175} phosphorylation, even though $pThr^{231}$ normally it is an innocuous epitope due to tight regulation.

That tau pathology is inducible by traumatic brain injury brings into question the origin of tau pathology in tauopathies as a whole. In instances where disease is induced by tau protein mutations such as FTDP-17 it clear that tau protein is the primary cause of the disease. In other states such as Alzheimer's disease where tau tangles are observed concomitantly with other pathologies, it is much more difficult to discern what the origin of neuronal toxicity may have been. In the case of CTE it is thought that tau pathology is induced by shear forces and axonal injury brought about by traumatic brain injury (Gavett et al., 2011). The same may be true of progressive age related tauopathy (PART). Beginning in the entorhinal cortex, tau tangles have been observed to increase with age in patients without overt dementia, and it has recently been

shown that the burden of tau pathology in PART post-mortem correlates strongly with rate of cognitive decline displayed in life (Jefferson-George et al., 2017). It is possible that in the absence of other pathological processes, tauopathy induced by stress over time is a slowly developing cognitive deficit. Tau deposition can therefore be thought of not as a function of normal ageing, but as a toxic byproduct of wear and tear on the brain over the lifespan. There is nothing physiological about tau deposition as it is a departure from homeostasis.

6.2.3 Affected cell populations

The pathology associated with Thr^{175} tau was contained primarily within neuronal cells suggesting that there may be divergent toxic processes responsible for tau pathology in neuronal and glial cell populations and is consistent with the hypothesis that glial tau pathology is a separate process influenced by the cellular environment within glia, which is distinct from the neuronal cellular environment due to differential protein expression (Kahlson and Colodner, 2015). Indeed, it has been suggested that neuronal and glial tauopathic processes are driven by separate mechanisms and kinases (Ferrer et al., 2001). One example of differential kinase expression affecting tau pathology is casein kinase 1 (CK1). Although the δ isoform of CK1 has been implicated in tau pathology in multiple neurodegenerative diseases, this isoform is not expressed in glial cells (Lohler et al., 2009). It is thus not surprising that CK1 associated tau pathology is not observed in glial cells (Li et al., 2004; Schwab et al., 2000). Additionally, glial and neuronal cells have been shown to exhibit opposite responses in MAPK activation after exposure to external signals such as cAMP due to differential B-raf receptor expression (Dugan et al., 1999). Of note, the B-raf signaling cascade has been shown to induce JNK activity when activated, and inhibition of this pathway reduces JNK activation (Park et al., 2005). Additionally, kinases that have been shown to phosphorylate tau protein such as CaM kinase II have much

lower expression in glia than neurons (Ferrer et al., 2001). If Thr^{175} phosphorylation is a stochastic process driven by cellular stress, it is conceivable that differential kinase activation or expression patterns would have a completely different likelihood for Thr^{175} phosphorylation. This may explain the disparity in phosphorylated residues between glial tau and neuronal tau.

The preferential layer pathology expression between superficial layers in the entorhinal cortex and deeper layers in frontal and anterior cingulate cortex (ACC) suggests an origin in entorhinal cortex which spreads along connections to the ACC. Superficial deposition throughout the entorhinal cortex with preferential localization to layer III may indicate that this is driven by mechanical cellular stress as observed in CTE. The occurrence of tau in superficial regions in CTE has brought about the hypothesis of a "wear and tear" source of origin due to mechanical disturbance to the neurons. This has also been suggested in hippocampal sclerosis of ageing (Nelson et al., 2013) and PART (Crary, 2016). Superficial neurons are characterized by more laterally oriented projections connecting neighbouring cortical columns. If tau protein spread occurs trans-synaptically as has been suggested, (Wang and Mandelkow, 2016) it is plausible that this process would spread laterally through the entorhinal cortex between columns remaining largely in layer III, with a lower likelihood of spread to other regions. Tau protein deposition in deep layers however, is more likely to spread inter-regionally. It is possible therefore that there is a critical mass of tau pathology needed to be reached before the spread will occur to other layers by chance and then to other regions. Layer IV and V neurons in the ACC receive inputs from the perforant pathway via thalamic hubs. These ACC neurons then send projections to distant brain structures, providing a route for tau spread to distant structures. Therefore, once tau protein accumulation in deep layers begins, it is possible for this process to perpetuate itself further in a

seemingly random process. That tau is consistently observed in the entorhinal cortex and other limbic structures, and then next most commonly in the ACC, is consistent with this finding.

ACC pathology was most frequently observed in diseases associated with cognitive impairment and has been observed to a greater extent in ALSci than ALS (Yang and Strong, 2012). Indeed, dysfunction of the ACC has been implicated in cognitive impairment in ALS through multiple studies (Ambikairajah et al., 2014; Byrne et al., 2012; Kew et al., 1993; Mohammadi et al., 2009; Van Deerlin et al., 2008). This region has been linked to language processing, emotion (Bush et al., 2000), motor imagery (Grezes and Decety, 2002), motor function (Basha et al., 2013), pain processing (Naro et al., 2015), error detection (Hyman et al., 2013; Wang et al., 2005) and conflict monitoring (Kerns et al., 2004; Wang et al., 2005). This may imply that the variability of frontal symptom progression in ALSci could result from differential ACC regional involvement. Furthermore, the ACC is also a highly connected region of the brain with the midcingulate cortex (MCC) subregion alone having cortical projections with amygdala, parietal cortex, insula, dorsolateral prefrontal cortex (dlPFC) (Vogt, 2016) and direct connections to spinal motor neurons (Dum and Strick, 1991).

Of further interest to neuronal pathology of the ACC, there are two unique classes of neurons known as spindle neurons and fork cell neurons which may be linked to social behavior localized specifically to the ACC and insula. These neurons appear to be affected preferentially in frontotemporal dementia (FTD) (Kim et al., 2016; Santillo et al., 2013; Seeley et al., 2006). Fork cells and spindle neurons are large neurons, localized to layer V of the anterior cingulate cortex, analogous to pyramidal upper motor neurons (Kim et al., 2016). It may be no coincidence then that ALS and FTD share such pathology and comorbidity. They show physical similarities (size), similar predisposition to pathological processes, and are contained within structurally

connected regions. If neurodegenerative disease is even partially stochastic in nature, the link shared by these cell populations would suggest that a higher than expected number of cases should develop both diseases especially in diseases bearing more aggressive pathology.

In the case in both Guamanian ALS as well as CTE, which bear intense tau pathology through the brain and spinal cord, the critical mass hypothesis may be relevant. The reduced frequency of tau pathology in spinal cord in other variants of ALS and tauopathies may be because motor neurons have a different molecular sequalae making tau inclusions harder to drive in them even though tau dysfunction can occur. In fact it has been suggested that neurofilament may serve as a phosphorylation sink in spinal motor neurons which may hold off tau pathology resulting in a higher "barrier to entry" (Nguyen et al., 2001). Nevertheless, animal models of tauopathy and even SOD1 (Nguyen et al., 2001; Spittaels et al., 1999) have been shown to exhibit abnormal tau protein metabolism and pathology in the spinal cord, suggesting that there is a possible role of tau in these neurons as well when dysfunction does occur in motor neurons. The spinal cord tau pathology I observed in CTE cases that did not exhibit motor impairment may indicate an early stage of tau pathology with phosphorylation preceding sufficient neuronal death to initiate clinical findings. It has been observed in ALS-Parkinsonism dementia complex (ALS-PDC) of Guam that spinal cord pathology was present even in 35% of cases with Parkinosnism without motor symptoms (Rodgers-Johnson et al., 1986). Tau dysfunction therefore may be relevant to motor neuron toxicity in diseases bearing a heavy tau burden to a much greater extent than those with relatively limited tauopathy. It is possible that it is one of the key toxic mediators of motor neuron degeneration in these cases.

6.2.4 Recognition of tauopathy

I noted divergence between AT8 and pThr^{175} tau pathology in the frontal cortex. Whereas AT8 only recognized frontal tau pathology in Alzheimer's cases selected on the basis of late stage tauopathy, pThr^{175} recognized tau pathology in the frontal cortex in 80% of ALSci cases studied. This is notable because AT8 is a primary antibody used in neuropathological study and the detection of phosphorylated tau protein in tauopathies. If tau can be toxic without phosphorylation at Thr^{202} (the AT8 epitope), AT8 may miss tau pathology resulting in false negative reporting. This highlights a need for sensitive methods to detect tau pathology in non-AD neurodegenerative diseases, using antibody panels or other methods.

Alternatively to antibody specificity, the thinness of paraffin embedded tissue sections routinely used for neuropathological analysis presents an additional limitation of tau detection. One method that has been applied recently to both Pick's disease (Irwin et al., 2016) and FTD-TDP (Brettschneider et al., 2014) is to use 70 μm sections (Feldengut et al., 2013) allowing for more detailed anatomical characterization. However, this technique also has a higher false negative rate as more cells and structures are present on each slide. Braak staging has been used since 1991 (Braak and Braak, 1991) as the gold standard in staging of neurofibrillary pathology related to AD. Recently (Irwin et al., 2016), a series of Pick's disease cases were stained using the thick section method. Their traditionally identified thin section Braak stage was a maximum of I/II, corresponding with what is considered to be a mild tau pathology which may be associated with ageing. However, upon further inspection using this sensitive technique it was apparent that tau pathology was widespread and found in regions spanning from limbic and frontal structures, to visual cortex. This paired with our finding that tau pathology may be

underestimated by one tau antibody, or silver staining alone, indicates that thorough investigation may be required before writing off tau pathology as a contributing factor to disease.

6.2.5 Tau as a central player in NDGs

Tau protein phosphorylation can be induced as an initiator of or response to a stressor. Thr^{175} phosphorylation is observed in neuronal pathology in all tauopathies investigated thus far and may play a role in the toxicity of tau protein. It may be that Thr^{175} phosphorylation can be induced by multiple factors such as axonal stress, other protein-induced stress, or other cellular dysmetabolism. In fact, it has been suggested that within neurodegenerative disease, the rate of mixed pathologies and number of combined neuropathological substrates may be very high (Rabinovici et al., 2017; Rahimi and Kovacs, 2014). Therefore, aberrantly phosphorylated tau protein may be one part of a pathological network of disease-related proteins capable of re-enforcing pathology in each other, propagating the toxic processes underlying neuronal death. Tau protein may therefore be a central player in many neurodegenerative disorders, contributing to pathology and toxicity. It is possible that it interacts with other components of this pathological network in a synergistic manner. This is discussed further in the Future Directions section.

6.3 Caveats

The results from my studies have shown that $pThr^{175}$ tau protein is pathogenic and associated with multiple disease states. However, these studies do not shed light on the induction of this process. That traumatic brain injury was capable of inducing this pathology suggests that other forms of cellular stress may be capable of inducing it as well. This suggests that Thr^{175} phosphorylation happens downstream of cellular stress, raising the issue of whether or not this is

a cause or consequence of neurodegenerative disease. Regardless, it may be a specific marker of neurodegeneration and it is a clear contributor to toxicity which may require intervention even if other contributors to toxicity are present in the diseased brain. Alternatively it may be an aberrant response to stress representing a tipping point, driving a physiological stress response to a pathological, out of control, toxic state of cellular dysfunction.

Another limitation of these studies is the inability to determine the order of phosphorylation of Thr^{175} and Thr^{231}. The cell culture and rat model expresses a construct mimicking $pThr^{175}$, and we therefore were only able to determine the downstream changes required for toxicity. It is possible that Thr^{175} phosphorylation locks $pThr^{231}$ into place, or pushes equilibrium of phosphorylation to dephosphorylation in the favour phosphorylation as it is likely that Thr^{231} would be phosphorylated already as tau may need to be off the microtubule to be phosphorylated at Thr^{175}. Future studies in the TBI model can be used to shed light on this sequence of events.

The lack of behavioural or imaging correlate in the *in vivo* studies of $pThr^{175}$ pathogenicity appeared to be related to insufficient levels and regional spread of expression. This suggests that a different mechanism of delivery or a longer period of time is required to induce widespread pathological effects. In other words, $pThr^{175}$ requires more time to induce pathology in the brain and needs to be expressed in multiple areas at higher levels to induce behavioural or large-scale changes. It may very well be that multiple hits are required, and that the toxicity of $pThr^{175}$ tau is made worse by another contributor. This will be the focus of future studies.

6.4 Future directions

One current study is addressing the synergistic toxicity of tau protein and transactive response DNA binding protein of 43 kDa (TDP-43). I am using the same somatic gene transfer as in chapter 5 to express 1) Thr^{175}Asp-tau, 2) wild-type human tau, 3) rAAV9-GFP in the hippocampus of either wild-type or transgenic rats expressing human mutant TDP-43 (NEF-tTA/TRE-TDP-43^{M337V}) (Huang et al., 2012; Zhou et al., 2010). Interactions between these two pathological proteins may be synergistic in the neurodegenerative process, as individual pathology is rarely observed, and comorbid pathologies are frequent (Amador-Ortiz et al., 2007; Josephs et al., 2014a; Josephs et al., 2014b; Smith, 2017). The association of TDP-43 pathology to ALS and ALS-FTD spectrum diseases is well described (Neumann et al., 2006) and comorbid TDP-43 pathology is frequent in tauopathies such as AD and CTE (Josephs et al., 2014a; Josephs et al., 2014b; McKee et al., 2010). Both TDP-43 and tau protein may contribute to neuronal death in ALSci, and tau protein toxicity has previously been shown to act synergistically with other neurodegeneration-associated toxic proteins (Zabrocki et al., 2005). It is possible that one pathological process primes the other, in which case the toxicity of Thr^{175}Asp expression may be enhanced by the coexpression of TDP-43 serving as a second hit to the CNS.

The induction of $pThr^{175}$ by traumatic brain injury in wild type rats provides an opportunity to study the upstream events leading to Thr^{175} phosphorylation. A time series study where pathological analysis is conducted at the time of injury, hours, days, and weeks after injury would allow for the characterization of tau fibrillization relative to phosphorylation status. Investigating this along with kinase activation status and co-localizing phospho-tau with these kinases would allow for the characterization of underlying kinases. Administering inhibitors of the identified kinases at the time of injury and following injury could be utilized to confirm

mechanistic involvement as well as determine the efficacy in blocking fibrillization of tau in this model. Additionally, GSK3β inhibition should be conducted in this model to characterize its therapeutic potential for blocking pThr^{175} toxicity as observed *in vitro*. This is particularly attractive as lithium is currently used in the treatment of psychological disorders (Geddes et al., 2004) and would therefore be easily repurposed for clinical use, expediting therapeutic use if they prove efficacious.

A detailed characterization of pThr^{175} in the tauopathies would elaborate further on my findings. This should be extended to tau mutation carriers including spicing modifying mutants and MPR affecting mutations. A more detailed account of the types of inclusions and pathology, as well as a further quantification with a larger cohort for each disease would shed light on the extent of pThr^{175} pathology in the disease process.

The mechanism by which pThr^{175} tau induces GSK3β activity remains unknown. As mentioned above, it is possible that this occurs through opening of the hairpin conformation of tau protein to expose the PAD domain. Expressing a pThr^{175} construct lacking the first 18 amino acids of tau should therefore abolish the upregulation of GSK3β activity and should not lead to fibril formation, cell death, or Thr^{231} phosphorylation.

The redistribution of activated GSK3β likely represents changes in the location and homogeneity of GSK3β activity in the cell. This warrants further, detailed investigation, and assays of GSK3β activity should be conducted. *In situ* activity assays of GSK3β in the presence and absence of pThr^{175} could be used to further delineate the nature of tau based GSK3β activation, (whether it is direct or indirect). Cellular fractionation could be used to investigate the activation status of GSK3β in multiple organelles. Finally, GSK3β isolation from different states

(injured/ uninjured) and organelles could be used to assay catalytic activity of the kinase in each subcellular region.

Tau protein pathology has been observed in a plethora of neurodegenerative diseases. Regardless of whether tau is a primary or secondary cause of disease, the clear demonstration of tau protein toxicity in many *in vitro* and *in vivo* models suggests that once tau pathology is induced, it is a contributor to neuronal death. Therefore, tau protein based toxicity may need to be considered for future attempts at effective therapeutic interventions for neurodegenerative disease. It is also therefore likely that effective therapies will need to be based on individual molecular contributors possibly recognized through *in vivo* imaging of different pathological markers to account for co-morbid underlying molecular diseases. Therapies attempted thus far have failed, possibly due to multiple contributing sources of toxicity not being addressed in strategies attempted. Future attempts may require one inhibitor per molecular contributor. The concept of combination therapy has been applied with great success to treatment of cancer (Bayat et al., 2017) and has been suggested as a possible path for neurodegenerative disease (Bredesen and John, 2013; Schmitt et al., 2004).

6.5 Conclusions

pThr^{175} tau protein is toxic and may play a role in neurodegeneration. The extent to which this phosphoepitope is involved in tauopathies appears widespread and requires further investigation. Thr^{175} phosphorylation and tau pathology is induced by traumatic brain injury. This warrants further investigation to examine the upstream mechanism underlying pThr^{175} phosphorylation as well as whether other stressors can induce this event. Therapeutic

intervention may be effective in mitigating this source of toxicity, and may be required in conjunction with other toxicity blockers for comorbid pathologies.

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Appendix A:

Animal Use Protocols

FW: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2015-004::2

Arthur Brown <abrown@robarts.ca>

Thu 2017-07-20 12:23 PM

To:Alexander Moszczynski <amoszczy@uwo.ca>; Natalie Maria Ossowsk <nossowsk@uwo.ca>;

From: Animal Care Committee [mailto:auspc@uwo.ca]
Sent: Wednesday, July 19, 2017 5:32 PM
To: Arthur Brown <abrown@robarts.ca>
Subject: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2015-004::2

From: eSiriusWebServer [mailto:esiriusadmin@uwo.ca]
Sent: May 2, 2017 2:48 PM
To: abrown@robarts.ca
Cc: Animal Care Committee <auspc@uwo.ca>; Animal Care Committee <auspc@uwo.ca>
Subject: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2015-004::2

Western

2015-004::2:

AUP Number: 2015 004
AUP Title: Investigations of CNS Injury and Regenerative Therapies
Yearly Renewal Date: 05/01/2017

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2015-004 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Schoelier, Marianne
on behalf of the Animal Use Subcommittee

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
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approval date Dec 12/13
renewal date Jan 1/15
expiry date Dec 31/17
YES - 3 special monitoring sheets

Submit - Animal Use Protocol - AUP Form

Table of Contents

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AUP NUMBER	2013-008
AUP TYPE	New
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Appendix B:

Relevant co-authored work

Chapter 9

Cortical Manifestations in Amyotrophic Lateral Sclerosis

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BACKGROUND

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset neurodegenerative disease of the motor system. Although the earliest clinical description of ALS appeared in the thesis of Aran (1850), it was Charcot J.M. and Joffroy A (1869) who coalesced the findings of progressive degeneration of both upper (descending supraspinal) and lower motor neurons into a single diagnostic entity. The net effect of this degeneration is a progressive loss of motor function, culminating in paralysis and death generally within 3–5 years of symptom onset (Strong, 2003).

Although neuropsychological changes in ALS were historically considered to be rare (Hudson A.J., 1993), the contemporary view is that 45–55% of patients with ALS will develop a neuropsychological syndrome reflective of frontotem-poral dysfunction, including a frontotemporal dementia (FTD), behavioral or cognitive impairment (ALSbi and ALSci, respectively), language impairment, or deficits in social cognition (Abrahams, Newton, Niven, Foley, & Bak, 2014; Elamin et al., 2011; Montuschi et al., 2015; Oh et al., 2014; Strong et al., 2009; Strong, Grace, Orange, & Leeper, 1996). The presence of a neuropsychological syndrome in ALS is prognostically relevant because affected patients will have a significantly shorter survival than if ALS occurs in isolation (Elamin et al., 2011; Hu et al., 2013).

NEUROPSYCHOLOGICAL MANIFESTATIONS OF FRONTOTEMPORAL DYSFUNCTION IN AMYOTROPHIC LATERAL SCLEROSIS

The neuropsychological manifestations of ALS can range from impairments in cognition or behavior, deficits in social cognition or theory of mind (ToM), or as an FTD consistent with either the Neary or Hodges criteria (Hodges & Miller,

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2001; Neary et al., 1998; Strong, 2008; Strong et al., 2009). Rare presentations can include progressive nonfluent aphasia or semantic dementia, suggesting a continuum with FTD. Approximately 2–4% of patients who have ALS will develop concomitant Alzheimer disease (AD) (Consonni et al., 2013).

Impairments in language, including deficits in naming, comprehension, and spelling, occur in upwards of 35% of patients (Abrahams et al., 2014). Defi-cits can be further subdivided into impairment in action verbs but not cognitive verbs, with the former showing a positive association with impairments in exec-utive functioning (York et al., 2014). These latter findings are associated with significant gray matter atrophy in the left precentral gyrus, left cingulate gyrus, and right medial frontal gyrus. As will be discussed, these observations begin to highlight the regional selectivity of the frontotemporal dysfunction in ALS.

Impairments in verbal fluency are commonly observed. In a meta-analysis of published studies, Raaphorst and colleagues observed that among those indi-viduals with cognitive impairment, impairments in verbal fluency, visual mem-ory, and immediate verbal recall each had a significant effect size (Raaphorst, De, Linssen, De Haan, & Schmand, 2010).

Behavioral dysfunction has been observed in upwards of 40% of patients with ALS and can include apathy, behavioral disinhibition, irritability, loss of sympathy/empathy, perseverative or stereotypic behavior, or changes in eat-ing behavior (Abrahams et al., 2014; van der Hulst, Bak, & Abrahams, 2014; Lomen-Hoerth et al., 2003). An increased incidence of psychotic symptoms has been observed in those individuals with ALS-FTD (Lillo, Garcin, Hornberger, Bak, & Hodges, 2010). Deficits in ToM have been described in a significant proportion of patients who have ALS and are characterized as an inability to represent others' intentions and beliefs and thus the ability to predict others' behavior by attributing independent mental states to them (Adenzato, Cavallo, & Enrici, 2010). These deficits can be observed even in the absence of overt evidence of dementia (Meier, Charleston, & Tippett, 2010). Consistent with pathology of the orbitofrontal cortex, impairments range from apathy through to greater difficulty in identifying emotional expression or reductions in emotional attributions while sparing intentional attributions (and thus a reduced ability to recognize others' emotional states) (Cerami et al., 2014). ToM deficits cor-relate with diffuse cortical atrophy [determined by magnetic resonance imaging (MRI)] with a specific accentuation in the left superior precentral gyrus, left paracentral gyrus, and right precentral gyrus (Agosta et al., 2012).

MOLECULAR, CLINICAL, AND NEUROPATHOLOGICAL CORRELATES OF FRONTOTEMPORAL DYSFUNCTION IN AMYOTROPHIC LATERAL SCLEROSIS

Approximately 10% of ALS cases are genetic in origin (Al-Chalabi et al., 2012; Renton, Chio, & Traynor, 2014) (Table 9.1). Although the mechanism(s) by which many of these mutations induce neuronal degeneration are uncertain,

TABLE 9.1 Genes Associated With Amyotrophic Lateral Sclerosis and Their Overlap With Frontotemporal Dementia

Protein	Gene	OMIM	Functional Changes	FTD	ALS	ALS-FTD	PLS/ Other	References
Superoxide dismutase 1	*SOD1*	147450	Oxidative stress		+		+ (SBMA, PMA)	Rosen et al. (1993)
Senataxin	*SETX*	608465	DNA/RNA processing		+		+	Chen et al. (2004)
Spastin	*SPAST*	604277	NFL, cytoskeleton, microtubule deficits		+		+	Munch, Rolfs, and Meyer, 2008; Wharton et al. (2003)
Fused in sarcoma	*FUS*	137070	Cell death (closely related to TDP)	+	+	+		Mackenzie, Rademakers, and Neumann, 2010; Vance et al. (2009)
Vesicle-associated membrane protein–associated proteins B and C	*VAPB*	605704	Altered axonal transport		+		+ (SMA)	Nishimura et al. (2004)
Angiogenin, ribonuclease, ribonuclease A family	*ANG*	105850	DNA/RNA processing		+	+	+ (PBP)	van Es et al. (2009)

Continued

TABLE 9.1 Genes Associated With Amyotrophic Lateral Sclerosis and Their Overlap With Frontotemporal Dementia—cont'd

Protein	Gene	OMIM	Functional Changes	FTD	ALS	ALS-FTD	PLS/ Other	References
TAR DNA binding protein (TDP-43)	*TARDBP*	605078	DNA/RNA processing	+	+	+		Davidson et al. (2007), Sreedharan et al. (2008)
Factor-Induced gene 4 *(FIG4)* homolog, *SAC1* lipid phosphatase domain containing (*Saccharomyces cerevisiae*)	*FIG4*	609390	Cell death/ protein degradation		+		+	Chow et al. (2009)
Optineurin	*OPTN*	602432	Cell death/ protein degradation		+		+ (PDB)	Maruyama et al. (2010)
Ataxin 2	*ATXN 2*	601517	Oxidative stress		+		+ (SCA2)	Elden et al. (2010)
Valosin-containing protein	*VCP*	601023	Protein degradation	+	+	+	+	Forman et al. (2006), Johnson et al. (2010), Weihl, Pestronk, and Kimonis (2009)
Ubiquilin 2	*UBQLN2*	300264	Protein degradation	+	+	+		Gellera et al. (2013), Ugwu et al. (2015)

Sigma nonopioid intracellular receptor 1	*SIGMAR1*	601978	Ion channel regulation		+	–		Al-Saif, Al-Mohanna, and Bohlega (2011), Belzil et al. (2013)
Profilin 1	*PFN1*	176610	NFL, cytoskeleton, microtubule deficits	+	+			Smith et al. (2015), van Blitterswijk et al. (2013)
Chromosome 9 open reading frame 72	*C9orf72*	614260		+	+	+		Renton et al. (2011)
Charged multivesicular body protein 2B	*CHMP2B*	609512	Vesicle trafficking	+	+			Cox et al. (2010)
Unc-13 homologue A (*Caenorhabditis*	*UNC13A*	609894	Synaptic neurotransmitter	+	+	+		Shatunov et al. (2010)
d-amino-acid oxidase	*DAO*	124050	Oxidative stress		+			Mitchell et al. (2010)
Dynactin 1	*DCTN1*	601143	Altered axonal transport		+		+ (Perry syndrome)	Farrer et al. (2009), Munch et al. (2004)
Neurofilament, heavy polypeptide	*NEFH*	162230	NFL, cytoskeleton, microtubule deficits		+			Al-Chalabi et al. (1999)

Continued

TABLE 9.1 Genes Associated With Amyotrophic Lateral Sclerosis and Their Overlap With Frontotemporal Dementia—cont'd

Protein	Gene	OMIM [a]	Functional Changes	FTD	ALS	ALS-FTD	PLS/ Other	References
Peripherin	*PRPH*	170710	NFL, cytoskeleton, microtubule deficits		+			Corrado et al. (2011)
Sequestome 1	*SQSTM1*	601530	Protein degradation	+	+	+	+ (PDB)	Le Ber et al. (2013)
TAF15 RNA polymerase II, TATA box binding protein (TBP)–associated factor, 68 kDa	*TAF15*	601574	DNA/RNA processing		+			Hand et al. (2002)
Spastic paraplegia 11	*SPG11*	610844	DNA damage repair		+		+ (HSP)	Daoud et al. (2012)
Elongator acetyltransferase complex subunit 3	*ELP3*	612722	Projection neuron maturation		+			Simpson et al. (2009)

ALS, amyotrophic lateral sclerosis; *FTD*, frontotemporal dementia; *HSP*, hereditary spastic paraplegia; *NFL*, neurofilament; *OMIM*, Online Mendelian Inheritance in Man; *PBP*, progressive bulbar palsy; *PDB*, paget disease of bone; *PLS*, primary lateral sclerosis; *PMA*, progressive muscular atrophy; *SBMA*, spinal-bulbar muscular atrophy; *SCA2*, spinocerebellar ataxia type 2; *SMA*, spinal muscular atrophy; *TAR*, transactive response; *TDP*, TAR DNA-binding protein.

there are three general themes including the induction of oxidative stress (eg, mutations in *SOD1*, *ATXN2*, *DAO*), alterations in the cytoskeleton and/or impairments in axonal transport (eg, *VAPB*, *SPAST*, *DCTN1*, *NEFH*, *PRPH*), and alterations in RNA metabolism (eg, *TARDP*, *ANG*, *FUS*, and pathological hexanucleotide expansions of *C9orf72*). However, a group of genetic mutations cannot be readily bundled into these potential mechanisms including genes thought to directly give rise to ALS and those thought to be genetic modifi-ers (*CHMP2B*, *VCP*, *UBQLN2*, *SIGMAR1*, *PFN1*, *UNC13A*, *SQSTM1*, *TAF15*, *SPG11*, *ELP3*). Ultimately, however, there are few clinical features that are unique to any of the genes associated with ALS, suggesting that the motor degeneration and potentially the neuropsychological deficits are syndromic or, in the latter, reflective of specific neural network dysfunction that is indepen-dent of the underlying pathological mutation.

The theme of ALS being syndromic is supported by neuropathological studies. Consistent with the primary manifestation as a progressive loss of motor function, the hallmark of ALS is a loss of both spinal and bulbar motor neurons with degeneration of descending supraspinal innervation pathways. Affected motor neurons demonstrate a range of nuclear and cytoplasmic neuronal inclusions (NNIs and NCIs, respectively). In a blinded analysis of both sporadic and familial ALS motor neuron pathology, it was not possible to identify (by light microscopy) a “signature” pattern of neuronal inclusions of either cytoskeletal proteins or RNA-binding proteins that would allow differentiation amongst individual genotypes of ALS (Keller et al., 2012). The exception to this was *SOD1* mutations. The presence of frontotemporal dysfunction in ALS is typically indistinguishable from that occurring as an isolated FTD in which diffuse frontal and anterior temporal atrophy is accompanied by a vacuolar appearance consistent with superficial linear spongiosis throughout affected regions (Wilson, Grace, Munoz, He, & Strong, 2001). Somewhat in contrast to the pathology of affected motor neurons in ALS, cortical and subcortical neurons in cases with a syndrome of frontotemporal dysfunction tend to display an increase in transactive response DNA-binding protein 43 (TDP-43) cytosolic expression and a range of both NCIs and NNIs (Neumann et al., 2006).

Although an up-regulation of TDP-43 expression can also be seen as a response to neuronal injury (Moisse et al., 2009), the presence of both an increased expression of neuronal TDP-43 and TDP-43 immunoreactive NNIs and NCIs as major neuropathological features of both ALS and FTD suggests a common pathogenic process across the two diseases. TDP-43 has a range of activities that map to the regulation of gene expression, including such diverse functions as anchoring of chromatin, participation in splicing and RNA granule formation, the regulation of RNA translation, and participation in RNA deg-radation through the Dicer complex (Droppelmann, Campos-Melo, Ishtiaq, Volkening, & Strong, 2014).

The hypothesis of a continuum encompassing both ALS and FTD has been further reinforced by the discovery of a pathological expansion of a hexanucleotide repeat (GGGGCC) in *C9orf72* in both familial and sporadic ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011). The RNA associated with this expansion undergoes a unique type of repeat-associated non–ATG-initiated translation to give rise to dipeptide repeat proteins that can function as sinks for a range of RNA-binding proteins, effectively sequestering them from participating in RNA metabolism (Ash et al., 2013; Souza, Pinto, & Oliveira, 2015). It remains to be fully clarified as to whether the pathological RNA alone or the presence of dipeptides alone, or a combination of both, are sufficient to induce cell death (Hukema et al., 2014; Mizielinska et al., 2014). The neurodegeneration associated with pathological hexanucleotide expansions in *C9orf72* is typically more symmetrical than that observed with other variants of FTD and includes frontal and temporal cortices and the hippocampus, as well as deeper structures such as the striatum and thalamus (Mahoney et al., 2012).

No single pathological inclusion describes all variants of frontotemporal dysfunction in ALS. Indeed, there is increasing evidence to suggest the coexistence of several pathological protein inclusions within the same case, including the presence of both *C9orf72* and TDP-43 (Mackenzie et al., 2013) or *C9orf72* and the microtubule-associated protein tau in pathological inclusions (Bieniek et al., 2013). In lumbar spinal motor neurons, the coexistence within the same inclusion of the RNA-binding proteins TDP-43, fused in sarcoma/translocated in liposarcoma, and Rho guanine nucleotide exchange factor has been described (Keller et al., 2012). The critical point here is that although there is a tendency to describe the various neurodegenerative syndromes using nomenclature that reflects either the underlying genetic basis or the preponderance of a single proteinaceous inclusion, upon critical evaluation the syndromic nature of ALS and its associated frontotemporal syndromes is evident.

The clinical expression of pathological expansions of *C9orf72* is heterogeneous, ranging from a rapidly progressive variant with marked neuropsychological abnormalities to an atypically slow progression that may last decades (Chester et al., 2013; Kandiah et al., 2012; Khan et al., 2012). Such a range of survival is not only consistent with the syndromic nature of ALS, but also suggests that the phenotypic expression of a pathological expansion of *C9orf72* can be modified either by the presence of a second genetic mutation (the basis of oligogenic inheritance) or alternatively by either exogenous or environmental factors.

Perhaps the most controversial aspect of the pathogenesis of frontotemporal dysfunction in ALS is whether or not alterations in the metabolism of tau are present. However, distinct from the presence of a tauopathy among the previously hyperendemic focus of ALS in the Western Pacific, we have observed that tau immunoreactive glial and neuronal inclusions are a significant feature of ALSci (Yang, Sopper, Leystra-Lantz, & Strong, 2003; Yang &

Strong, 2012). Tau isolated from the frontal cortex of patients with ALSci is typically insoluble with (in contrast to AD tau) all six tau isoforms being expressed in the insoluble fraction and abnormally phosphorylated at threonine 175 (pThr175-tau) (Strong et al., 2006). Both the pattern of tau deposition and this phosphorylation state render the tau deposition of ALSci different from primary age-related tauopathy (Crary et al., 2014; Jellinger et al., 2015) and from normal tau deposition as a function of aging (Yang, Ang, & Strong, 2005). Moreover, pseudophosphorylated tau mimicking pThr175-tau forms pathological intracellular inclusions in vitro and leads to cell death (Gohar et al., 2009; Moszczynski et al., 2015).

These observations suggest that the phenotypic expression of both the motor neuron and cortical or subcortical neurodegeneration of ALS can be driven by a wide range of pathological processes, sometimes occurring as isolated meta-bolic syndromes or at times as a confluence of metabolic derangements. If this is the case, then the motor neuron phenotype would be expected to be uniform across all biological variants because there is a limited phenotypic reserve with which to manifest motor neuron dysfunction, specifically as a loss of motor function. The converse cannot be held for the neuropsychological manifesta-tions because the phenotypic reserve upon which to draw for the clinical expres-sion of a specific pathological process will be considerably greater. However, these latter manifestations are not limitless and, as discussed, are reflected in a discrete number of well-defined syndromes of frontotemporal dysfunction. As will become evident, our postulate is that these syndromes do in fact draw on a limited phenotypic reserve, but in this case, the reserve is defined by neural networks.

NEUROIMAGING CORRELATES OF IMPAIRED NEURAL NETWORK FUNCTION AS THE BASIS OF FRONTOTEMPORAL DYSFUNCTION IN AMYOTROPHIC LATERAL SCLEROSIS

The postulate that the frontotemporal syndromes of ALS are based on perturbations in neural networks finds support across a number of neuroimaging modalities, but most specifically resting state functional MRI (RS-fMRI) and diffusion tensor imaging (DTI) (see Chapter 3). RS-fMRI correlates brain regions that are activated concomitantly and has been used to compare functional network alterations in ALS and the behavioral variant of FTD (bvFTD) (Trojsi et al., 2015). This latter study highlighted the involvement of three major neural networks in both ALS and bvFTD: the salience network (SN), the default mode network (DMN), and the central executive network (CEN) (Fig. 9.1).

To visualize the networks more directly and, more specifically, to assess the integrity of neuronal pathways, DTI can be applied. The basis of DTI is the measurement of the diffusion of water along neuronal projections. Given the narrow diameter of neuronal tracts, water is more able to diffuse along the tract than across it, having an anisotropic motion, which when measured,

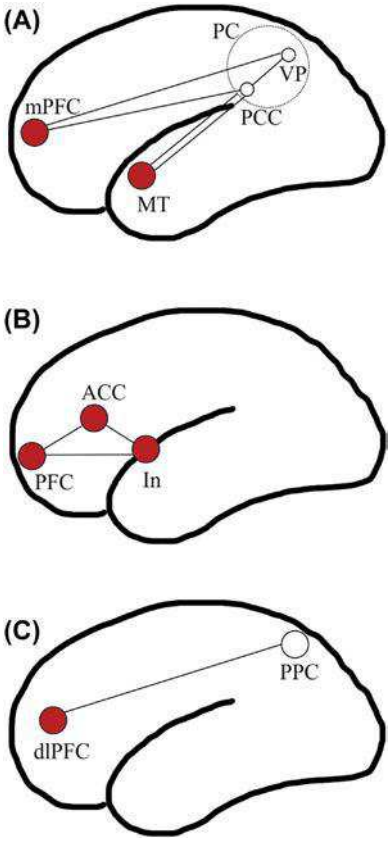

FIGURE 9.1 Three major networks affected in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). *Red nodes* have been shown to be dysfunctional in FTD. (A) Default mode net-work (DMN) areas affected in FTD include the medial prefrontal cortex *(mPFC)* and medial tem-poral (MT) lobes, whereas the posterior cingulate cortex *(PCC)*, ventral precuneus *(VP)*, and parietal cortex *(PC)* are less commonly implicated. The *dashed circle* indicates that the PC is superficial to the contained structures. (B) Salience network (SN) nodes including the anterior cingulate cortex *(ACC)*, insula *(In)*, and prefrontal cortex *(PFC)* are all implicated in FTD-related dementia processes.
(C) Central executive network (CEN) areas affected include the dorsolateral prefrontal cortex *(dlPFC)*, whereas the posterior parietal cortex is less com-monly implicated.

allows for an approximation of the tract direction to be generated. More myelination of tracts increases the signal because of higher water content. Therefore a reduced signal is likely to indicate reduced integrity of the connective pathways. Although not yet applied to understanding the frontotemporal dysfunction of ALS, DTI is being applied to understanding the degeneration of the corticospinal tracts as part of the neural network subserving motor function (Brettschneider, Petzold, Sussmuth, Ludolph, & Tumani, 2006; Hendrix et al., 2015; Karlsborg et al., 2004).

The concept that neural networks mediate not only the phenotypic expression of the neurodegenerative process but also can serve as "highways of disease propagation" has been supported by staging the spread of either tau protein or α-synuclein pathosis in AD and Parkinson disease (Braak & Braak, 1995; Braak et al., 2003). These observations suggest that whereas network connectivity may be affected as a whole, there are specific patterns of vulnerability within these networks. This approach to the study of neural networks has provided insight into the dysfunctional network systems in a variety of

disease states in which syndrome-specific patterns of dysfunction are observed (Seeley, Crawford, Zhou, Miller, & Greicius, 2009). Extending these methodol-ogies to evaluate the integrity of white matter tracts within the brain (structural network imaging), mathematical models in conjunction with fMRI has yielded the capacity to evaluate differences in activated brain areas comprising nodes in these networks (functional network imaging) and to ascribe differences to individual disease states (Zhou et al., 2010).

The understanding of neural networks gained through such studies can be applied to understanding the neuroanatomical origins of the frontotemporal syndromes observed in ALS. The DMN consists of regions in the medial temporal lobe (memory), medial prefrontal cortex (involved in ToM), posterior cingulate cortex, ventral precuneus, and medial, lateral, and inferior parietal cortices. The DMN is active largely during periods of wakeful rest, while the patient is not focusing on anything occurring in the outside world (ie, daydreaming) (Yan et al., 2009). Importantly, the DMN has been implicated in social cognition (Schilbach, Eickhoff, Rotarska-Jagiela, Fink, & Vogeley, 2008). The SN has been implicated in a number of psychotic disorders (Palaniyappan & Liddle, 2012). In cases of young-onset FTD, many are first diagnosed as psychotic disorders up to 5 years before FTD because of the similarity of presentation (Velakoulis, Walterfang, Mocellin, Pantelis, & McLean, 2009). It is noteworthy then that increased psychotic symptoms have been observed in ALS with FTD (Lillo et al., 2010). The SN is thought to act as a switch between the DMN and the CEN (Menon & Uddin, 2010; Sridharan, Levitin, & Menon, 2008), allow-ing for the focus of attention to the external world and one's inside thoughts to be prioritized and maintained. This network consists of the anterior cingu-late, insula, and prefrontal cortices. The CEN is implicated in executive control (D'Esposito, 2007; Koechlin & Summerfield, 2007). The CEN (also referred to as the *frontoparietal network*) consists of the dorsolateral prefrontal cortex and posterior parietal cortex, with particular activity along the intraparietal sul-cus. Importantly, the DMN and CEN have anticorrelated activations such that activation of one leads to inhibition of the other (Fox et al., 2005). Supporting dysfunction in these networks in ALS, the presence of protein inclusions in the anterior cingulate cortex paired with the signs of both ToM (van der Hulst et al., 2014) and executive control dysfunction (York et al., 2014) may indicate an SN abnormality because there are dysfunctions across all three network activities that may indicate a switching and control abnormality. Consistent with this, both ALS and FTD brains have reduced SN functioning, whereas patients with AD have an enhancement in this network and a reduction in activity of the DMN (Zhou et al., 2010).

Beyond describing the basis of the neuropsychological manifestations of ALS, the analysis of neural networks has provided insight into neural network dysfunction in ALS before the detection of executive dysfunction (Trojsi et al., 2015). When both ALS and bvFTD were compared with controls, reduced right supramarginal gyrus connectivity (reflecting CEN dysfunction) and decreased

medial prefrontal cortex and insular activation (reflecting SN dysfunction) was observed in both ALS and bvFTD, although it occurred in bvFTD more than in ALS. Of note, divergence between disease states was observed, because ALS cases showed reduced posterior cingulate connectivity (reflecting DMN dysfunction), whereas bvFTD cases showed an increase in connectivity of this region along with decreased connectivity in the frontal regions of this network, indicating more widespread connectivity changes when the cognitive phenotype was present. The convergence in network dysfunction may indicate common processes at work in these separate phenotypes. Conversely, the divergence in DMN activity, along with more severe deficits in other network connectivity may be responsible for the lack of change in social cognition in some patients with ALS.

Additional network connectivity studies in patients with bvFTD have shown a reduced connectivity throughout the brain, including the anterior cingulate cortex, temporal poles, frontal gyri, and insular cortices (Agosta et al., 2013). This reduced connectivity has been determined to represent a reduction in connection efficiency and may represent a reduced ability to transfer and therefore process information (Agosta et al., 2013). Additionally, white matter integrity is compromised in the same regions as major gray matter loss, with extension to other regions with no measured gray matter atrophy (Mahoney et al., 2012). A reduction in the overall connectivity of the uncinate fasciculus has been implicated in bvFTD in distinction from other dementias, namely AD, which shares some network connectivity–change overlap with bvFTD (Mahoney et al., 2012).

Apart from the cognitive involvement, structural brain network imaging studies of patients with ALS has revealed a motor network dysfunction that correlates with the severity of disease to a larger extent than total measured atrophy (Verstraete et al., 2014). Expansion of these deficits is seen with disease progression, suggesting a spread of pathosis reminiscent of the spread of protein inclusions (reviewed in Jucker & Walker, 2013). Such a postulate would also explain a progressive diversification of symptoms, implying that this spread of dysfunctional activity along brain network paths is a key component of the disease process.

MODELS OF NEUROPSYCHOLOGICAL DYSFUNCTION IN AMYOTROPHIC LATERAL SCLEROSIS

Whereas a number of models of the motor dysfunction of ALS have been identified, there are very few that recapitulate the neuropsychological dysfunction described, and essentially none that address the integrity of neural networks. Thus although the most commonly utilized murine model for ALS harbors the G93A *SOD1* mutation seen in familial ALS, little is known regarding its impact on cognitive function in the mouse, although these mice do possess shorter dendrites in the prefrontal cortex and have reduced fear extinction (Sgobio et al., 2008).

The latter precedes the development of motor deficits. Mice harboring the G37R *SOD1* mutation have spontaneous alternation deficits on a T maze task (Filali, Lalonde, & Rivest, 2011).

Murine models of TDP-43 pathology have been developed, including the overexpression of wild-type TDP-43 (Wils et al., 2010). These mice develop spinal and cortical TDP-43 pathosis reminiscent of ALS-FTD. In a separate study, the overexpression of human wild-type TDP-43 in mice led to memory deficits in the Morris water maze as well as fear conditioning tasks (Tsai et al., 2010). The expression of mutant TDP-43 (A315T) induces both cortical and spinal motor neuron death in mice in the absence of pathological cytoplasmic TDP-43 aggregates (Wegorzewska, Bell, Cairns, Miller, & Baloh, 2009). To attempt to more precisely reflect the human disease state, Swarup and colleagues designed mouse models of human wild-type TDP-43 as well as A315T and G348C mutants that expressed TDP-43 at levels that more closely resemble that in the human CNS (Swarup et al., 2011). They found that along with motor deficits, mice developed cytoplasmic TDP-43 pathosis resembling that of ALS-FTD. Affected mice developed learning deficits on the Barnes maze test, indica-tive of cognitive abnormalities.

The discovery of *C9orf72* is relatively recent, and thus the development of models of cognitive dysfunction lags behind that of *SOD1* and TDP-43 models.

THERAPEUTIC STRATEGIES

Given the relatively recent increase in our understanding of both the incidence of frontotemporal dysfunction in ALS and its probable phenotypic basis in dysfunction of neural networks seemingly independent of the underlying proteinopathy, little is known regarding its treatment. Indeed, at this time, there are no studies that have specifically addressed pharmacotherapies for this aspect of the disease process.

CONCLUSIONS AND FUTURE DIRECTIONS

ALS is a clinical presentation of a group of pathologies that happen to affect the same cells through potentially different mechanisms. When patients exhibit dysexecutive syndrome, it is the result of specific network activity malfunc-tion, such as the insula in the SN. Spread of pathosis through these networks is likely to be responsible for disease progression. Further insight into the appar-ent selective vulnerability of the motor and frontal cortical neurons will also be important in determining the etiology of the disease. Patient imaging with molecule-specific ligands and genotypic analysis to determine which patholo-gies are the most likely causes of the phenotype will be crucial for developing strategies to stop disease progression in individual patients and stratify cases based on possible mechanisms of cell death such as oxidative, RNA processing, or cytoskeletal abnormalities.

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Curriculum Vitae

Curriculum Vitae

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4. Queen Elizabeth II Graduate Scholarship in Science and Technology, 2015-2016. Amount: $15, 000.
5. Western Graduate Research Scholarship, 2015-2016. Amount: $7, 000.
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7. International Conference on Frontotemporal Dementia student travel bursary, $1000.
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Manuscripts published: 8
Manuscripts in press: 0
Manuscripts under review: 2
Manuscripts in preparation: 1
Manuscripts as first author: 7

1. Moszczynski AJ, Mccunn P, Gopaul J, Volkening K, deOliviera C, Bartha R, Schmid S, Strong MJ. Somatic gene transfer using a recombinant adenoviral vector (rAAV9) encoding pseudophosphorylated human Thr^{175} tau in adult rat hippocampus induces a pathological tauopathy. [In preparation].
2. Moszczynski AJ, McKee AC, Strong W, Strong MJ. Tau protein isoform composition and phosphorylation sites are shared in CTE and ALSci. [Submitted to *Neurology*, under review].
3. Deshaies J, Shkreta L, Moszczynski AJ, Flamier A, Sidibé H, Semmler S, Fouillen A, Bennet E, Bekenstein U, Destroismaisons L, Toutant J, Delmotte Q, Volkening K, Stabile S, Aulas A, Khalfallah Y, Soreq H, Nanci A, Bernier G, Strong MJ, Chabot B, Vande Velde C. TDP-43 regulates hnRNP A1 alternative splicing to generate an aggregation prone isoform in amyotrophic lateral sclerosis. [Submitted to *Brain*, under review].
4. Moszczynski AJ, Yang W, Strong MJ. Threonine 175, a novel pathological phosphorylation site on Tau protein linked to multiple tauopathies. *Acta Neuropath Comms*. 2017 Jan; 5(1):6.
5. Moszczynski AJ, Strong MJ. Cortical manifestations in amyotrophic lateral sclerosis. Chapter in: The Cerebral Cortex in Neurodegenerative and Neuropsychiatric Disorders: Experimental Approaches to Clinical Issues. Edited by David Cechetto. *Elsevier* 2016.
6. Moszczynski AJ, Gohar M, Volkening K, Strong W, Strong MJ. Thr^{175} phosphorylated tau induces pathological fibril formation via GSK3β mediated phosphorylation of Thr^{231} *in vitro*. *Neurobiol Ageing*. 2015 Mar; 36(3):1590-9.
7. Masuko A, Villa TR, Pradella-Hallinan M, Moszczynski AJ, De Souza Carvalho D, Tufik S, Do Prado GF, Coelho FMS. Higher prevalence of bruxism during sleep in children with episodic migraine. *BMC Res Notes*. 2014 May 14; 7(298): doi: 10.1186/1756-0500-7-298.
8. Moszczynski AJ, Tandon A, Coelho FMS, Zinman L, Murray BJ. Mortality Associated with Periodic Limb Movements of Sleep in Amyotrophic Lateral Sclerosis. *Einstein*. 2012 Dec; 10(4):428-32.
9. Moszczynski A, Murray BJ. Neurobiological Aspects of Sleep Physiology. *Neurol Clin*. 2012 Nov; 30(4):963-85.
10. Rasquinha RJ, Moszczynski AJ, Murray BJ. A modern artifact in the sleep laboratory. *J. clin sleep med.* 2012 Apr 15; 8(2):225-6.
11. Coelho FMS, Moszczynski A, Narayansingh M, Parekh N, Predalla-Hallinan M. Sexual hypnagogic hallucinations and narcolepsy: a case report. *Sleep science*. 2011;4(3):110–112.

International Presentations:

1. Moszczynski AJ, McKee AC, Strong, W, Strong MJ. Phosphothreonine175 and Phosphothreonine231 Expression in Chronic Traumatic Encephalopathy (CTE) and Chronic Traumatic Encephalomyelopathy - Therapeutic Implications. American Academy of Neurology, Vancouver, British Columbia, Canada. Neurology 86 (16 Supplement), S11. 004.

Scientific Abstracts Presented:

1. Moszczynski A, Yang W, Strong M, pThr175 tau is associated with tau pathology in a spectrum of tauopathies. 10th international conference on Frontotemporal Dementias. Munich, Germany. *J. Neurochem*. 2016; (Suppl 1): 333.
2. Moszczynski A, McKee A, Strong W, Strong M. pThr175 tau is a toxic phosphoepitope in CTE, CTEM and ALSci. 10th international conference on Frontotemporal Dementias. Munich, Germany. *J. Neurochem*. 2016; (Suppl 1): 334.
3. Moszczynski A, Gopaul J, Mccunn P, Volkening K, Harvey M, Bartha R, Schmid S, Strong M. An AAV9 mediated rat model of pThr175 tau protein toxicity. 10th international conference on Frontotemporal Dementias. Munich, Germany. *J. Neurochem*. 2016; (Suppl 1): 334.
4. Moszczynski AJ, Yang W, Gopaul J, Mccunn P, Volkening K, Bartha R, Schmid S, Strong MJ. The role of pThr175 tau in neurodegeneration: evidence of a common contributing mechanism in multiple diseases. London Health Research Day. March 29, 2016. London, Ontario. Canada.
5. Moszczynski AJ, Volkening K, Strong MJ. Thr175- phosphorylated tau induces pathologic fibril formation via GSK3β-mediated phyosphorylation of Thr^{231} *in vitro*. 5th International Research Workshop on Frontotemporal Dementia in ALS. June 7-10 2015. London, Ontario, Canada. 3rd place prize.
6. Moszczynski AJ, Volkening K, Strong MJ. Thr175- phosphorylated tau induces pathologic fibril formation via GSK3β-mediated phyosphorylation of Thr^{231} *in vitro*. 11th annual ALS Canada Research Forum. May 1-4, 2015. Toronto, Ontario, Canada.
7. Moszczynski AJ, Volkening K, Brown A, Strong MJ. Thr175- phosphorylated tau induces pathologic fibril formation via GSK3β-mediated phosphorylation of Thr^{231} *in vitro*. London Health Research Day. April 1, 2015. London, Ontario. Canada.
8. Moszczynski AJ, Volkening K, Strong MJ. Thr175 phosphorylated tau induces pathological fibril formation via GSK3β mediated phosphorylation of Thr^{231} *in vitro*. 9th international conference on Frontotemporal Dementias. Vancouver, British Columbia, Canada. *Am J Neurodegener Dis*. 2014; 3(Supplementary Issue 1):1-375.
9. Moszczynski AJ, Gohar M, Volkening K, Strong MJ. Thr^{175} Phosphorylation Regulates GSKβ Activity and Tau Pathology *in vitro.* Southern Ontario Neuroscience Association. May 5, 2014. London, Ontario, Canada.

10. Moszczynski AJ, Gohar M, Volkening K, Strong MJ. Thr^{175} Phosphorylation Regulates GSKβ Activity and Tau Pathology *in vitro.* 10^{th} annual ALS Canada Research Forum. May 3-5, 2014. Toronto, Ontario, Canada.
11. Moszczynski AJ, Gohar M, Volkening K, Strong MJ. Thr^{175} Phosphorylation Regulates GSKβ Activity and Tau Pathology *in vitro.* London health research day. March 18, 2014. London, Ontario, Canada.
12. Moszczynski AJ, Gohar M, Volkening K, Strong MJ. Thr175 phosphorylation regulates GSK3 beta activity and tau fibril formation *in vitro*. 43rd Annual Meeting of the Society-for-Neuroscience. San Diego, CA, USA. Society for Neuroscience. November 09 -13, 2013. Volume: 43.
13. Moszczynski AJ, Gohar M, Volkening K, Strong MJ. Thr175 Phosphorylation May Regulate GSK3β Activity and Tau Pathology *in vitro*. Fourth International Research Workshop on Frontotemporal Dementia in ALS. June 3-5 2013. London, Ontario, Canada.
14. Moszczynski AJ, Gohar M, Volkening K, Strong MJ. Inhibition of GSK3β activity may inhibit pathological tau fibril formation *in vitro*. London health research day. March 19, 2013. London, Ontario, Canada.
15. Rasquinha R, Moszczynski A, Murray B. Automated Quantification of Axial EMG Tone in Sleep Identifies Patients with REM Sleep Behavior Disorder. 64th Annual Meeting of the American- Academy-of- Neurology. *Neurology*. Apr 2012:78 (S1): P05005.
16. Moszczynski AJ, Tandon A, Coelho FMS, Zinman L, Murray BJ. Mortality associated with periodic limb movements of sleep in amyotrophic lateral sclerosis. University of Toronto Neurology Faculty Research Day. Nov 30, 2010. Toronto, Ontario, Canada.
17. Moszczynski AJ, Tandon A, Coelho FMS, Zinman L, Murray BJ. Restless Legs Syndrome and Periodic Limb Movements of Sleep in Amyotrophic Lateral Sclerosis. Best Summer Research Competition. Aug 6, 2010. Sunnybrook Hospital. University of Toronto, Ontario Canada.

Undergraduate research experience:

Honours Thesis (2011-2012): Effects of anesthetic drugs on the hypoglossal motor pool of anaesthetized rats. Under supervision of Dr. Richard Horner, PhD.

Summer Student (2010 and 2011): Sunnybrook Hospital sleep lab, under supervision of Dr. Brian J. Murray, MD.

Undergraduate volunteer (2009-2010): Centre for the analysis of genome evolution & function (CAGEF) under supervision of Dr. David Guttman, PhD.

Extracurricular Leadership positions:

Founder/ coordinator of collaboration of practitioners and researchers (CPR) seminar series (2016-present): A seminar initiative for graduate students and medical students to come together and collaborate on current gold standard in healthcare and the state of the field in research. Starting this type of communication as trainees, we hope to create a culture of open talk between tomorrow's clinicians and researchers to work together, increase understanding of each others' roles on the front lines of medicine and ultimately generate disease treatment faster.

Chair, Schulich Graduate Student Council (2015-16; elected position): Direct Student council for all 700 graduate students in the Schulich School of Medicine and Dentistry. This involves holding and running council meetings, helping plan academic and social events, speaking to the incoming graduate students at their orientation event, writing monthly newsletter articles, sitting on the Schulich graduate affairs committee with the Chair of each department, and meeting with University administration to represent student perspectives in decisions.

Graduate Neuroscience Program student representative (2014-2015; elected position): Attend Schulich council meetings and Neuroscience program faculty committee meetings to keep up to date on program details and voice concerns of students within the program.

Gradcast Radio show founder, chair (2013-2015; elected) and host (2013-present): Founding member and chair of GradCast, the official radio show of the Society of Graduate Students (SOGS). This bi-weekly show interviews graduate students about their research and discusses current issues. My role as chair is to organize the show and other members' duties, as well as write annual reports to present at SOGS meetings. I was re-elected as chair by a second committee for an additional year after its first year. Stepped down for the third year due to another leadership role on the Schulich Graduate Council.

Neuroscience Assosiation of Undergraduate Students, Vice president of social affairs (U of T; 2011-2012): Plan any and all events held by NAUS including student socials, seminars, and assist with running the Canadian Undergraduate Life Sciences Challenge (CULSC) held at U of T each year.

Senior Undergraduate Neuroscience Student Advisory Committee (SUN-SAC; 2011; invited position): Meet with Dr. Bill Ju and other upper year undergraduate students to discuss undergraduate neuroscience program courses and provide feedback.

Volunteer Experience:

Let's talk science (2013-present), teach science in a fun and exciting format to gradeschool classes.

Making Waves London (2012-2015), volunteer swim instructor for children in the community with special needs.

Kensington Gardens Long-term care home (2012), Friendly visitor for residents with special needs (ie: read to visually impaired patient).

Undergraduate volunteer (May 2009-April 2010), Guttman lab, University of Toronto, Dept. of Cell and Systems Biology.

Brain Day volunteer presenter (through the University of Toronto; 2010), present importance of brain function and safety to children aged 9-12.

Assistant martial arts instructor (2004-2007), for Sifu John Yee at Bedford Park community center. Helped teach basic self defense skills to new students while John taught more advanced students. Taught entire classes when John was unable to do so.

Sunnybrook Health Sciences Centre Volunteer (October 2004- June 2005), Assisted with recreation therapy for veterans, helping them play bingo and brought them to and from their rooms.

Non-academic Awards and Achievements:

- Ivey-Deloitte Entrepreneurship bootcamp course winner, June 11, 2017
- Proteus innovation challenge winner ($7,500), March 3, 2017
- National Lifeguard Service (NLS), May 26, 2013
- Standard First Aid with CPR-C (December 12, 2010)
- Ontario Federation of School Athletic Associations (OFSAA) swim competitor (2006, 2007)
- Cardinal Carter Athlete of the Year (2006) for outstanding motivation and hard work in athletics
- Cardinal Carter Academy for the Arts senior chamber orchestra, performed at Midwest clinic (Chicago December 2006), first Canadian high school orchestra to do so
- Black belt in John Yee's Walk Tall Martial Arts Program (2005)
- Wrote and recorded original song for inter-faith unity documentary (2008)