The Thesis Committee for Julia Gerson certifies that this is the approved version of the following dissertation:

Characterization of Tau Oligomeric Strains: Implications for Disease Phenotypes

Committee:

Rakez Kayed, PhD, Mentor, Chair

Fernanda Laezza, PhD, MD

Marc Morais, PhD

Volker Neugebauer, PhD, MD

Yogesh Wairkar, PhD

Jose Abisambra, PhD

David Niesel, Dean, Graduate School

Characterization of Tau Oligomeric Strains: Implications for Disease Phenotypes

by

Julia Elise Gerson, B.S.

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Characterization of Tau Oligomeric Strains: Implications for Disease Phenotypes

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Julia Elise Gerson, PhD

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Abstract: Neurodegenerative diseases are prevalent, costly and debilitating conditions that are commonly characterized by the aggregation of tau protein. While original studies of Alzheimer's disease noted the presence of tau aggregated into large, insoluble fibrillar structures known as neurofibrillary tangles, our lab and others found that small, oligomeric aggregates are likely the most toxic species. I found that toxic tau oligomers are elevated in progressive supranuclear palsy, traumatic brain injury and synucleinopathy models and reversal of toxicity can be achieved with treatment of a tau oligomer-specific monoclonal antibody. Tau oligomers have prion-like properties, enabling them to seed the misfolding and aggregation of natively folded, functional tau protein endogenous in the brain. Moreover, the spread of pathology from early affected regions to unaffected areas may be driven by propagation of tau oligomers. However, tau oligomers are not a homogenous population and the formation of different prion-like strains may underlie diversity of pathophysiology in different diseases. In this series of experiments, I have shown that tau oligomeric strains can be prepared in the laboratory and isolated from human disease brain tissue. Brain-derived tau oligomeric strains seed the aggregation of tau *in vitro* and display distinguishable characteristics based on techniques from the prion field. For the first time, I show that tau oligomeric strains can enter the brain when injected in the eye in mice and induce diverse outcomes. These results lay a critical foundation for the identification of specific tau oligomeric strains and combinations of strains that could be used as biomarkers for different neurodegenerative diseases. Moreover, these results could be used for future development of personalized therapeutics against neurodegenerative disorders by specifically targeting the most toxic strains initiating disease in different individuals.

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List of Abbreviations

3R tau	Tau with three microtubule binding repeats
4R tau	Tau with four microtubule binding repeats
AAALAC	American Association for the Accreditation of Laboratory Animal Care
ABS	Advanced Blast Simulator
AD	Alzheimer's disease
AFM	Atomic force microscopy
ANOVA	Analysis of Variance
APP	Aβ precursor protein
ATPZ	Aminothienopyridazines
Aβ	Amyloid-β
BBB	Blood brain barrier
BDTO	Brain-derived tau oligomer
BTA	Benzothiazole aniline
CaMKII	Ca2+/Calmodulin-dependent protein kinase II
CBD	Corticobasal degeneration
CDK5	Cyclin-dependent kinase-5
CFA	Freund's complete adjuvant
CHIP	Carboxyl terminus of the Hsp70-interacting protein
CK1a/1d/1e/2	Casein kinase 1a/1d/1e/2
CNS	Central nervous system

CSF	Cerebrospinal fluid
Ctrl IgG	Control IgG
DLB	Dementia with Lewy bodies
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DYRK1A	Dual-specificity tyrosine phosphorylation and regulated kinase 1A
EGCG	(−)-epigallocatechin-3-gallate
ELISA	Enzyme-linked immunosorbant assay
EM	Electron microscopy
ER	Endoplasmic reticulum
ERK	Extracellular-signal-related-kinases
FPI	Fluid percussion injury
FTD	Frontotemporal dementia
FTDP-17	FTD and Parkinsonism linked to chromosome 17
FTLD	Frontotemporal lobar dementia
GFAP	Glial fibrillary acid protein
GSK3β	Glycogen synthase kinase-3β
HCl	Hydrochloric acid
HDAC6	Histone deacetylase 6
HFIP	Hexafluoroisopropanol
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
Hsf1	Heat shock transcription factor 1

HSP	Heat shock protein
Htau mice	Mice expressing non-mutated human tau
ICV	Intracerebroventricular
IFA	Freund's incomplete adjuvant
IV	Intravenous
K18	Microtubule binding repeat domain fragment of 4R human tau
kD	Kilodalton
Kd	Dissociation constant
KO	Knockout
LB	Lewy body
LBD	Lewy body dementia
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinases
MARK	Microtubule affinity-regulating kinases
MSA	Multiple systems atrophy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide mononucleotide
NFT	Neurofibrillary tangle
NGase	N-acetylglucosaminidase
NMDA	N-methyl-D-aspartate
NMNAT	NAD adenylyl transferase
NOR	Novel object recognition
OCT	Optimal cutting temperature medium

p75NTR	p75 neurotrophin receptor
PBS	Phosphate-buffered saline
PcTS	Pthalocyanine tetrasulfonate
PD	Parkinson’s disease
PDI	Protein disulfide isomerases
PDPK	Proline-directed protein kinases
PET	Positron emission tomography
PHF	Paired helical filament
PiD	Pick’s disease
PK	Proteinase K
PKA	Protein kinase cAMP-dependent
PKB/Akt	Protein kinase cAMP-dependent B
PKC	Protein kinase cAMP-dependent C
PKN	Protein kinase cAMP-dependent N
PP2A	Protein phosphatase-2A
PrPc	Cellular prion protein
PS1	Presinilin 1
PSP	Progressive supranuclear palsy
RNA	Ribonucleic acid
RR	Righting reflex
RT	Room temperature
SDS	Sodium dodecyl sulfate
SEC	Size-exclusion chromatography

SF	Straight filament
TBI	Traumatic brain injury
TBST	Tris-buffered saline with low Tween
TDP43	TAR DNA-binding protein 43
TH	Tyrosine hydroxylase
TMB-1	3,3',5,5',-tetramethylbenzidine
TNAP	Tissue-nonspecific alkaline phosphatase
TOMA	Tau oligomer-specific monoclonal antibody
TTBK1/2	Tau-tubulin kinase ½
UPS	Ubiquitin proteasome system
UTMB	University of Texas Medical Branch
WB	Western blot
WT	Wildtype

CHAPTER 1

INTRODUCTION

Tau Aggregation in Disease

Tau in Neurodegeneration

Neurodegenerative disease is a leading cause of death and disability in the elderly population. There are currently no effective therapeutics for any neurodegenerative disorders and as life expectancy continues to rise, the number of those affected will only grow. A number of neurodegenerative disorders are associated with the microtubule-associated protein, tau. These diseases are collectively termed tauopathies and include Alzheimer's Disease (AD), Parkinson's Disease (PD), Lewy Body Dementia (LBD), Progressive Supranuclear Palsy (PSP), Pick's Disease (PiD), Frontotemporal Dementia (FTD), Corticobasal Degeneration (CBD), and even Traumatic Brain Injury (TBI). A common histopathological hallmark of these diseases is the aggregation of tau protein, leading to accumulation of fibrillar tau deposits or neurofibrillary tangles (NFTs) (Ballatore, Lee et al. 2007).

Currently, the only therapeutics on the market for the most prevalent neurodegenerative tauopathy, AD, are acetylcholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists, which are only capable of treating the symptoms of AD, but are ineffective at slowing the progression of the disorder. Much of the research investigating therapeutics for AD thus far has been focused primarily on the accumulation of amyloid-β (Aβ) aggregates, however, tau pathology correlates with

disease symptoms far better than Aβ. Therefore, tau represents one of the most promising therapeutic targets for a number of neurodegenerative diseases (Iqbal, Liu et al. 2014).

Tau Oligomers are the Toxic Species in Neurodegenerative Disease

In its native state, tau exists as an unfolded monomeric protein, primarily bound to microtubules. It has an important role in the cell, stabilizing microtubules, controlling neurite growth and axonal transport (Binder, Frankfurter et al. 1985, Drubin and Kirschner 1986). In addition, recent studies suggest tau may be involved in learning during development (Sapir, Frotscher et al. 2012). *Tau* is alternatively spliced in exons 2, 3, and 10, forming six different tau isoforms. Exon 10 splice products are particularly prone to mutations that may affect the aggregation of tau, forming three isoforms with three microtubule binding repeats (3R tau) and three isoforms with four microtubule binding repeats (4R tau) (Goedert, Spillantini et al. 1989). While NFTs have historically been considered the main hallmark in tauopathies, they do not appear to be the main toxic species in disease. Cell death occurs in disease prior to the formation of NFTs (Gomez-Isla, Hollister et al. 1997, Vogt, Vogt et al. 1998, Terry 2000, van de Nes, Nafe et al. 2008) and NFT-containing neurons have been shown to be functionally intact *in vivo* (Kuchibhotla, Wegmann et al. 2014). Moreover, NFTs do not affect signaling cascades involved in long-term potentiation and memory formation (Rudinskiy, Hawkes et al. 2014). Tau transgenic animal models acquire behavioral deficits, synaptic dysfunction, and cell death in the absence of NFT formation (Wittmann, Wszolek et al. 2001, Andorfer, Kress et al. 2003, SantaCruz, Lewis et al. 2005, Spires, Orne et al. 2006, Berger, Roder et al. 2007, Yoshiyama, Higuchi et al. 2007, Cowan, Bossing et al. 2010).

Numerous researchers have investigated tau pathology using animal models and human tissue, yielding a better understanding of the toxicity of different tau structures. A study in aged mice expressing native human tau (Htau mice) found that while NFT formation occurred as animals aged, there was no correlation between the presence of tau filaments and cell death (Andorfer, Kress et al. 2003). Additionally, a study examining the P301S mouse model, which expresses mutant human tau, found that hippocampal synaptic dysfunction occurred prior to NFT formation (Yoshiyama, Higuchi et al. 2007). Studies using the rTg4510 mouse model, which conditionally expresses P301L mutant tau, found that cell death occurred prior to NFT formation and that cell loss and behavioral impairments could be suppressed by inhibiting tau expression without removing NFTs or preventing their continued accumulation (SantaCruz, Lewis et al. 2005, Spires, Orne et al. 2006). In accordance with this finding, it has been shown that NFTs are protective in the same mouse model (de Calignon, Fox et al. 2010), and only pro-aggregate human tau mice (TauRD) show behavioral deficits (Sydow, Van der Jeugd et al. 2011). Another study in the same mouse model characterized tau oligomers biochemically that appeared early and correlated with cognitive deficits (Berger, Roder et al. 2007, Sahara, DeTure et al. 2013). Similar results have also been seen in drosophila AD models, where expression of mutant tau causes neurodegeneration, synaptic dysfunction, and axonal transport deficiencies in the absence of NFTs (Wittmann, Wszolek et al. 2001, Cowan, Bossing et al. 2010). Usage of the protein nicotinamide mononucleotide (NAD) adenylyl transferase (NMNAT) was shown to decrease behavioral and morphological deficiencies in a frontotemporal dementia drosophila model by decreasing levels of tau oligomers (Ali, Ruan et al. 2012). Therefore, it is likely

that these intermediate tau species that form between tau monomers and NFTS—tau oligomers—are responsible for the onset of disease.

Biochemical analysis of human AD brain tissue has also yielded results suggesting that tau oligomers may initiate toxicity, rather than NFTs. When compared to control brains, levels of tau oligomers were found to be significantly increased in AD brains early in the disease, prior to when NFTs appear and clinical symptoms are evident (Maeda, Sahara et al. 2006, Maeda, Sahara et al. 2007, Patterson, Remmers et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012). In addition to correlative evidence for the importance of tau oligomers to toxicity, treatment with tau oligomers has also been shown to cause adverse effects in animals. Isolated tau oligomers, but not monomers or NFTs, induced memory impairments, synaptic dysfunction, and mitochondrial dysfunction when given intracerebrally to wild-type mice (Lasagna-Reeves, Castillo-Carranza et al. 2011). Therefore, it is possible that NFTs are actually neuroprotective, sequestering toxic forms of tau into large aggregates with less flexibility and surface area to interact with cells. All of these studies form the framework for the model of the progression of neurodegenerative tauopathies beginning with the seeding and propagation of toxic tau oligomers (Figure 1.1).

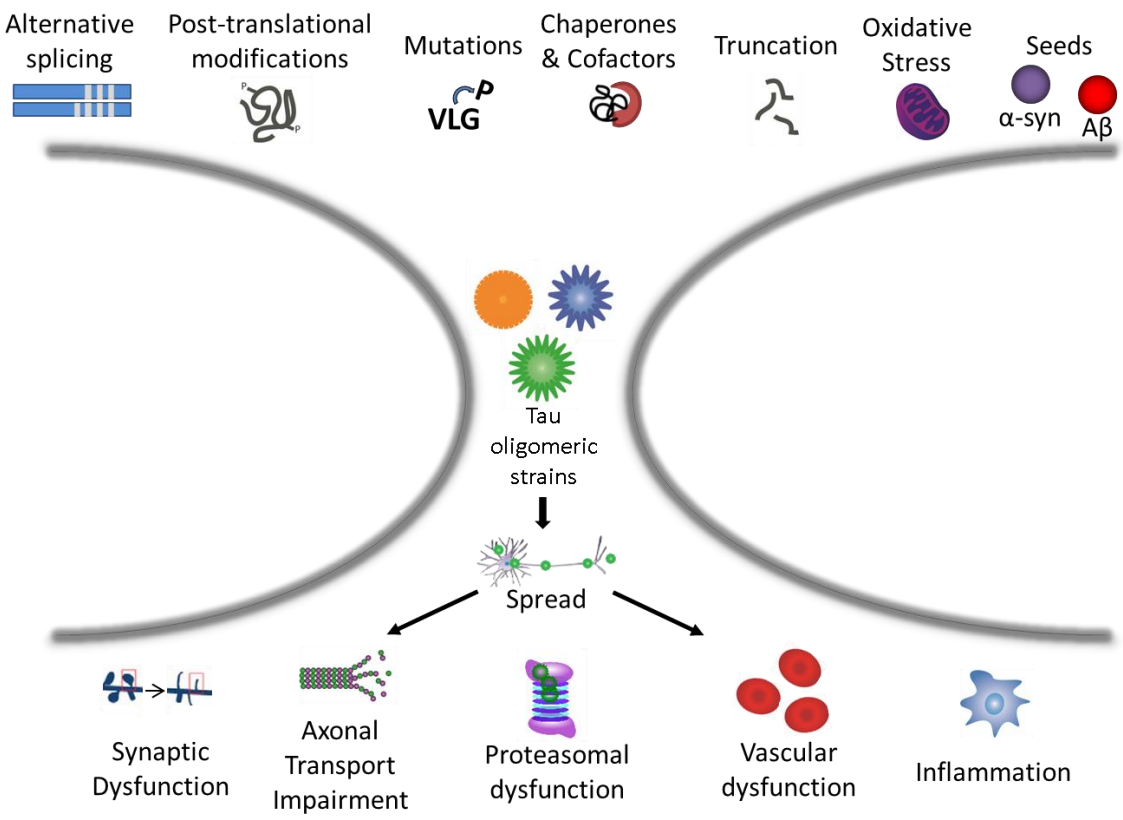

Figure 1.1 Schematic illustrating the central role of tau oligomers in tauopathies. Tau intermediate soluble aggregates (tau oligomers) are the toxic tau entities and initiators of tau pathology and propagation in tauopathies, rather than monomeric tau or hyperphosphorylated NFTs (p-NFTs). Many factors can induce the formation of tau oligomers in different conformational states (tau oligomeric strains), which can then spread and cause a host of downstream toxic effects. Thus, tau oligomers represent the ideal target for anti-tau therapeutic approaches.

Tau Aggregation Mechanism

In order for aggregation to occur, tau must be released from microtubules to reach a high concentration of free cytosolic tau in a disordered, high energy state, allowing for the exposure of hydrophobic patches and the formation of intermolecular contacts. Conformational changes must occur to allow for aggregation, possibly by increasing β sheet content, and dimerization must occur (Chang, Kim et al. 2008, Congdon, Kim et al. 2008). Tau fibrils are known to be capable of seeding the fibrillization of tau monomer by template assisted growth, whereby monomers that come into contact with the tau filament are integrated into parallel β-sheet structure (Margittai and Langen 2004). It has been difficult to find the experimental occurrence of fibrillization that occurs spontaneously without using fragments comprised of only the microtubule binding repeat region (Wille, Drewes et al. 1992) or applying polyanionic compounds and free fatty acids (Wilson 1997, King, Ahuja et al. 1999, King, Gamblin et al. 2000, Barghorn and Mandelkow 2002, Chirita, Necula et al. 2003, von Bergen, Barghorn et al. 2005). The addition of polyanions, such as heparin or RNA can induce fibrillization of tau (King, Ahuja et al. 1999), causing a conformational change from random coil structure to β sheet structure (von Bergen, Barghorn et al. 2005). Free fatty acids, such as arachidonic acid can also increase aggregation (Wilson 1997, King, Gamblin et al. 2000) due to the presence of an alkyl chain, which induces micellization, and a negatively-charged head group on the fatty acid to create a negatively charged surface on the micelle. In the presence of tau, the critical concentration for micelle formation is greatly decreased, allowing anionic micelles to attract tau to the negatively-charged surface and thereby

compensate for positive charges in tau and enable tau aggregation (Barghorn and Mandelkow 2002, Chirita, Necula et al. 2003).

Phosphorylation may also play a role in fibrillization. Paired helical filaments (PHFs) and straight filaments (SFs) that make up the NFTs found in the brains of patients with AD are comprised of hyperphosphorylated tau in cross β-sheet conformation common to amyloid fibrils (Grundke-Iqbal, Iqbal et al. 1986, Lee, Balin et al. 1991, von Bergen, Barghorn et al. 2005). Phosphorylated tau has a higher tendency towards aggregation than unphosphorylated tau and kinases involved in the phosphorylation of these sites in tau have been shown to be altered in AD (Pérez, Cuadros et al. 2000). Hyperphosphorylated tau has been shown to aggregate *in vitro,* possibly due to the addition of negative charge that would increase aggregation, similarly to the addition of polyanions and free fatty acids. Furthermore, this process can be inhibited by dephosphorylation, (Alonso, Zaidi et al. 2001, Alonso, Zaidi et al. 2001). Phosphorylation may also induce aggregation by reducing the interaction of tau with microtubules and allowing it to interact instead with unphosphorylated tau and form aggregates (Biernat, Gustke et al. 1993, Bramblett, Goedert et al. 1993, Sengupta, Kabat et al. 1998). Mutations, such as those that lead to FTD and Parkinsonism linked to chromosome 17 (FTDP-17), can increase tau aggregation through different mechanisms. Many mutations lead to a decrease in microtubule assembly kinetics, which could lead to more free cytosolic tau and increase aggregation (Hasegawa, Smith et al. 1998{Barghorn, 2000 #291{DeTure, 2000 #292{Poorkaj, 2002 #293, Barghorn, Zheng-Fischhöfer et al. 2000, DeTure, Ko et al. 2000, Poorkaj, Muma et al. 2002). Some mutations lead to a decrease in the dissociation constants (K_d) for dimer and filament formation, while others

increase the rate of nucleation without affecting K_d (Chang, Kim et al. 2008). Mutations that cause increased formation of β-sheets lead to heightened aggregation due to an increase in hydrophobic interactions, deviating from the highly hydrophilic native tau protein (Harada 1971, von Bergen, Barghorn et al. 2001). Fibrillar tau can thereby be recognized by dyes which interact with β-sheets, such as Congo Red and thioflavin S (Friedhoff, Schneider et al. 1998). However, at high concentrations, these dyes can induce fibrillization due to an attraction between positive charges formed in the core of PHFs and negative charges of the anionic dyes (Lira-De León, García-Gutiérrez et al. , Kim, Randolph et al. 2003, Congdon, Necula et al. 2007).

The seeding of tau with brain-derived tau oligomers (BDTO) results in highly toxic species capable of propagating from affected to unaffected regions in cells and mice, however fibrils are not able to propagate or induce toxic effects (Lasagna-Reeves 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012, Wu, Herman et al. 2013). These results suggest a prion-like mechanism for the spread of tau pathology dependent upon oligomeric tau (Gerson and Kayed 2013). In spite of evidence suggesting that tau fibrils are not the most toxic species in disease and may even be neuroprotective, much of the current research on tauopathy treatment has been focused on fibrillar tau. Nevertheless, new avenues for treatment of tau oligomers, in particular tau therapeutics targeting extracellular tau, are a novel and exciting route for treatment as they may be able to slow or stop the spread of disease (Castillo-Carranza DL 2013, Gerson and Kayed 2013, Guerrero-Muñoz, Castillo-Carranza et al. 2014).

Tau Oligomers are Seeds for the Propagation of Pathological Tau

Recently, researchers have begun to make comparisons between the spread of neurodegenerative disease and prion disease, as studies suggest that misfolded protein templating, known as seeding, may underlie disease progression (Walker Lc 2013). Understanding how tau seeds pathological forms of the protein that propagate to different brain regions is critical to devising a solution to stop the spread of disease.

In amyloid proteins in which seeding has been well-established, such as prion proteins and Aβ, oligomers have been shown to be the most potent seeds (Silveira 2005, Langer, Eisele et al. 2011), working by way of oligomer-nucleated conformational induction (Serio, Cashikar et al. 2000, Lee, Culyba et al. 2011). Due to the increased interest in the toxicity of tau oligomers, evidence has emerged in support of the oligomer-nucleated conformational induction model as more studies have begun to explore the importance of tau oligomers in the initialization of tauopathies. Oligomer-nucleated conformational induction entails oligomers or conformational changes irreversibly stabilizing the highest energy protein states, known as the nucleus, allowing stable monomers to aggregate into oligomeric structures. Oligomers are driven to further elongation to form lower energy, stable filaments (Ruschak and Miranker 2009). As opposed to template-assisted growth, monomers are not incorporated directly into fibrils, but are instead entirely aggregated into oligomers prior to filament formation (Lasagna-Reeves, Castillo-Carranza et al. 2010). Tau dimerization increases the tendency to aggregate and can be induced by oxidation (Mandelkow, Biernat et al. 1993), which suggests that tau oligomerization may be an important step in the fibrillization pathway. The appearance of oligomeric species of other amyloid proteins has been observed on the

path to fibril formation (Ahmad, Uversky et al. 2005, Bernstein, Dupuis et al. 2009, Frare, Mossuto et al. 2009).

While the addition of reagents and mutations used to induce fibrillization has been integral to understanding how tau aggregation occurs, it does not explain how fibril formation may occur spontaneously in sporadic disease. The mechanism by which tau aggregation occurs physiologically has not yet been elucidated, however there have been some advances in the understanding of how certain steps in the process may occur. Release of tau from microtubules may occur following post-translational modifications, such as phosphorylation (Biernat, Gustke et al. 1993{Bramblett, 1993 #289, Bramblett, Goedert et al. 1993). Localization to anionic surfaces, alternative splicing, and post-translational modifications stabilizing aggregated conformations may all act as enhancers to increase speed of nucleation (Chirita, Congdon et al. 2005). Under physiological conditions, nucleating cofactors can induce tau aggregation in a similar fashion to agents used *in vitro*. There is evidence that polyanionic species, such as tubulin, RNA, and α-synuclein can increase the tendency of tau to aggregate (Littauer, Giveon et al. 1986, Kampers, Friedhoff et al. 1996{Littauer, 1986 #298, Giasson, Forman et al. 2003). The formation of disulfide bridges is critical for the initial creation of dimers from monomers, as well as intermolecular crosslinking of the microtubule binding domain independent of cysteine to continue oligomerization of three-repeat tau (Sahara, Maeda et al. 2007). Prior to monomer aggregation into oligomers, the free energy of solvation decreases, causing a shift in preference for peptide-solvent interactions towards peptide-peptide interactions, as water is evacuated due to poor interaction with the peptide backbone and sidechains. Water release increases entropy of the solvent, thereby balancing the loss in

conformational entropy caused by aggregation. The interaction of side chains with the backbone in the form of hydrogen bonding leads to the creation of β-sheet structure and aggregate stabilization. While oligomers form a similar structure to fibrils, they are not as ordered, which likely increases their toxicity (Matthes, Gapsys et al. 2012). Proteolytic processing by endogenous proteases has also been shown to create self-aggregating fragments, which nucleate and co-aggregate with full-length protein effectively enough for a small amount of fragment to seed PHFs (Wang, Biernat et al. 2007). Direct interactions between misfolded tau and native protein may be the underlying mechanism of seeding as experiments have shown tau protein-protein interactions occur when tau aggregates enter cells containing native tau (Kfoury, Holmes et al. 2012).

Tau oligomers—identified with the tau oligomer-specific antibody, T22, which does not recognize monomers or fibrils (Lasagna-Reeves, Castillo-Carranza et al. 2010)—that have been seeded with oligomers derived from brain tissue have been shown to be highly toxic (Lasagna-Reeves 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012). When tested with Bis-ANS, which recognizes exposed hydrophobic patches, oligomers had higher affinity than PHFs, which may underlie toxicity. The toxic effects of tau oligomers formed by seeding recombinant tau with oligomeric seeds, however, can be prevented when pre-treated with T22 (Lasagna-Reeves 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012).

Some tauopathies, such as PSP, only have one pathogenic species involved in disease progression (Ballatore, Lee et al. 2007). However, most tauopathies contain other amyloid proteins in addition to tau, such as Aβ in AD and α-synuclein in PD. In such diseases, cross-seeding of heterologous protein species is an additional mechanism

that is important for tau seeding (Busciglio, Lorenzo et al. 1995, Gotz, Chen et al. 2001, Ferrari, Hoerndli et al. 2003, Giasson, Forman et al. 2003, Lasagna-Reeves, Castillo-Carranza et al. 2010, Waxman and Giasson 2011, Ono, Takahashi et al. 2012).

Tau Oligomers Propagate from Affected Brain Regions to Unaffected Regions

Aβ has been shown to propagate from affected brain areas to unaffected areas in mice over-expressing Aβ precursor protein (APP) that have been injected with Aβ isolated from the brains of AD patients and AD transgenic mice (Walker, Callahan et al. 2002, Meyer-Luehmann, Coomaraswamy et al. 2006), suggesting that perhaps tau could spread in a similar fashion. A few years later, a similar mechanism was demonstrated for the propagation of tau. When tau extracted from P301S mice was injected into the brains of mice over-expressing wild-type human tau (ALZ17 mice) that do not form tau aggregates, tau pathology was observed to have spread from the injection site to neighboring brain regions (Clavaguera, Bolmont et al. 2009). Additionally, in transgenic mice that differentially express pathological tau in the entorhinal cortex, where tau pathology is first observed in AD, human tau has been shown to spread to both neighboring and synaptically connected neurons, which do not express human tau mRNA. Translocated human tau was able to seed mouse tau misfolding (de Calignon, Polydoro et al. 2012, Liu, Drouet et al. 2012). However, these studies did not specifically investigate which tau species specifically induced seeding and propagation of tau pathology and the usage of transgenic mouse models is not analogous to sporadic forms of AD. When wild-type mice were injected with both tau oligomers and PHFs isolated from AD brains, tau oligomers induced the spread of tau pathology from the injection site to neighboring brain regions and impaired memory, as measured by object recognition.

Conversely, mice injected with PHFs only exhibited tau pathology near the injection site and did not exhibit any memory impairments on the behavioral task, suggesting that tau oligomers, but not fibrillar tau, are capable of seeding and propagating pathology (Lasagna-Reeves 2012). Furthermore, similar results have been found using primary neurons. Neurons were exposed to low molecular weight aggregates—recognized by the tau oligomer-specific antibody, T22, and examined via electron microscopy for oligomeric characteristics—as well as to fibrils formed *in vitro*, filaments formed *in vivo* and monomers. Low molecular weight aggregrates and short fibrils exhibited uptake into the cell, but monomers and filaments were not internalized (Wu, Herman et al. 2013). Other studies have shown tau aggregate uptake using cell culture, but did not specifically identify the type of aggregates being internalized. Neural stem cells treated with tau monomers and aggregates formed using the tau microtubule binding repeat region induced to fibrillize with arachidonic acid, exhibited significantly more tau aggregate uptake than monomer uptake. Additionally, aggregates, but not monomers, induced seeding of endogenous tau misfolding (Frost, Jacks et al. 2009).

On the other hand, Guo and Lee hypothesized that seeding of pathological tau in cultured cells would be able to occur more quickly by seeding with pre-formed tau fibrils, thereby omitting the step where monomer must be converted to oligomer prior to fibril formation. Fibrillization of recombinant tau was induced with the addition of heparin and was verified using thioflavin T. Fibril-treated cells exhibited seeding and propagation of aggregates via endocytosis. However, following fibril confirmation with thioflavin T and prior to cell treatment, fibrils used in this study were sonicated (Guo and Lee 2011). Previous research investigating Aβ seeding found that sonication increased seeding

ability by fragmenting fibrils into smaller, soluble species (Langer, Eisele et al. 2011) and sonicated prions have also been shown to have more potent seeding potentials than unsonicated fibrils (Silveira 2005). Since it has been shown that both prion and Aβ oligomers, rather than fibrils, are the seeds for pathological protein templating (Serio, Cashikar et al. 2000, Lee, Culyba et al. 2011), it is likely that sonication partially converts insoluble fibrils into soluble oligomeric forms. Sonication of tau fibrils has also been shown to cause shearing of filaments, particularly those in PHF form (Frost, Ollesch et al. 2009). Therefore, it is likely that sonicated tau fibrils used to treat cells in the previous study (Guo and Lee 2011) also contained tau in oligomeric form, which may explain why seeding and propagation was successful.

Recently, Wu et al. studied propagation of tau in primary neurons using microfluidic chambers which allow somatodendritic compartments to be isolated from axonal compartments, enabling not only the analysis of tau uptake from the extracellular space into the cell, but also propagation within the neuron. They found that low molecular weight tau aggregates specifically recognized by tau oligomer-specific antibody, T22, propagate between isolated neuronal compartments both anterogradely and retrogradely (Wu, Herman et al. 2013). Importantly, tau is primarily found in the axons of healthy neurons (Dotti, Banker et al. 1987), though tau may also be found in the dendrite where it colocalizes with the src kinase, fyn (Lee, Newman et al. 1998, Ittner, Ke et al. 2010). In AD, however, misfolded and hyperphosphorylated tau accumulates in the axon, dendrites, and the cell body (Avila, Lucas et al. 2004), suggesting that intracellular transport may also be important for the spread of disease. In lamprey neurons expressing low levels of tau, tau was primarily localized to the axon and proximal dendrites, both

regions consistent with tau functioning as a microtubule associated protein. However, in neurons expressing high levels of tau, tau was found in distal dendrites and near the soma membrane, both areas lacking microtubules. High-expressing tau cells showed more degeneration and secretion of tau. Moreover, as tau can modulate activity of microtubule-associated motor proteins involved in dendritic transport, tau localized to the dendrite may have implications for its propagation (Lee 2012). Phosphorylated tau localized at the synapse in AD brain samples appears to correlate with ubiquitin proteasome system (UPS) dysfunction, suggesting that tau oligomer accumulation at the synapse impairs the UPS, which is a crucial player in the breakdown of tau. Accumulation of tau at the synapse may also suggest a mechanism for trans-synaptic tau propagation (Tai, Serrano-Pozo et al. 2012).

Phosphorylation clearly plays a role in the toxicity and localization of tau, however, its exact role in neurodegenerative disease is unknown and appears to be quite complicated. While hyperphosphorylated tau has been shown to have toxic results in the cell, increasing aggregation and abnormal tau localization (Pérez, Cuadros et al. 2000, Avila, Lucas et al. 2004), dephosphorylated tau can also have harmful effects. Phosphorylated tau released into the medium of cultured neuroblastoma cells through muscarinic receptor activation that is dephosphorylated by tissue-nonspecific alkaline phosphatase (TNAP) led to excitotoxicity, increasing calcium levels in nearby cells. Additionally, levels of TNAP are heightened in AD brains compared to control brains (Díaz-Hernández, Gómez-Ramos et al. 2010). Another study of primary cortical neurons also found that extracellular tau is largely dephosphorylated (Pooler 2013). Conversely, one study found that phosphorylation of tau increased its secretion from HeLa cells

(Plouffe, Mohamed et al. 2012). Inflammation and activation of microglia has been shown to increase tau phosphorylation as well as aggregation, but is complicated by the fact that the opposite effect is seen in Aβ (Li, Liu et al. 2003{Bhaskar, 2010 #295, Bhaskar, Konerth et al. 2010, Ghosh 2013, Nash, Lee et al. 2013). The localization of tau in the cytosol, cell membrane, and the nucleus also appears to be important for tau toxicity, and is mediated by phosphorylation. Oxidative stress and heat shock induce the dephosphorylation of cytosolic tau and its transport into the nucleus. Once relocated to the nucleus, tau appears to protect neuronal DNA from damage under cell stress (Sultan, Nesslany et al. 2011), which may be important in AD where DNA damage has been shown to occur (Coppede 2009). One possibility is that abnormal phosphorylation of tau in AD may prevent tau from being dephosphorylated and translocated to the nucleus to protect against DNA damage. The localization of tau to the cell membrane may also depend upon its dephosphorylation, as tau with lower levels of phosphorylation in its proline region was shown to be associated with the cell membrane, while phosphorylated tau was found in the cytoplasm (Arrasate, Pérez et al. 2000, Pooler, Usardi et al. 2012). However, interaction with membrane bound proteins, such as aforementioned fyn may stabilize association of phosphorylated tau with the membrane.

Mechanism of Tau Propagation

The entry of prion proteins, Aβ, and other amyloid proteins into the cell via different mechanisms has been well-established (Kanu, Imokawa et al. 2002, Yang, Dunlap et al. 2002, Magalhães, Baron et al. 2005, Ren 2009). One hypothesis for amyloid oligomer toxicity and entrance into cells is through protein interaction with the cell membrane. One model suggests that oligomers embed themselves into the cell membrane and form

pores. However, it appears as though the formation of pore-like annular protofibrils occurs through a separate pathway from fibril formation (Lasagna-Reeves, Glabe et al. 2011). An alternative model suggests that oligomers interact with lipid rafts in the bilayer, causing membrane thinning and increased membrane permeabilization, which may play a role in oligomer toxicity, allowing non-specific ion entrance, as well as leakage of cellular compartments. Several types of amyloid oligomers have been shown to increase membrane permeability, including Aβ, α-synuclein, and prion protein (Kayed, Sokolov et al. 2004, Demuro, Mina et al. 2005, Ren, Lauckner et al. 2009, Stockl 2013). Tau has been reported to interact with the lipid rafts in the cell membrane and undergo conformational changes leading to membrane stress (Flach, Hilbrich et al. 2012, Jones, Dubey et al. 2012, Kunze, Barre et al. 2012{Jones, 2012 #37, Mondragon-Rodriguez, Trillaud-Doppia et al. 2012). Additionally, permeabilization of the membrane could mediate internalization of oligomers into the cell.

There has also been evidence for endocytosis as a route of amyloid entry into the cell. Propagation of α-synuclein, prion protein, Sup35, and Aβ has been shown to be associated with the endosomal pathway (Narayanan, Bosl et al. 2003, Lee, Suk et al. 2008, Yu 2010). One study found that tau aggregates co-localize with dextran in neural stem cells, implying that entry into the cell occurs via macropinocytosis. However, the aggregate type was not specifically tested (Frost, Jacks et al. 2009). Aggregates identified specifically as tau oligomers colocalized with fluid-phase endocytosis marker, dextran, as well as with early endosomal marker, Rab5, and late endosomal/lysosomal marker, Lamp1. When endocytosis was inhibited with dynamin inhibitor, Dynasore, tau uptake was blocked, while inhibition of clathrin-mediated endocytosis with Pitstop2B did

not impact internalization (Wu, Herman et al. 2013). These studies together suggest a mechanism for tau propagation in which tau is internalized via pinocytosis and enters the endosomal pathway. Tau can move through the endosomal pathway to the lysosome where toxic species may be degraded or recycled back to the cell membrane, where they may be released to be internalized by adjacent neurons. More research is needed to determine how membrane-enclosed tau oligomers are released inside of the cell, though it appears likely that the majority are degraded in the lysosome, while those that avoid degradation may cause the endosomal membrane to burst and be released in the cytoplasm, where they can seed aggregation of healthy tau (Wu, Herman et al. 2013). While clathrin-mediated endocytosis did not appear to be involved in tau propagation, endocytosis inhibitors are often found to be non-specific (Ivanov 2008), and therefore, the possibility of other types of endocytosis in tau spread bears more study.

Receptor-mediated endocytosis could be another route of entry into the cell as amyloids have been reported to bind to cell surface receptors. Internalization of α-synuclein has been shown to be dependent upon receptor-mediated endocytosis, potentially through caveolin-mediated endocytosis (Lee, Suk et al. 2008). Additionally, Aβ binds to NMDA, α7 nicotinic acetylcholine, and APOE receptors, inducing receptor endocytosis (Wang, Lee et al. 2000, Nagele, D'Andrea et al. 2002, Bu, Cam et al. 2006, Kurup, Zhang et al. 2010, Kessels, Nabavi et al. 2013). Aβ oligomers also bind cellular prion protein (PrP^c), which is complexed with the Src tyrosine kinase, Fyn. This interaction has been shown to increase tau dysfunction and prevents native tau from binding to fyn (Larson, Sherman et al. 2012). Under normal conditions, tau binds to fyn in oligodendrocytes (Klein, Krämer et al. 2002) and in neurons, activating the

Ras/MAPK pathway (Pooler, Usardi et al. 2012). Mutations to the microtubule binding region in tau lead to decreases in oligodendrocyte process number and length and disease-related missense mutations increase tau association with Fyn (Bhaskar, Yen et al. 2005). Results indicate that the interaction between tau and fyn may be important for neurodegeneration, both through a loss in native tau interaction and through a gain in toxic tau function. Interaction with PrP^c complexed to fyn could also mediate tau entry into the cell as the PrP^c complex is associated with and endocytosed with caveolin (Peters, Mironov et al. 2003). Tau may also enter the cell through a direct interaction with fyn. Lee et al. used a lamprey ABC tauopathy model in which tau is expressed in specifically identified ABC neurons to investigate the spread of tau. They report that tau phosphorylated at Y18, the site most commonly phosphorylated by fyn kinase, is associated with vesicular organelles. Additionally, when tau is overexpressed and localizes to the dendrite, dendritic vesicle accumulation is observed. Phosphorylated tau is colocalized with vesicles which bear resemblance to endosomes, as well as to fyn. Fyn has also been shown to colocalize with exosomes, suggesting a possible mechanism for fyn-tau transport in which fyn-associated tau is endocytosed, transported from early endosomes to late endosomal compartments, and then transported out of the cell via exosomes (Lee 2012).

While one mechanism for tau oligomer release is through oligomer toxicity leading to cell death, causing the cell to lyse and release its contents (Simón 2012), studies show that this likely does not account for the majority of tau release. In primary neurons treated with tau oligomers, extracellular tau only increases once levels as high as 40% cell death are reached, which does not correspond to physiological levels of cell

death during the initial spread of neurodegenerative disease (Karch, Jeng et al. 2012). Additionally, treatment with tau oligomers in primary neurons does not lead to significant levels of apoptosis (Wu, Herman et al. 2013). There has however been some evidence for non-apoptotic membrane blebbing as a possible secondary mechanism for tau release (Fackler and Grosse 2008, Lee 2012).

Exocytosis has been implicated as a mechanism of amyloid spread as prion proteins and α-synuclein have been shown to be associated with exosomes in cell culture (Fevrier, Vilette et al. 2004, Emmanouilidou, Melachroinou et al. 2010). However, investigations of a similar mechanism for tau release have been unclear. Simón et al. found that when tau was overexpressed in kidney-derived cell lines, secreted tau was contained within membrane vesicles (Simón 2012, Simón, García-García et al. 2012). While tau secreted by neuroblastoma cells and tau in human CSF was found to be associated with exosomes in one study (Saman, Kim et al. 2012), another reported that tau was not detected in isolated exosomes from neuroblastoma cells (Karch, Jeng et al. 2012). However, these studies used cell models where tau was overexpressed. In an attempt to approach more similar conditions to those seen physiologically, researchers cultured primary cortical neurons containing endogenous tau and found that tau was released by a mechanism unrelated to cell death and was regulated by AMPA receptor activation. Inhibition of synaptic vesicle release decreased extracellular tau, while tau was not found to be associated with exosomes, indicating that release of tau through traditional synaptic exocytosis following AMPA receptor activation may be one mechanism of tau release (Pooler 2013). Another study found that cells constitutively release tau that is not contained within a membrane under conditions inhibiting cell death

(Chai, Dage et al. 2012). Therefore, more research is warranted to investigate the conditions under which tau is associated with exosomes and the specific tau conformations found in exosomes. Tunneling nanotubes—long, temporary channels that allow for long-distance transport between cells—have recently been discovered as a transport mechanism for prion protein (Gousset, Schiff et al. 2009). While they have not yet been studied directly in the context of tau, similarities between the spread of prions and tau suggest that tunneling nanotubes may be another potential mode of tau propagation meriting study.

Determining how neurodegenerative tauopathies initiate and propagate toxic species will be crucial to finding a treatment for these diseases. Recent evidence suggests that tau oligomers, not NFTs, are the toxic tau species mediating the initiation, seeding, and propagation of neurodegenerative tauopathies and are the best target for anti-tau therapeutics. The mechanism by which tau seeding occurs remains to be elucidated, but oligomer nucleated conformational induction, whereby native tau monomers are entirely converted to oligomers prior to aggregation into fibrils, appears to be a likely model. Tau oligomers can effectively enter cells, be transported intracellularly, and be released from cells to affect others. However, the mechanism by which propagation occurs is unclear. Tau likely enters the cell in one of two main ways, stressing the cell membrane or entering via endocytosis. Entrance through interaction with the membrane may occur through formation of pores or by interacting with lipid rafts causing membrane stress. Both macropinocytosis and receptor-mediated endocytosis have been implicated as possible mechanisms for tau entry. Tau secretion is likely not due simply to cell death, but may occur within exosomes, through synaptic vesicle release, or a non-traditional

secretion pathway in which tau is not enclosed in a membrane. The elucidation of the mechanisms addressed will lead to a better understanding of neurodegenerative disease and may reveal new targets for treatment.

Tau Therapeutic Strategies

Molecular Chaperones

Proteostasis is largely mediated by the presence of proteins known as molecular chaperones, which aid in the folding and stabilization of protein conformation. As chaperones are often upregulated in stress, they are frequently referred to as heat shock proteins (Hsps) (Hartl FU 2011). Hsps have been found to be present in increased levels in the brains of AD patients (Hamos 1991, Perez, Sugar et al. 1991, Sahara, Murayama et al. 2005). In a healthy system, misfolded, non-functional proteins will generally either be targeted for refolding by chaperones or will be targeted for degradation by the UPS. The Hsp90, Hsp70, and small Hsp families are three classes of chaperones commonly implicated in protein folding relevant to neurodegenerative tauopathies. Hsp70 proteins interact with hydrophobic patches in proteins early in the folding process (Rüdiger S 1997), while Hsp90 proteins act in the late stages of the folding pathway (Pearl and Prodromou 2006). The carboxyl terminus of the Hsp70-interacting protein (CHIP) has a dual role in proteostasis, both as a co-chaperone for Hsp70 and as an E3 ubiquitin ligase, ubiquitinating Hsp70-bound proteins and allowing it to control the balance between folding and degradation in protein quality control (Ballinger, Connell et al. 1999, Jiang, Ballinger et al. 2001). Tau has been shown to be a substrate for Hsp70, Hsp90, and CHIP (Petrucelli, Dickson et al. 2004).

A wealth of research has been completed investigating the function of Hsps in regulating tau, in the context of potential therapeutics. CHIP has been found to mediate the ubiquitination of tau, acting in opposition to Hsp70. While CHIP leads to increased levels of insoluble tau aggregates, Hsp70 reduces levels of hyperphosphorylated, insoluble tau (Petrucelli, Dickson et al. 2004). In addition, both Hsp70 and Hsp90 were found to decrease levels of tau accumulation, while increasing the association of the protein with microtubules in cells. Moreover, in mice expressing mutant tau protein and in AD brains, levels of the chaperones were inversely correlated with levels of insoluble tau aggregates (Dou, Netzer et al. 2003). However, the results from these studies inspire the question of whether the targeting of large fibrillar aggregates affects levels of toxic soluble tau intermediates.

Using P301L mutant tau mice crossed with CHIP-knockout mouse, Dickey et al. showed that while levels of soluble phosphorylated tau and Caspase-3 activity, which leads to increased tau aggregation, were both increased when CHIP ubiquitination function was lost, NFTs do not accumulate in these mice, suggesting that CHIP may function to target soluble, tau oligomeric species and that NFT accumulation may actually be neuroprotective (Dickey, Yue et al. 2006, Cook and Petrucelli 2013). CHIP has also been shown to lower levels of histone deacetylase 6 (HDAC6) which, when inhibited, increases the ubiquitination of Hsp90-bound proteins (Cook, Gendron et al. 2012). Direct inhibition of HDAC6 with tubastatin lowered levels of total tau, correlating with memory improvement in tau transgenic mice (Selenica, Benner et al. 2014). Hsp27 was also found to induce clearance of aberrant phosphorylated tau by a ubiquitin-independent mechanism (Shimura, Miura-Shimura et al. 2004). Hsp90 can

both stabilize tau binding to microtubules and lead to the degradation of tau. Therefore, while Hsp90 was initially considered to be beneficial for inhibiting tau aggregation, evidence that it may induce the selective degradation of non-toxic insoluble tau aggregates suggests it may lead to stabilization of toxic, disordered conformations of tau (Karagöz, Duarte et al. 2014). This hypothesis led to the testing of the therapeutic potential of inhibiting Hsp90 activity, allowing for the release of toxic tau species from the chaperone, thereby allowing improved targeting for degradation, as well as upregulating CHIP and Hsp27 activity.

As Hsp90 inhibitors are frequently too large for adequate penetration through the blood brain barrier (BBB), multiple low molecular weight inhibitors were derived for testing. In cellular models, inhibitors were shown to lower levels of tau phosphorylated at specific sites and in a misfolded conformation associated with toxicity (Dickey, Dunmore et al. 2006). Additionally, peripheral administration of Hsp90 inhibitor, EC102, in Human tau (Htau) mice (overexpress non-mutated human tau with no endogenous mouse tau expression) led to targeted degradation of phosphorylated tau (Dickey, Kamal et al. 2007). The most thoroughly investigated Hsp90 inhibitor, geldanamycin, was shown to decrease levels of insoluble tau through targeting to the proteasome (Petrucelli, Dickson et al. 2004, Opattova, Filipcik et al. 2013), as well inhibit tau phosphorylation through downregulation of extracellular-signal-related-kinases (ERK)(Dou, Yuan et al. 2005). However, clinical testing of geldanamycin was halted due to the finding that it induced liver toxicity (Kitson and Moody 2013), leading to the development of the analog, 17-AAG which was shown to lower levels of tau, as well as its phosphorylation *in vitro* (Dou, Yuan et al. 2005, Dickey, Koren et al. 2008). In tau-expressing drosophila

larvae, 17-AAG and another Hsp90 inhibitor, radicicol, which also increases Hsp70 and Hsp40 activity were shown to decrease levels of total tau. However, treatment had no effect on tau-dependent locomotor deficits (Sinadinos, Quraishe et al. 2013), though it has been shown to reverse cognitive deficit in mice by lowering soluble Aβ (Chen, Wang et al. 2014). Other Hsp90 inhibitors, novomiocin and KU-32, which was found to be capable crossing the BBB and inducing fewer toxic side effects, mediate neuroprotection against Aβ in cells, suggesting they may be effective against tau, but this has yet to be evaluated (Ansar, Burlison et al. 2007, Lu, Ansar et al. 2009, Li, Ma et al. 2012). Alternatively, FK506 binding protein, which complexes with Hsp90 to prevent the degradation of tau yields increased oligomerization of tau and accelerated onset of neurodegeneration in tau transgenic mice, suggesting that therapeutics inhibiting this interaction may be worth investigation (Blair, Nordhues et al. 2013).

A challenge in the therapeutic application of Hsp90 inhibitors is the wide range of effects these drugs may have as Hsp90 is ubiquitously expressed. Inhibiting Hsp90 leads to upregulation of the heat shock transcription factor, Hsf1, which in turn leads to increased expression of multiple different Hsps (Kim, Kang et al. 1999, Westerheide and Morimoto 2005) and could be beneficial (Jiang, Wang et al. 2013), but could also lead to unintended side effects that could act in opposition to the inhibition of Hsp90. Therefore, much of the upcoming research is focused on targeting cochaperones of Hsp90 in an attempt to yield more specific effects (Jinwal UK 2013). Withaferin A, which inhibits the co-chaperone of Hsp90, Cdc37, was shown to decrease tau aggregates in mice, as well as increase Hsp70 and Hsp27 levels (Sinadinos, Quraishe et al. 2013). Additionally, the co-chaperones FKBP51, BAG2, and CHIP may be effective targets, though

pharmacological agents targeting them have yet to be thoroughly tested (Petrucelli, Dickson et al. 2004, Carrettiero, Hernandez et al. 2009, Jinwal, Koren et al. 2010). Hsp90 and Hsp70 appear to compete for binding to tau (Thompson, Scaglione et al. 2012) and most Hsp90 inhibitors also modulate Hsp70, which may underlie beneficial effects. Therefore, the specific modulation of Hsp70 has also been investigated.

Similarly to Hsp90, the interaction of tau with Hsp70 is complex. Hsp70 has been shown to reduce tau fibril accumulation (Dou, Netzer et al. 2003), but it has also been found to preferentially bind to oligomeric tau (Voss, Combs et al. 2012), inhibiting fast axonal transport deficiencies in squid axons (Patterson, Ward et al. 2011). Therefore, the therapeutic potential of both Hsp70 inhibitors and activators has been investigated. In contrast to studies showing that decreases in tau correlated with increased activation of Hsp70 (Dou, Netzer et al. 2003, Petrucelli, Dickson et al. 2004, Sinadinos, Quraishe et al. 2013), Hsp70 inhibitors, YM-08 and methylene blue, were shown to decrease tau levels and toxicity both *in vitro* and *in vivo* (Congdon, Wu et al. 2012, Miyata, Rauch et al. 2012, Thompson, Scaglione et al. 2012, Miyata, Li et al. 2013). The systematic evaluation of both activators and inhibitors of Hsp70 shed some light on these apparently contradictory results. Activating Hsp70 may lead to an apparent reduction in levels of tau due to increased tau bound to the chaperone, while inhibiting Hsp70's ability to refold tau may lead to increased degradation of tau complexed to the protein, thereby suggesting that using a combination treatment in which Hsp70 is first increased in order to sequester tau, then its ATPase activity is inhibited in order to target tau-Hsp70 complexes for breakdown may be most effective (Jinwal, Miyata et al. 2009). Moreover, different members of the Hsp70 family appear to process tau differently, with Hsp72 targeting tau

for degradation and Hsc70 retaining tau and aiding in its native function stabilizing microtubules (Abisambra, Jinwal et al. 2013, Jinwal, Akoury et al. 2013). Tau clearance may also depend upon the DnaJ-binding domain of Hsp70 chaperones (Abisambra, Jinwal et al. 2012). Therefore, cochaperones that guide tau to be degraded, rather than sustained, may be useful.

While animal Hsps are useful in the degradation of amyloids, they are ineffective at rapid disaggregation. Hsp104 is a chaperone specific to bacteria, fungi, protozoa, chromista, and plants, and has been shown to be efficacious at disaggregating amyloid proteins, including toxic oligomers (Shorter and Lindquist 2004, Shorter and Lindquist 2006). Hsp104 has also been shown to be safe for use in animal models and is capable of disassembling α-synuclein oligomers and protecting dopaminergic neurons in a rat model of Parkinson's disease (Lo Bianco, Shorter et al. 2008). Hsp104 could theoretically be optimized for use against any amyloid protein, including tau (Vashist, Cushman et al. 2010) as it dissolves aggregates from different proteins using varied mechanisms (DeSantis, Leung et al. 2012). Therefore, it may be an exciting option for specific targeting of toxic tau in future therapeutics.

In addition to the clearance of tau by the UPS, autophagy and lysosomal pathways may also degrade toxic tau protein (Wang, Martinez-Vicente et al. 2009). Trehalose is a molecular chaperone present in invertebrates, and is an autophagy enhancer in vertebrates. The disaccharide was found to effectively decrease soluble tau levels through the upregulation of autophagy, as well as to directly inhibit aggregation, resulting in a decrease in toxicity (Krüger, Wang et al. 2012). The use of other non-Hsp chaperones has also been tested for tau clearance. Protein disulfide isomerases (PDIs) are

molecular chaperones specific to the endoplasmic reticulum that have been shown to be upregulated in neurodegenerative disease. PDIs induce proper protein folding through the breakage and formation of disulfide linkages (Andreu, Woehlbier et al. 2012). PDI has been shown to be colocalized with NFTs in AD brain (Honjo, Ito et al. 2010). Analysis of PDI effects *in vitro* show that it inhibits tau fibrillization (Xu, Liu et al. 2013). However, the problem remains that the effects of the chaperone on oligomerization are unknown. A molecular chaperone identified in drosophila, NMNAT, degraded phosphorylated tau oligomers through the UPS, as well as decreased apoptosis and reversed behavioral and morphological deficiencies in a drosophila tauopathy model (Ali, Ruan et al. 2012).

Naturally-Occurring Small molecules

Research of the health benefits of naturally occurring products has led to the discovery of multiple small molecules that affect tau, known as polyphenols. Polyphenols appear to interfere with the misfolding of multiple amyloid proteins through the disruption of Π-stacking and β-sheet formation important for fibrillization (Porat, Abramowitz et al. 2006). Polyphenols include a class of chemicals known as flavonoids, of which myricetin, curcumin, and (−)-epigallocatechin-3-gallate (EGCG) have been demonstrated to interact with tau, as well as with Hsps. Myricetin lowers tau levels through inhibition of Hsp70 (Jinwal, Miyata et al. 2009), while curcumin, a compound found in turmeric, may act independently of Hsp activity. Curcumin was found to inhibit Aβ aggregation, as well as decrease levels of tau hyperphosphorylation in cells and mice through the Akt/GSK-3β pathway and the upregulation of proteins shown to clear tangles, BAG2 and LAMP1 (Hoppe, Coradini et al. 2013, Patil, Tran et al. 2013, Huang, Tang et al. 2014).

Curcumin was shown to be capable of binding to fibrillar tau in brain sections from multiple tauopathies with comparable binding to common markers of NFTs, Thioflavin S, AT8, etc (Mohorko, Repovš et al. 2010). However, none of these studies specifically assessed the effects of curcumin on toxic tau oligomers. A recent study showed that Htau mice treated with curcumin exhibited improvement in cognitive tasks, synaptic dysfunction, and alterations in Hsps associated with a decrease in tau dimers, but not tau monomer or insoluble tau (Ma, Zuo et al. 2013), suggesting that curcumin's effects may be specific to toxic intermediates. Curcumin's ability to cross the BBB makes it a potential candidate for therapeutics (Purkayastha, Berliner et al. 2009). However it does have limitations, including low bioavailability and poor solubility in water. In a clinical trial of curcumin in AD patients, no evidence of improvement was seen (Ringman, Frautschy et al. 2012). In order to overcome these shortcomings, curcumin derivatives were created, showing increased efficacy in inhibiting tau fibrillization at a 10-1000x lower concentration than curcumin alone. The addition of a sugar group was able to increase solubility in water (Narlawar, Pickhardt et al. 2008, Dolai, Shi et al. 2011). ECGC, a flavonoid found in green tea, has also been found to decrease tau phosphorylation, cognitive deficit, and toxicity in APP transgenic mice (Rezai-Zadeh, Arendash et al. 2008).

In cellular and mouse models of tauopathies, resveratrol, a polyphenol found in grapes and red wine, reverses cognitive deficits and toxicity (Kim, Nguyen et al. 2007) through the activation of SIRT-1, a de-acetylase that inversely correlates with tau accumulation in disease (Julien, Tremblay et al. 2009). Resveratrol inhibits tau hyperphosphorylation and prevents memory deficits *in vivo* through the inhibition of

GSK3-β activity (Porquet, Casadesús et al. 2013, Varamini, Sikalidis et al. 2013, Du, Xie et al. 2014, Lee, Shin et al. 2014). Resveratrol has also been shown to be capable of converting other amyloid oligomers into non-toxic conformations and inducing their degradation (Marambaud, Zhao et al. 2005, Ladiwala, Lin et al. 2010). While resveratrol appears to be safe for use in human subjects, its bioavailability is very low (Almeida, Vaz-da-Silva et al. 2009).

Olive oil intake has been known to be associated with health benefits for many years, leading to the investigation of polyphenols found in olives, including oleuropein, oleocanthal, oleuropein aglycone, and hydroxytyrosol. All three were capable of inhibiting tau aggregation *in vitro* likely through the binding of aldehydes to the microtubule binding repeat region of tau responsible for aggregation, thereby locking tau in an unfolded monomeric state incompetent to fibrillization (Li, Sperry et al. 2009, Daccache, Lion et al. 2011). While oleuropein does not appear to be capable of crossing the BBB, its metabolite hydroxytyrosol efficiently enters the brain (Wu, Lin et al. 2009).

Aggregation Inhibitors

It has been suggested that aggregation inhibitors and molecular chaperones could work synergistically in therapeutics, creating a more effective treatment together than individually. Alone, aggregation inhibitors may lead to a dramatic increase in total monomeric tau, which could potentially mediate toxicity separately from aggregation by disrupting axonal transport and destabilizing microtubules (Stamer, Vogel et al. 2002). By allowing the two treatments to work synergistically, aggregation inhibitors could increase levels of low molecular weight tau species, which may bind more efficiently to chaperones, allowing them to be targeted for degradation (Blair LJ 2013).

Phenothiazines have been tested for therapeutic potential in a variety of medical fields. Investigations of the aggregation inhibitory properties of phenothiazine, methylene blue, have yielded conflicting results which may be due to its pleiotropic nature. Many studies have shown anti-aggregation effects of methylene blue through the stabilization of monomeric conformation by modulation of cysteine residues (Hattori, Sugino et al. 2008, Akoury, Pickhardt et al. 2013, Crowe, James et al. 2013). Methylene blue also decreases tau pathology *in vivo* when administered to P301L mice (Hosokawa, Arai et al. 2012) and attenuates toxicity in a *C. elegans* tauopathy model (Fatouros, Pir et al. 2012). However, behavioral deficits and tau pathology were unaffected in a P301L zebrafish model treated with methylene blue (van Bebber, Paquet et al. 2010). Notably, in contrast to many other aggregation inhibitors, when administered to multiple tauopathy mouse models methylene blue was shown to reduce levels of tau oligomers specifically, without affecting levels of NFTs (O'Leary, Li et al. 2010, Congdon, Wu et al. 2012, Spires-Jones, Friedman et al. 2014). The pleiotropic nature of methylene blue may explain conflicting results and calls into question whether benefits are solely due to inhibition of aggregation, or whether they may also be due to other factors, including antioxidant, energy metabolism, and inflammation benefits (Stack, Jainuddin et al. 2014). In addition, methylene blue has been shown to modulate levels of tau through increasing autophagy (Congdon, Wu et al. 2012) and proteasome activity (Medina, Caccamo et al. 2011). Methylene blue has progressed to Phase II clinical trials under the name *Rember*, showing evidence of cognitive improvement (T. 2008).

N-phenylamines inhibit tau aggregation, as well as dissolve aggregates in cellular models, leading to reduction in toxicity. As toxicity in this cellular model was found to

correlate best with levels of oligomeric tau, it is possible that N-phenylamines are capable are interfering with tau aggregation prior to the formation of fibrils, however this has not been directly tested (Khlistunova, Biernat et al. 2006, Pickhardt M 2007). Phenylthiazolylhydrazides attenuate tau fibrillization and disassemble aggregates *in vitro* and in cells, leading to increased cell viability (Pickhardt, Larbig et al. 2007, Park 2010).

Anthraquinones, including emodin, daunorubicin, adriamycin, and others are effective tau aggregation inhibitors, both preventing the formation of filaments and dissolving pre-formed filaments. The abundant ring structures in these compounds are thought to interfere with the formation of β-sheet structure. Furthermore, anthraquinone compounds do not interfere with native tau microtubule binding and protect against cytoxicity in cellular models of tauopathy (Pickhardt, Gazova et al. 2005).

Aminothienopyridazines (ATPZ), including a class of compounds derived based on anti-aggregatory properties, as well as ability to cross the BBB when orally administered in mice, have been proposed for treatment. ATPZ compounds effectively inhibit the formation of tau fibrils *in vitro* similarly to methylene blue, through cysteine oxidation and the inhibition of the formation of disulfide linkages, rendering tau incapable of fibrillization, as well as reverse the motor phenotype in a *C. elegans* tauopathy model (Ballatore, Brunden et al. 2010, Ballatore, Crowe et al. 2012, Fatouros, Pir et al. 2012, Crowe, James et al. 2013). However, analysis of levels of specific tau species treated with ATPZ compounds shows that they may be more effective at preventing fibrillization than oligomerization and may actually increase tau oligomer levels due to breakdown of larger aggregates (Crowe, Huang et al. 2009).

Rhodanines are a group of compounds that are well-tolerated in humans (Hotta, Akanuma et al. 2006) and may have clinically relevant anti-tau aggregation properties. Rhodanines were shown to both inhibit tau aggregation and disassemble tau filaments *in vitro*, without yielding any toxic effects in cells (Bulic, Pickhardt et al. 2007). The rhodanine, bb14, prevented tau pathology development in a hippocampal slice model, as well as protected against Ca^{+2} dyshomeostasis, dendritic spine loss, and cell death (Messing, Decker et al. 2013).

Porphyrins, including pthalocyanine tetrasulfonate (PcTS) have been studied for therapeutic and anti-aggregatory effects in prion diseases (Caughey, Raymond et al. 1998). PcTS was proposed as a viable therapeutic in AD, having been shown to inhibit Aβ oligomer formation (Park, Ahn et al. 2008), as well as block formation of tau aggregates and disassemble tau filaments (Taniguchi, Suzuki et al. 2005). However, research into the mechanism of PcTS interaction with tau fibrils showed that the compound stabilizes soluble oligomeric tau species, suggesting that the compound may actually increase toxicity (Akoury, Gajda et al. 2013).

Benzothiazole aniline (BTA) compounds were originally recognized for their protection against Aβ toxicity, but have recently been suggested for potential anti-tau aggregation efficacy (Song, DiBattista et al. 2014).

Exebryl-1, created by Proteotech, was approved to enter Phase I clinical trials and was found to inhibit the aggregation of both Aβ and the microtubule-binding repeat domain of tau, apparently through the binding to tau monomer and inhibition of the conversion to β-sheet structure from random coil (Snow, Cummings et al. 2009).

Intermolecular disulfide bonds formed by tau cysteine residues have repeatedly been shown to induce tau oligomerization (Schweers, Mandelkow et al. 1995, Mo, Zhu et al. 2009, Daebel, Chinnathambi et al. 2012, Walker, Ullman et al. 2012). Mutations inhibiting the formation of disulfide bridges prevented the formation of oligomers (Barghorn and Mandelkow 2002), suggesting that disulfide cross-linking may be a valid target for blocking tau aggregation. A rosamine derivative specific for tau cysteine thiol groups, TR-2, effectively inhibits the formation of disulfide bonds necessary to oligomer and fibril formation *in vitro* (Haque, Kim et al. 2014), suggesting that small molecules inhibiting disulfide cross-linking bear further study using cellular models and *in vivo* experiments for their efficacy as aggregation inhibitors. Targeting the paired edges of β sheets in fibrils has also been suggested as an anti-aggregation therapeutic approach (Zheng, Liu et al. 2011), though this would be ineffective in the disassembly of oligomers.

The use of aggregation inhibitors for the treatment of neurodegenerative tauopathies appears promising, but should be approached with caution. As the large majority of identified aggregation inhibitors stabilize soluble oligomeric tau species (Schafer, Cisek et al. 2013), when used alone, they could result in more toxicity and cognitive impairment. Therefore, more focus on the compounds that stabilize unfolded monomer or potentially large filamentous species at the expense of the toxic oligomers may be warranted. Adaptation of approaches used for Aβ to accelerate the fibrillization in order to decrease levels of tau oligomers may be an important alternative to approaches targeting the degradation of fibrils (Cheng, Scearce-Levie et al. 2007). Alternatively, approaches in which aggregation inhibitors are combined with molecular chaperones in

order to first dissolve filaments into small aggregates that are more effectively recognized by chaperones, and subsequently targeting these complexes for degradation, may be useful.

Tau Post-Translational Modification Modulators

Post-translational modifications have previously been implicated in the toxic processing of tau, leading to its aggregation. The most prevalent post-translational modification of tau is phosphorylation, which is an important modulator of tau's native function. Increased phosphorylation is known to decrease tau's affinity for microtubules (Martin, Latypova et al. 2011). However, phosphorylation has a complex relationship with tau. While many studies have found that aberrant phosphorylation is associated with tau aggregation, un-phosphorylated tau aggregates are also present and induce toxicity. Additionally, some researchers show that phosphorylation may not increase tau aggregation and may even inhibit aggregation (Schneider, Biernat et al. 1999, Khlistunova, Biernat et al. 2006). However, these conflicting results could be explained by the multitude of tau phosphorylation sites, which can yield different effects. Studies investigating tau phosphorylation are inconsistent in the analysis of various phosphorylation sites. Evidence suggests that phosphorylation at certain sites preceding residue 208 may actually inhibit aggregation, while phosphorylation at the C terminal region likely increases aggregation (Abraha, Ghoshal et al. 2000, Haase, Stieler et al. 2004, Liu, Li et al. 2007). Phosphorylation at certain sites is believed to induce tau toxicity both by inhibiting tau affinity for microtubules and by promoting its aggregation.

Tau kinases can be divided into three groups: proline-directed protein kinases (PDPK)—glycogen synthase kinase-3β (GSK3β), cyclin-dependent kinase-5 (CDK5), and mitogen-

activated protein kinases (MAPK); non-PDPK kinases—tau-tubulin kinase ½ (TTBK1/2), casein kinase 1a/1d/1e/2 (CK1a/1d/1e/2), dual-specificity tyrosine phosphorylation and regulated kinase 1A (DYRK1A), microtubule affinity-regulating kinases (MARKs), protein kinase cAMP-dependent/B/C/N (PKA, PKB/Akt, PKC, PKN) and Ca2+/Calmodulin-dependent protein kinase II (CaMKII); and tyrosine kinases (Sergeant, Bretteville et al. 2008). Inhibition of tau kinases is one of the best-studied therapeutic approaches in neurodegeneration. GSK3β inhibition is thought to be the most promising tau kinase target for therapeutics. Blocking GSK3β with either lithium or NP12 treatment leads to decreases in tau pathology, toxicity, cognitive deficits, and neuronal death in tau transgenic mouse models (Nakashima, Ishihara et al. 2005, Noble, Planel et al. 2005, Engel, Goñi-Oliver et al. 2006, Caccamo, Oddo et al. 2007, Serenó, Coma et al. 2009). Tau hyperphosphorylation could also result from an inhibition of tau phosphatases in disease. Levels of the most prevalent phosphatase, protein phosphatase-2A (PP2A), are diminished in AD (Liu, Grundke-Iqbal et al. 2005) and treatment with PP2A leads to a restoration of tau binding to microtubules (Wang, Grundke-Iqbal et al. 2007), making PP2A a viable therapeutic target to reduce toxic tau phosphorylation.

The DYRK1A kinase has been shown to directly phosphorylate tau as well as interact with phosphorylation activity of GSK3-β and phosphatase activity of calcineurin to increase tau hyperphosphorylation and NFT formation (Woods YL 2001, Liu, Liang et al. 2008, Jung, Park et al. 2011). DYRK1A is also involved in the regulation of tau alternative splicing, potentially leading to toxicity due to an imbalance in the ratio of 3R:4R tau (Shi, Zhang et al. 2008, Yin, Jin et al. 2012). These results, combined with the ability of DYRK1A to interact with regulators of Aβ cleavage (Ryoo, Cho et al. 2008,

Ryu, Park et al. 2010) and evidence of its elevation in neurodegenerative disease (Ferrer, Barrachina et al. 2005), have made its inhibition a promising therapeutic target for AD. However, due to the fact that DYRK1A plays a critical role in the modulation of multiple signaling pathways, its inhibition should be approached with caution and the goal of treatment must be to decrease levels of DYRK1A only to those seen in healthy controls (Smith, Medda et al. 2012). The most potent and bioavailable known inhibitor of DYRK1A is harmine, a natural compound derived from tropical plants. However, while harmine has been shown to enhance cognition in rodents, it results in hallucinogenic and psychoactive side effects that may make its clinical application difficult (Frost, Meechoovet et al. 2011, Mennenga, Gerson et al. 2015). The polyphenol, ECGC, has also been shown to inhibit DYRK1A activity and may result in fewer side effects (Bain J 2003, Guedj, Sébrié et al. 2009). Quinalizarin 3 is a potent inhibitor of protein kinase C which was also found to inhibit DYRK1A, but its low promiscuity score suggests it may not be useful for targeting DYRK1A (Cozza G 2009). Other potential natural DYRK1A inhibitors that may be useful as starting points for therapeutic agents include the peltogynoid flavonoids, benzocoumarin 5a, staurosporine, and analogue 8. Additionally, synthetic compounds, pyrrazolidine-3,5-diones, quinazolines, benzothiazoles, meriolins, meridianins, 3-(6-hydroxyindol-2-yl)-5-(phenyl)-pyridine and pyrazine analogues, chromenoindoles, and 3,6-diamino-1H-pyrazolo[3,4-b]pyridines, have all shown anti-DYRK1A activity (Smith, Medda et al. 2012).

The Src kinase Fyn is known to be associated with Alzheimer's disease and is well-documented to phosphorylate tau at tyrosine residues associated with disease (Bhaskar, Hobbs et al. 2010). Moreover, Aβ oligomers have been shown to bind cellular

prion protein, PrP^c complexed with Fyn, increasing tau dysfunction (Larson, Sherman et al. 2012). Tau has also been shown to bind to fyn in oligodendrocytes (Klein, Krämer et al. 2002) and in neurons (Pooler, Usardi et al. 2012). Disease-related missense mutations increase tau association with Fyn (Bhaskar, Yen et al. 2005). As the PrP^c complex is associated and endocytosed with caveolin (Peters, Mironov et al. 2003) and under membrane stress, such as that induced by tau oligomers, caveolin dissociates and flattens, leading to an increase in endocytosis following relaxation of stress (Sinha, Köster et al. 2011), Fyn could potentially be involved in uptake of toxic tau. Therefore, targeting of Fyn could alleviate toxicity in disease by multiple mechanisms. Currently, there are a few pharmacologic compounds targeting Fyn that have shown promising results pre-clinically and clinically. Tyrosine kinase inhibitor, masitinib, was effective in reducing cognitive deficits in AD patients in a Phase II clinical trial (Piette, Belmin et al. 2011). Inhibition of Src family kinases with saracatinib is another possibility. It is currently in Phase I clinical trials and has been shown to be well-tolerated in cancer research patients (Nygaard HB 2014).

The p75 neurotrophin receptor ($p75^{NTR}$) is abnormally modulated in AD, leading to the activation of tau kinases and Aβ toxicity. Small molecule inhibitors of $p75^{NTR}$, LM11A compounds, inhibit the induction of multiple different tau kinases *in vitro*, including GSK3β, cdk5, and c-Jun (Yang, Knowles et al. 2008), and lower tau phosphorylation, correlating with cognitive benefits in an AD mouse model (Nguyen, Shen et al.).

Disruptions to insulin signaling are associated with neurodegeneration and heightened phosphorylated tau levels in AD (Clodfelder-Miller, Zmijewska et al. 2006,

Jolivalt, Lee et al. 2008). This led to the testing of a Type 2 Diabetes drug, the glucagon-like peptide, liraglutide for the modulation of tau phosphorylation. Liraglutide successfully protects against cognitive deficit in an AD mouse model, accompanied by a decrease in levels of phosphorylated tau, likely through the inhibition of mitogen-activated protein kinases, ERK and JNK (Xiong, Zheng et al. 2013).

Phosphorylation of tau can also be inhibited by another post-translational modification, O-GlcNAcylation (Kang MJ 2013), a form of glycosylation involving the transfer of β-N-acetylglucosamine, which is a glucose metabolism sensor reduced in AD brains (Liu, Shi et al. 2009). A balance between O-GlcNAcylation and phosphorylation has been shown to exist for tau protein, impacting its cellular localization (Lefebvre, Ferreira et al. 2003). Inhibition of N-acetylglucosaminidase (NGase), the enzyme catalyzing the removal of β-N-acetylglucosamine, leads to decreased hyperphosphorylation of tau in mice (Yu, Zhang et al. 2012). O-GlcNAcylation also directly interacts with the aggregation of tau, inhibiting oligomerization and fibrillization, without affecting monomeric conformation, likely either by increasing the solubility of tau monomer or by destabilizing aggregated tau (Yuzwa, Cheung et al. 2014) and protects against cognitive deficit in an AD rat model (Diwu 2013). Inhibition of deglycosylation therefore may be a viable therapeutic target (Fischer 2008).

Another post-translational modification which appears to be important to tau pathology in neurodegenerative disease is glycation with glucose and ribose. Filamentous tau in AD brains has been found to be glycated (Ko, Ko et al. 1999). While glycation does not induce fibrillization, it does shift the equilibrium towards increased fibril formation (Necula and Kuret 2004, Kuhla, Haase et al. 2007). Glycated tau induces

toxic effects, including the production of reactive oxygen species and Aβ (Yan SD 1995). Ribosylation was shown to induce oligomerization and eventual formation of globular aggregates that are toxic to neuroblastoma cells (Chen, Wei et al. 2009). Therefore, the inhibition of glycation may effectively modulate toxic tau aggregation.

Recently, the possible acetylation sites of tau were mapped and the effects of acetylation on heparin-induced fibril formation were determined. Although acetylation of tau was found to inhibit aggregation, it is incapable of preventing fibrillization entirely (Kamah, Huvent et al. 2014). In addition to its role in the modulation of Hsps, HDAC6 is capable of modulating tau acetylation, which has also been found to inhibit tau phosphorylation and aggregation. Therefore, a BBB permeable HDAC6 inhibitor was tested *in vivo*, yielding promising results for the inhibition of tau aggregation (Cook, Carlomagno et al. 2014). Furthermore, HDAC6 inhibition rescues defects in microtubules due to tau toxicity in drosophila models through increases in microtubule acetylation (Xiong, Zhao et al. 2013). The non-specific HDAC inhibitors, crebinostat and BBB-permeable, sodium 4-phenylbutyrate, enhance memory and neuroplasticity (Ricobaraza A 2009, Fass, Reis et al. 2013).

However, similarly to phosphorylation, contradictory results for acetylated tau have been seen. This may be due to the heterogeneity of acetylated sites in tau samples used in different studies. Opposing results showed that inhibition of HDAC6 increased both acetylation and hyperphosphorylation of tau, leading to slower degradation of aggregates (Noack, Leyk et al. 2014). Moreover, acetylated tau is associated with tau aggregates in human tauopathy tissue, as well as tauopathy mouse models. Acetylation at specific lysine sites also impaired tau stabilization of microtubules and increased tau

fibrillization (Cohen TJ 2011). In another study, acetylation was shown to inhibit phosphorylated tau degradation (Min, Cho et al. 2010). Collectively, these results suggest that while acetylation does appear to be important in modulating tau in disease, the effect may differ drastically based on undetermined factors, including the residue. Further investigation is needed to determine the best way to target toxic acetylation. Additionally, it may be important to differentiate between effects of acetylation on fibrillization versus oligomerization, which may account for some discrepancies in results.

It has recently been reported that sumoylation (binding of small ubiquitin-like modifier proteins) is associated with tau in neurodegenerative disease (Takahashi, Ishida et al. 2008). While the relationship has not yet been solved, alterations in sumoylation and ubiquitination may affect degradation of tau aggregates and prove a viable therapeutic target in the future. Likewise, tau nitration, the addition of nitrogen dioxide to tyrosine, has been implicated in tau aggregation. Peroxynitrite, which induces nitration at specific tau residues, inhibits the ability of tau to stabilize microtubules and increases the formation of tau oligomers (Zhang, Xu et al. 2005, Reynolds, Lukas et al. 2006). Therefore, strategies inhibiting tau nitration may reduce tau toxicity in disease.

Tau Fragmentation

Proteolytic cleavage of tau was suggested as a potential mechanism for tau aggregation early on, as the minimal component required for the formation of tau filaments is the microtubule binding repeat fragment. However, the exact mechanism of tau cleavage is unknown (Kolarova, García-Sierra et al. 2012). Tau contains many caspase cleavage sites. Caspases are known to be upregulated in neurodegenerative disease (Rissman, Poon

et al. 2004), making them a likely candidate. The formation of tau fragments has been shown to preclude aggregation of tau more effectively than phosphorylation (Khlistunova, Biernat et al. 2006, Pickhardt, Larbig et al. 2007, Pickhardt M 2007). Errors in the lysosomal processing of tau can lead to the accumulation of tau fragments, which form toxic oligomers that interact with the lysosome and inhibit its ability to degrade aggregated tau (Wang, Martinez-Vicente et al. 2009). Therefore, therapeutics that aim to decrease fragmented tau, possibly through inhibition of caspases and other enzymes, as well as upregulate lysosomal processing, may be effective in neurodegenerative tauopathies.

Post-translational modifications clearly play an important role in the processing and aggregation of tau, though much more clarification is needed to understand exactly how to alter the process to reverse or prevent toxicity (Figure 1.2).

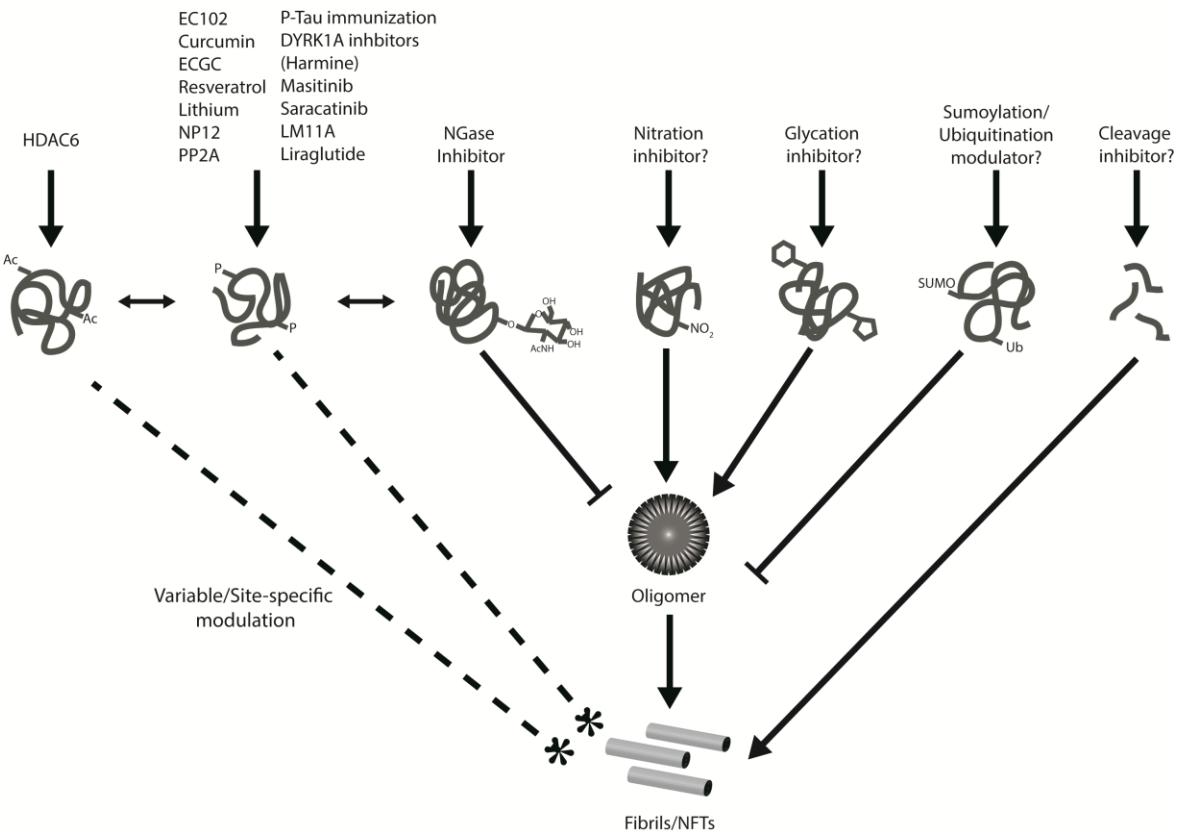

Figure 1.2 Summary of Post-translational Modification Effects on Tau and Potential Therapeutic Agents.

Immunotherapy

Immunotherapeutic approaches for the treatment of AD and other tauopathies are perhaps the most promising disease-modifying intervention (Castillo-Carranza DL 2013). Despite the fact that tau pathology correlates better with brain atrophy (Hampel, Burger et al. 2005), most studies have been concentrated on the removal of Aβ pathology from the brain. However, the lack of positive results obtained by targeting Aβ has stimulated a growing interest in tau pathology. Several lines of investigation highlight the importance of tau protein in the pathogenesis of the disease. One of the most convincing arguments comes from previous findings indicating that Aβ toxicity is mediated by tau pathology

(Rapoport, Dawson et al. 2002, SantaCruz, Lewis et al. 2005, Roberson, Scearce-Levie et al. 2007). This could explain why the removal of Aβ is insufficient to stop the disease progression. Therefore, immunotherapy against abnormal tau protein seems to be an important therapeutic alternative. In the past few years, preclinical studies have shed some light on the use of tau pathology as an immunotherapeutic target. However, most of the studies thus far have been directed for specific phosphorylated tau epitopes, rather than the oligomeric tau that has been demonstrated to be the most toxic species. Moreover, research into the native function of tau protein has raised concerns about targeting it as a whole. Removing tau entirely may affect neuronal function as tau stabilizes microtubules and is of great importance for neurite growth.

Immunotherapy may be divided into active immunization in which the antigen of interest in isolated and administered to activate the immune system to create its own antibodies to fight the toxin and passive immunization in which antibodies are developed and administered to patients to fight the antigen of interest. While both strategies hold merit, initial active immunotherapy against amyloid-β (Aβ) caused encephalitis, forcing clinical trials to be halted, suggesting that similar strategies for tau protein should be approached with caution and careful evaluation of potential autoimmune effects (Orgogozo, Gilman et al. 2003). Indeed, pre-clinical studies have found that active tau immunization induces dangerous levels of inflammation (Rosenmann, Grigoriadis et al. 2006, Rozenstein-Tsalkovich, Grigoriadis et al. 2013). Therefore, passive immunotherapy may be one of the most promising strategies for neurodegenerative tauopathy treatment. Numerous researchers are involved in this exciting field, determining what the most efficacious targets may be. Though many initial

immunotherapy studies for AD focused on Aβ, the tau field is now advancing rapidly, with many new targets being tested pre-clinically and a number of treatments commencing clinical trials (Castillo-Carranza DL 2013, Gerson, Castillo-Carranza et al. 2014, Pedersen and Sigurdsson 2015, Wisniewski and Goñi 2015).

Immunotherapy Targeting Non-Specific Tau

In spite of the fact that tau is known to have an important function in the healthy brain, many studies have investigated whether immunotherapy against all forms of tau—including those that are not specific to disease conditions—may be effective. This strategy is supported by evidence that genetic ablation of tau in Alzheimer's disease (AD) models can have neuroprotective effects (Rapoport, Dawson et al. 2002, Roberson, Scearce-Levie et al. 2007, Ittner, Ke et al. 2010, Vossel, Zhang et al. 2010, Shipton, Leitz et al. 2011) and that other proteins can compensate for the loss of tau allowing for normal development in tau knockout mice (Harada, Oguchi et al. 1994, Dawson, Ferreira et al. 2001, Tucker, Meyer et al. 2001, Fujio, Sato et al. 2007). These studies led to the testing of treatments that target all forms of tau protein, including the functional non-aggregated form. Passive immunotherapy against tau in the triple transgenic AD mouse model expressing mutated APP and tau led to cognitive benefits while lowering both total and hyperphosphorylated tau. However, lowering total tau did not decrease levels of toxic Aβ (Dai CL 2015). As promising as these results are, conflicting studies showing negative effects in AD models with the lowering of total tau have been seen (Dawson, Cantillana et al. 2010). An early attempt at targeting total tau by immunotherapy utilized an active immunization approach with full-length tau in wild-type mice. Unexpectedly, the injection resulted in the induction of NFT pathology and memory deficits. However,

treated mice also exhibited signs of inflammation (Rosenmann, Grigoriadis et al. 2006), which bears the question of whether the negative consequences were due to the targeting of total tau or the danger in using an active vaccination approach. The failure of initial clinical trials using an active immunization approach against Aβ for AD led to increased awareness of the potential problems with using amyloidogenic peptides as a vaccination approach (Gilman S 2005). Therefore, passive immunotherapy has been suggested to be a safer route of treatment. However, a recent study that targeted tau with an antibody recognizing all aggregation states of the protein in a mouse model lacking tau overexpression, but overexpressing APP also found reason to question strategies that diminish levels of all forms of tau. Researchers found that decreases in total tau did not provide any cognitive protection and actually led to sudden death in a large number of animals (Mably, Kanmert et al. 2015). The authors cautioned that prior studies showing efficacy of genetic and antibody-based alteration of total tau levels may only be reflective of tau transgenic mouse models, suggesting that in healthy animals, reducing levels of tau may actually induce toxicity. Indeed, further study of tau knockout mice revealed that once mice were aged, neurological deficits did appear (Ikegami, Harada et al. 2000, Ke, Suchowerska et al. 2012, Lei, Ayton et al. 2012) and increased levels of microtubule binding protein, MAP1A, that was thought to compensate for the lack of tau in development was not present in aged mice (Dawson, Ferreira et al. 2001). Similar to models of tauopathy, tau-ablated mice had memory deficits, as well as parkinsonism (Ikegami, Harada et al. 2000, Lei, Ayton et al. 2012), suggesting that further study of the role of tau natively may be needed prior to utilizing strategies aimed at decreasing the functional form of the protein.

Immunotherapy Targeting Phosphorylated Tau

One of the first identified factors associated with pathological tau in disease was hyperphosphorylation. Therefore, phosphorylated tau has been the main target of interest in most treatments devised against tau, including immunotherapeutics. Many initial studies targeting hyperphosphorylated tau with both direct and antibody-mediated immunotherapy have shown promising results. However, the relationship between tau toxicity and phosphorylation has been revealed to be much more complicated than initially believed and remarkably different effects seem to occur between different phosphorylation sites.

Initially, researchers used active immunization strategies to target phosphorylated tau, as had been previously completed for Aβ. Research into the staging of tau phosphorylation in AD has shown that phosphorylation at Ser396/404 occurs early in disease onset and may be present prior to the formation of tau fibrils (Mondragón-Rodríguez, Perry et al. 2014). Asuni et al, demonstrated that immunization using a peptide containing the phospho-tau Ser396/404 diminishes NFT load in a transgenic mouse expressing the P301L mutation (JNPL3)(Lewis, McGowan et al. 2000). Sensorimotor deficits associated with tau pathology were reduced after treatment. Evidently, the antibodies produced by active immunization were able to enter the cell and bind to NFTs. This was demonstrated by the labeling of purified antibodies injected into other animals (Asuni, Boutajangout et al. 2007). In a later study, the same research group carried out preventive immunization using the same immunogenic peptide in Htau/PS1 mice. This mouse model expresses human tau and the presenilin 1 (PS1) M146L mutation in a mouse tau knockout background. The early onset usually observed at two

months of age was prevented when mice were immunized in the early stages. As demonstrated previously, a reduction of NFTs was observed in the brains of these mice (Boutajangout, Quartermain et al. 2010). In a separate study targeting the same phospho-sites (Ser396/404) described above, a mouse model expressing the tau P301L mutation, pR5 mice, were immunized at 4, 8 and 18 months and were evaluated to determine the benefits of tau removal at different ages. Treatment at early ages slowed the progression of pathology, whereas progression was unaffected in the oldest group of mice, where NFTs appeared to be targeted. Importantly, an increase in activated astrocytes was observed only in the group of aged mice. This indicates astrocytes may be responsible for NFT clearance (Bi, Ittner et al. 2011).

A recent immunotherapeutic approach incorporated a tau peptide containing the phospho-sites (Ser396/404) into liposomes for the treatment of transgenic mice carrying tau P301L mutation. The long-term vaccination seemed to be effective at removing phospho-tau Ser396 in the soluble and insoluble brain fractions. However, the reduction of tau could not be confirmed in tissue since the immunostaining failed to detect a difference between groups (Theunis, Crespo-Biel et al. 2013). A similar approach was conducted in a double mutant mouse (K257T/P301S) that develops NFT-pathology. Mice were immunized with a combination of three peptides containing the phospho-tau epitopes Ser202/Thr205, Ser214/212 and Thr231. The robust decrease in NFT pathology observed in the cortex, hippocampus and brain stem, was accompanied by an increase in microglial cells, although no evidence of phagocytosis was found. It seems likely that NFT reduction is mediated by a lysosomal pathway (Boimel, Grigoriadis et al. 2010). A subsequent immunization against the pathological epitope, phospho-Ser422 was

conducted in THY-Tau22 mice. This mouse model exhibits neurofibrillary tangle-like inclusions in the hippocampus and abnormal tau phosphorylation. Some of the therapeutic effects found in treated mice included a decrease in insoluble phosphorylated tau, which correlated with cognitive improvement in treated mice. Importantly, following immunization, the levels of tau were high in the blood, suggesting that a peripheral sink mechanism was involved in antibody-mediated tau clearance (Troquier, Caillierez et al. 2012).

As was true with concerns for active immunization against total tau, increased inflammation in multiple studies against phosphorylated tau led to the question of whether the approach would be safe. Therefore, a study specifically addressing these safety concerns was completed. The phospho-tau epitopes Ser202/Thr205, Ser212/214 and Thr231 were administered in a NFT mouse model with E257T/P301S tau mutations and in wild-type mice. Repeated phospho-tau immunization induced encephalitogenicity in both transgenic and wild-type mice. Their findings suggest that repeated phospho-tau immunization using recombinant peptides can have safety issues (Rozenstein-Tsalkovich, Grigoriadis et al. 2013).

In this regard, passive immunization, the use of antibodies to treat tau pathology, seems to be superior to active immunization. Antibodies against phospho-tau (Ser396/404) have been used to immunize the JNPL3 mice described above. Repeated doses of the PHF1 (Ser396/404) antibody decreased functional impairment, while reducing levels of insoluble tau from the dentate gyrus of the hippocampus as well as the cortex (Boutajangout, Ingadottir et al. 2011). In a similar manner, immunization of two tauopathy mice, JNPL3 and mice expressing the P301S mutation, were immunized using

PHF1 and the conformational antibody, MC1, which detects early misfolded tau conformation. As demonstrated in previous immunizations, the treatment with both antibodies reduced a 64 kilodalton band from brain homogenate. The NFT burden was reduced in cortex/forebrain in the JNPL3 model, but showed variable results in the P301S model. However, the spinal cord in P301S mice showed a reduction in neurospheroids in the treated group, correlating with locomotor improvement (Chai, Wu et al. 2011).

Passive immunization against other phosphorylated sites associated with tau pathology has been completed. Researchers found decreased levels of pathological tau when transgenic mice were treated with a tau antibody specific to the pSer404 epitope (Ittner, Bertz et al. 2015). A recent study from d'Abramo et al. investigating passive immunotherapy in P301L mutated tau transgenic mice using antibodies against three different tau phosphorylation sites revealed that the efficacy of phosphorylated tau immunotherapy is drastically different depending on the epitope. Only one antibody (pSer202) out of the three tested lowered levels of both soluble and insoluble tau aggregates in the brain, however, evaluation of the hippocampus showed that tau aggregation was increased following treatment (d'Abramo, Acker et al. 2015). A previous study targeting phospho-tau Ser202/Thr205 with the AT8 antibody reduced levels of tau at the synapse in triple transgenic AD mice, though the treatment was unable to reduce pathological Aβ (Walls, Ager et al. 2014). Remarkably, the second of the three antibodies tested (pThr231) actually led to an increase in soluble phosphorylated tau aggregates in the hindbrain, while the third (pSer409) induced a massive rise in soluble tau aggregates in the cortex (d'Abramo, Acker et al. 2015). While it is difficult to make conclusions on the implications of each treatment to a phenotypic outcome without

behavioral analysis, it is clear from the results of this study that targeting phosphorylated tau by passive immunotherapy is not straightforward and may be a very difficult strategy. Potential explanations for the lack of success in the selected targets of the study can be derived from our knowledge of these phospho-epitopes.

Research on pSer202 has shown that it is associated with NFTs that form late in disease (Zheng-Fischhöfer, Biernat et al. 1998) and this phosphorylation site is critical early in life during normal mammalian development and the shift with age is poorly understood (Goedert, Jakes et al. 1993, Rösner, Rebhan et al. 1994). Moreover, pThr231 is also associated with tau fibril formation (Moszczynski, Gohar et al. 2015) and pSer409 tau is found in NFTs (Jicha, Weaver et al. 1999). A recent study investigated phosphorylation sites for tau implicated in early stages of disease based on mouse models expressing both 3R and 4R human tau isoforms. An antibody directed against a site identified to be of importance for early toxicity (Ser413) was used for passive immunotherapy and discovered to decrease levels of both hyperphosphorylated tau and tau oligomers. Moreover, treatment recovered levels of synaptic protein and decreased neuronal death (Umeda, Eguchi et al. 2015). The success of a strategy targeting phosphorylated tau specific to early toxicity that is capable of specifically reducing tau oligomers may inform future attempts to treat tauopathies with phospho-tau specific strategies. It is clear that all phosphorylation sites do not yield the same results and if a phosphorylated tau strategy is to be successful it will likely need to be dependent on a focus on early disease toxicity as well as those sites that lead to the formation of tau oligomers, rather than inert hyperphosphorylated tangles. Though there is evidence supporting a role for phosphorylation and other post-translational modifications in the

misfolding and aggregation process of tau, at this time the specific mechanism is unclear. Moreover, our previous work showed that both phosphorylated and unphosphorylated tau oligomers are present in AD brains (Lasagna-Reeves, Castillo-Carranza et al. 2012), suggesting that tau phosphorylation is likely not a requirement for tau oligomer formation. Therefore, a strategy that may be more effective for reducing levels of pathological tau is targeting the oligomers that form downstream of post-translational modifications that alter the folding and conformation of tau.

Immunotherapy Targeting Tau Aggregates

In a recent study, three antibodies with differing abilities to block tau aggregate seeding were used to immunize P301S mice. The chronic infusion of the antibodies, HJ9.3 (residues 306-320), HJ9.4 (residues 7-13) and HJ8.7 (residues 25-30), into brain lateral ventricle by osmotic pumps improved cognition in mice. All antibodies markedly reduced hyperphosphorylated, aggregated, and insoluble tau. These findings are expected, since these antibodies have affinity for monomeric and fibrillar recombinant tau and stain neurofibrillary tangles and neuropil threads in AD brain (Yanamandra, Kfoury et al. 2013).

Immunotherapy Targeting Tau Oligomers

Our group has led studies on the benefits of removing tau oligomers in multiple mouse models. A single dose of our anti-tau oligomer-specific monoclonal antibody (TOMA) reduced cognitive and motor deficits associated with tau pathology in mice overexpressing P301L-mutated tau. Importantly, the reduction of tau oligomers had no effect on NFT load. Our findings indicated that tau oligomers were cleared from the

extracellular space since the antibody did not enter the cell. It is possible that clearance of extracellular oligomers by antibodies may inhibit further uptake and thereby indirectly clear intracellular tau aggregates (Castillo-Carranza, Sengupta et al. 2014). In a second study from our group, we found that cognitive deficit induced by injection of brain-derived tau oligomers can be prevented in an Htau mouse model. A single injection, as well as multiple doses of TOMA, was demonstrated to be effective as a preventative therapy inhibiting oligomeric tau and preserving memory function (Castillo-Carranza, Gerson et al. 2014). Our findings confirmed that removal of tau oligomers, without lowering levels of hyperphosphorylated NFT load or functional monomeric tau, conferred benefits to memory. However, the previously mentioned study finding massively detrimental effects with targeting total tau in an APP overexpressing mouse highlighted the importance of testing tau immunotherapy in additional animal models, not only in tau transgenic mice (Mably, Kanmert et al. 2015). Moreover, even successful immunotherapeutic approaches against tau in AD mouse models were previously only able to reduce levels of tau, but were ineffective against Aβ (Walls, Ager et al. 2014, Dai CL 2015). Crucially, we found that targeting tau oligomers in Tg2576 mice overexpressing mutated APP resulted in protection against memory deficits without evidence of side effects or inflammation. Moreover, treatment with tau oligomer-specific antibody not only lowered levels of tau oligomers, but also led to a decrease in the toxic aggregate, Aβ*56 (Castillo-Carranza, Guerrero-Muñoz et al. 2015) which has been shown to be present early in AD and correlate with tau toxicity and may play a role in synaptic dysfunction (Sokolow, Henkins et al. 2012, Handoko, Grant et al. 2013, Lesne 2013).

The ability of a tau oligomer-specific antibody to mediate toxicity from Aβ as well suggests that passive immunotherapy against oligomeric tau may be able to reduce toxicity in mixed pathology diseases more effectively that targeting proteins that aggregate upstream of tau alone, such as Aβ and α-synuclein. We have previously shown that oligomers specifically, but not fibrils, are capable of cross-seeding between different amyloidogenic proteins and that tau and α-synuclein may co-aggregate in disease (Lasagna-Reeves, Castillo-Carranza et al. 2010, Guerrero-Muñoz, Castillo-Carranza et al. 2014, Sengupta, Guerrero-Muñoz et al. 2015). Therefore, depleting tau oligomers may disrupt amyloid structures formed from multiple proteins.

Mechanism of Tau Clearance by Immunotherapy

With the growing success of passive immunotherapeutic approaches to treat neurodegenerative disease, many are attempting to determine the mechanism by which antibodies can clear pathological tau protein from the brain. Currently, this process is unknown, however there have been studies that may shed light on the process. Classically, tau aggregates were thought of as solely occurring intracellularly, begging the question of how an antibody approach would be capable of influencing the toxic protein. One group has shown that tau antibodies are capable of entering the cell through the clathrin-mediated endocytic pathway. Moreover, the successful treatment of tau transgenic mice depended upon antibody entry into the cell (Asuni, Boutajangout et al. 2007, Krishnamurthy, Deng et al. 2011, Congdon, Gu et al. 2013). A second group recently showed that a phosphorylated tau-specific antibody also binds intracellular tau and targets its degradation in the lysosome (Collin, Bohrmann et al. 2014).

However, tau-specific antibodies utilized by our group and others have not shown a similar ability to enter the cell and target intracellular tau aggregates (d'Abramo, Acker et al. 2013, Castillo-Carranza, Sengupta et al. 2014, Yanamandra, Jiang et al. 2015). Therefore, it was hypothesized that extracellular tau aggregates important for seeding the spread of tau pathology throughout the brain may in fact be targeted by tau immunotherapy rather than the intracellular aggregates (Yanamandra, Kfoury et al. 2013). It has long been known that the tau staging of AD follows a stereotypic pattern of spreading (Braak H 1991), however only more recently have more in-depth studies supported the hypothesis that extracellular tau actually may be able to transfer between different brain regions. In a transgenic mouse model conditionally expressing tau in the entorhinal cortex, it was shown that tau was able to spread between synaptically connected areas in the brain (de Calignon, Polydoro et al. 2012, Liu, Drouet et al. 2012). Moreover, tau aggregates injected into the brain can be found to expand from the injection site to other brain regions (Ahmed, Cooper et al. 2014). Extracellular tau aggregates applied to cells in culture are able to be taken up into the cells, specifically in the oligomeric conformation (Wu, Herman et al. 2013) and stimulation of neuronal firing leads to an elevation in levels of extracellular tau (Pooler 2013). All of these data suggest that extracellular tau aggregates are an important component to the decline in neurodegenerative disease and a good target for treatment. Entry of antibodies into the cell versus targeting of extracellular tau may depend upon the antibody and the form of tau that is targeted. Those that target tau oligomers and soluble tau do not appear to enter the neuron and may be more likely to target the seeds responsible for the spread (Figure 1.3), while some antibodies targeting hyperphosphorylated tau associated with NFTs

have been found to act intracellularly. This may be partially explained by the efficiency of seeding and spreading of oligomers when compared to fibrils.

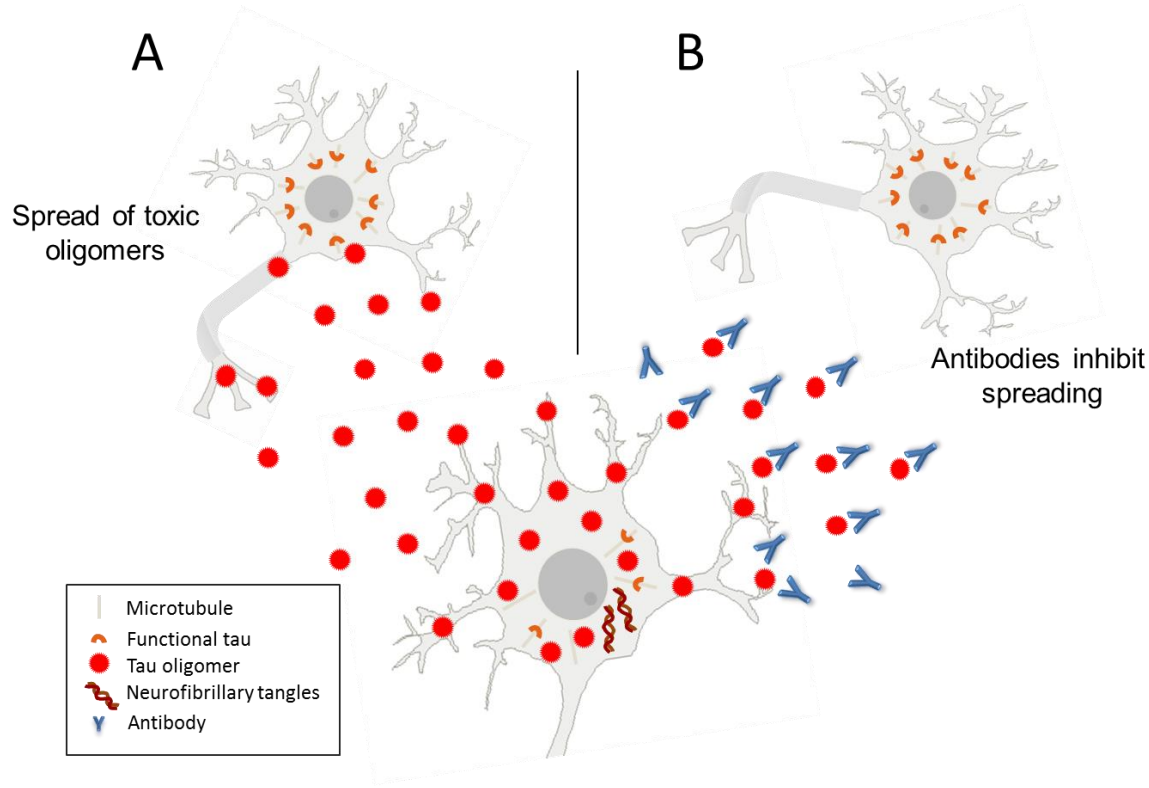

Figure 1.3 Mechanism of the prevention of tau oligomer spreading by antibodies.

A) Tau oligomers released from affected cells can propagate to neighboring or synaptically connected neurons, inducing the spread of disease. B) Passive immunization with tau oligomer-specific antibodies prevent the uptake of extracellular tau oligomers responsible for the spread of pathology.

In a study testing the process by which tau antibodies may inhibit the spread of aggregates from one cell to another, it was shown that a minority of antibodies are

capable of binding to tau in the extracellular space and preventing its uptake into the neuron (Funk, Mirbaha et al. 2015). This mechanism appears to be dependent on the specific form of tau that is targeted by the antibody however. Specifically, an antibody that affected the smaller, oligomeric aggregates was able to inhibit neuronal entry of extracellular tau oligomers. Therefore, there may be a size-dependent mechanism by which antibodies that target oligomers may act by stopping the spread of seeds (Castillo-Carranza, Gerson et al. 2014, Castillo-Carranza, Sengupta et al. 2014, Castillo-Carranza, Guerrero-Muñoz et al. 2015, Funk, Mirbaha et al. 2015). However, antibodies that target the large, stable, fibrillar tau aggregates do not appear to act by inhibiting the entry of fibrils to the cell. Rather, these antibodies activate microglial cells that promote the clearance of fibrillar tau (Funk, Mirbaha et al. 2015, Yanamandra, Jiang et al. 2015). Furthermore, using an *ex vivo* brain section culture model, degradation of hyperphosphorylated tau and NFTs by microglia is accentuated when sections are treated with conformation-specific antibody MC1 that is specific to misfolded tau (Luo 2015). These results could also partially explain the activation of inflammation in immunotherapy studies that target hyperphosphorylated tau and tau non-specifically (Rosenmann, Grigoriadis et al. 2006, Boimel, Grigoriadis et al. 2010, Bi, Ittner et al. 2011).

Another suggested mechanism for the removal of tau aggregates from the brain is the peripheral sink hypothesis, first proposed for the clearance of Aβ, whereby a dynamic interplay between the central nervous system and the periphery leads to the removal of aggregates from the brain to the blood (DeMattos, Bales et al. 2001). We have previously shown that following administration of tau oligomer-specific antibodies in tau transgenic

mice, levels of tau oligomers are lowered in the brain but increase in the serum (Castillo-Carranza, Sengupta et al. 2014). Other groups have also found elevated levels of antibody-bound tau aggregates in the plasma following tau passive immunotherapy treatment (Yanamandra, Jiang et al. 2015). Further, a recent study aimed at optimizing the measurement of tau in the serum following immunotherapy found that after treatment with multiple different tau antibodies, the level of tau protein in the serum was elevated. However, the experiment did not show any relationship between levels of tau in the brain. Therefore the authors caution that the process leading to elevated levels of tau in the serum following tau immunotherapy may be more complex than the initial process proposed by the peripheral sink hypothesis and further study is required to come to a better understanding of the mechanism (d'Abramo, Acker et al.).

Other Potential Strategies

Targeting tau loss of function, in addition to the toxic gain of function, through the use of microtubule stabilizers may be beneficial for therapeutics as neurodegeneration is likely partially dependent upon aberrant axonal transport (Brunden, Trojanowski et al. 2014). Microtubule stabilizers have previously been used clinically for cancer treatment. Drugs that stabilize microtubules have shown benefits for toxicity, cognition, synaptic function, and tau pathology in cells and in multiple tauopathy animal models (Vulih-Shultzman, Pinhasov et al. 2007, Matsuoka, Jouroukhin et al. 2008, Shiryaev, Jouroukhin et al. 2009, Brunden, Zhang et al. 2010, Shemesh and Spira 2011, Barten, Fanara et al. 2012, Brunden KR 2012, Das and Miller 2012, Zhang, Carroll et al. 2012, Quraishe S 2013, Erez, Shemesh et al. 2014). These results suggest that the stabilization of microtubules may be a beneficial route of treatment. However, microtubule stabilizers frequently have

undesired side effects. Additionally, the majority of mutations inducing FTD affect tau splicing, leading to a higher ratio of 4R:3R tau and suggesting that therapeutics targeting tau alternative splicing may also be effective (Wolfe 2014).

Thus far, techniques that have been studied for the treatment of tauopathies include molecular chaperones, small molecules and aggregation inhibitors, post-translational modification modulators, immunotherapy, microtubule stabilizers, and alternative splicing modulators (Figure 1.4).

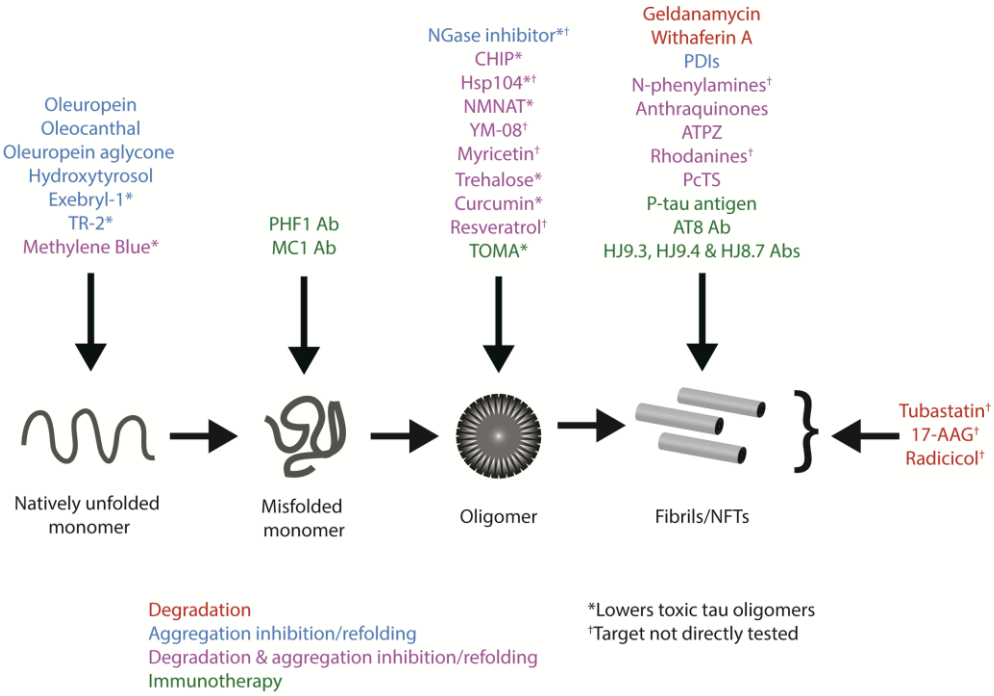

Figure 1.4 Summary of potential tauopathy therapeutics which have been tested and their hypothesized modes of action.

Passive immunotherapy studies appear to be very promising in terms of safety when compared to active immunization and in efficacy, particularly conformation-specific antibodies for misfolded tau and tau oligomers. The most efficacious treatment will

likely result from the combination of different effective therapies, possibly through the combination of different techniques for targeting tau, as well as targets for other amyloid proteins involved in diseases with the presentation of tau as a secondary amyloidosis. Information included in this chapter has previously been published and may be referred for further information (Gerson and Kayed 2013, Gerson, Castillo-Carranza et al. 2014).

CHAPTER 2

CHARACTERIZATION OF TAU OLIGOMERIC SEEDS IN PROGRESSIVE SUPRANUCLEAR PALSY

Introduction

Progressive supranuclear palsy (PSP) is a common parkinsonian neurodegenerative tauopathy which is characterized by the development of dementia, changes to personality, visual and speech deficiencies, and gait alteration (Steele 1964). It affects about 6.5 in 100,000 people (Nath, Ben-Shlomo et al. 2001). Pathologically, PSP is primarily defined by the deposition of tau into neurofibrillary tangles (NFT). Tau in its native form has important functions for microtubule stabilization and neurite growth. *Tau* undergoes alternative splicing in exons 2, 3, and 10 which results in six different isoforms of tau protein in the adult central nervous system (Goedert, Spillantini et al. 1989). Products of splicing on exon 10 are the most prone to mutations and result in three isoforms with three microtubule binding repeats (3R tau) and three isoforms with four microtubule binding repeats (4R tau). As the sequence corresponding to exon 10 appears to be of great importance in increasing the affinity of tau for microtubules (Panda 1995), differences between 3R and 4R tau may have implications for microtubule dynamics, as well as for localization of tau to the microtubules. This is of great importance because tau with low affinity for microtubules may be released into the cytosol, where it is free to form aggregates. It is unknown whether the seeding potential of oligomers from 4R tau differs from that of 3R tau and whether this may underlie some of the differences between various tauopathies specific to the number of microtubule-binding repeats.

PSP, unlike tauopathies such as Alzheimer's disease (AD) that form aggregates from both tau forms, has a shifted ratio of 4R:3R tau (Ingelsson, Ramasamy et al. 2007) and specifically forms NFTs from 4R tau (Sergeant, Wattez et al. 1999). Similarly, corticobasal degeneration (CBD) also exhibits aggregates from 4R tau alone (Sergeant, Wattez et al. 1999). However, tau isolated from CBD and PSP display different fragmentation due to variation in cleavage at the amino terminal, suggesting that the two diseases have different mechanisms of proteolytic processing for tau aggregates (Arai 2001, Arai, Ikeda et al. 2004). Additionally, though most of the phosphorylation sites of misfolded tau in PSP are similar to the other tauopathies, there are some differences (Delacourte, Sergeant et al. 1998, Arai 2003). These differences may underlie the unique pathological features and spatial organization of tau aggregation between each disease, which likely lead to differences in functional outcome. The two main pathological hallmarks of PSP include globose-type NFTs and tuft-shaped astrocytes, while other neurodegenerative tauopathies display different histological tau features (Steele 1964, Hauw 1990, Yamada, McGeer et al. 1992). The presence of tau aggregation in glial cells is a prominent feature in PSP. In addition to the presence of tufted astrocytes, oligodendrocytes may be affected in PSP as well. Argophylic threads and coiled bodies comprised of 4R tau have been found in oligodendrocytes in PSP cases (Arima 2006). Tau pathology is also located in different regions in PSP than in other diseases. NFTs in PSP are seen in the brainstem, basal ganglia, and the prefrontal and precentral cortex and hippocampus (Hauw 1990, Hof 1992). Therefore, while tauopathies do share many common features, it is important to study the mechanism of each individually.

While NFTs are the main histological hallmark in PSP, tau, like other disease-associated amyloids, can form oligomers which then go on to form fibrils. Recent studies from our laboratory and others have shown that tau oligomers, not NFTs, are the most toxic species *in vitro* and *in vivo* and may seed the pathological spread of tau (Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012, Gerson and Kayed 2013). Tau oligomers have been shown to be present in the brains of patients with Alzheimer's disease before NFTs can be detected (Maeda, Sahara et al. 2006, Maeda, Sahara et al. 2007, Patterson, Remmers et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012), correlating with dysfunction of the ubiquitin proteasome system and mitochondria (Kopeikina, Carlson et al. 2011, Tai, Serrano-Pozo et al. 2012). Moreover, cognitive and motor deficits in animal models of tauopathies correspond to levels of tau oligomers, but not to levels of NFTs (SantaCruz, Lewis et al. 2005, Berger, Roder et al. 2007, Yoshiyama, Higuchi et al. 2007, Polydoro, Acker et al. 2009, Lasagna-Reeves, Castillo-Carranza et al. 2011, Sahara, DeTure et al. 2013). However, the role of oligomeric tau has never been investigated in PSP, though the importance of oligomeric tau in the spread of pathology in PSP has been suggested (Golbe 2014). In this study, we characterized tau oligomers for the first time in human PSP brain samples and showed the potential for them to seed the oligomerization of both 3R and 4R tau, implicating oligomeric tau as an important component of PSP disease progression.

Methods

Preparation of Brain Homogenate

Frozen brain tissue from the pons was attained from subjects with PSP and age-matched control subjects from the Institute for Brain Aging and Dementia (University of California–Irvine, Irvine, California, USA) and the Brain Resource Center at Johns Hopkins. All postmortem brain tissue used were randomized. PSP tissue cases were collected with patient consent and handled under protocols approved by the Johns Hopkins Institutional Review Board. PSP samples were examined at the Division of Neuropathology at John Hopkins University. Brains were homogenized in PBS with a protease inhibitor cocktail (catalog no. 11836145001; Roche Applied Science, Indianapolis, IN, USA), using a 1:3 dilution of brain: PBS (w/v). Samples were centrifuged at 10000 rpm for 10 min at 4°C. Supernatants were aliquoted, snap-frozen, and stored at -80°C until use.

Immunofluorescence

Sections used for fluorescent immunohistochemistry were deparaffinized, rehydrated, and blocked in normal goat serum for 1 hr and incubated overnight with anti-tau oligomer-specific polyclonal antibody, T22 (1:300). The next day, sections were washed three times for 10 min in PBS and incubated with goat anti-rabbit IgG Alexa-568 (1:500; Invitrogen) for 1 hr. Sections were again washed three times for 10 minutes in PBS and were either incubated overnight with Tau-5 (1:300) for total tau, AT8 (1:100) for Ser202/Thr205 phosphorylated tau, or PHF13 (1:250) for Ser396 phosphorylated tau. The following day, sections were washed in PBS three times for 10 minutes, then incubated with donkey anti-mouse IgG Alexa-Fluor 488 (1:500; Invitrogen). Sections were washed and incubated in DAPI (Invitrogen), then mounted using Fluoromount G (Southern Biotech) mounting medium. Sections were imaged using a Zeiss LSM 510

Meta confocal system. Six images were taken from each sample and cells were randomly selected from each image for quantification using Image-J. The total level of fluorescence was measured for each cell, as well as the level of background from three different regions around the cell without fluorescence. In order to correct the level of fluorescence for background and cell size, the background multiplied by the area of the cell was subtracted from the total fluorescence. The corrected cell fluorescence was analyzed via One-way Analysis of Variance (ANOVA).

Western Blot

Pre-cast NuPAGE 4-12% Bis-Tris Gels for SDS-PAGE (Invitrogen) were loaded with 20-25 μg of protein for each sample per well, run under reducing conditions, and then transferred to nitrocellulose membranes. Membranes were then blocked overnight at 4°C with 10% nonfat dried milk. The next day membranes were incubated with T22 (1:250) for tau oligomers, Tau-5 (1:1000) for total tau, and GAPDH (1:1000; Sigma) as a loading control, diluted in 5% nonfat dried milk for 1 hr at room temperature. Tau-5 and GAPDH immunoreactivity were detected with horseradish peroxidase-conjugated IgG anti-mouse secondary antibody (1:3000, GE Healthcare) and T22 was detected with horseradish peroxidase-conjugated IgG anti-rabbit secondary antibody (1:3000, GE Healthcare). For signal detection, ECL plus (GE Healthcare) was used. Densitometry of each band was quantified and normalized with GAPDH using Image-J and analyzed by one-way ANOVA.

ELISA

For ELISA analysis, 96-well plates were coated with 15 µl of samples (PBS soluble fractions of brains) using 0.05 M sodium bicarbonate (pH 9.6) as the coating buffer and incubated overnight at 4°C. Plates were washed once with TBST (0.01% Tween 20), then blocked for 2 hrs at RT with 10% non-fat milk. Plates were then washed once with TBST. T22 (1:250) or Tau-5 (1:1000) diluted in 5% nonfat milk was added and allowed to react for 1 hr at RT. Plates were washed three times with TBST. T22 and Tau-5 immunoreactivity was detected using 100 µl of HRP-conjugated anti-rabbit IgG (GE Healthcare) or 100 µl of HRP-conjugated anti-mouse IgG (GE Healthcare) respectively diluted 1:3000 in 5% nonfat milk and incubated for 1 hr at RT. Lastly, plates were washed three times with TBST and incubated with 100ul of 3,3,5,5-tetramethylbenzidine (TMB-1 component substrate, from Dako) for 1 hr in the dark. To stop the reaction, 100 µl 2M HCl was applied and plates were read at 450 nm in a Polar Star Omega plate reader (BMG Labtech). Each sample was measured in triplicate and results were analyzed by student's t-test.

Preparation of PSP Brain-Derived Tau Oligomers (BDTO)

The immunoprecipitation of tau oligomers from PSP pons was completed as previously described (Lasagna-Reeves, Glabe et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012). Thirty µL of tosyl-activated magnetic Dynabeads (Dynal Biotech, Lafayette Hill, PA) were coated with 20 µg of anti-tau oligomer-specific polyclonal antibody, T22 (1.0 mg/ml) diluted in 50 µL of 0.1 M borate, pH 9.5, keeping end concentration of beads at 20 mg/mL overnight at 37°C. Beads were washed (0.2 M Tris-HCl, 0.1% bovine serum albumin, pH 8.5) and then incubated with 100 µl of PSP brain homogenate (PBS soluble fraction) with rotation at room temperature for 1 hr. Beads were washed three times with

PBS and eluted using 0.1 M glycine, pH 2.8. The pH was adjusted using 1 M Tris-HCl pH 8.0 and then fractions were centrifuged in a microcon centrifugal filter device with a molecular weight cut-off of 25 kDa (Millipore, Cat # 42415) at 14000 g for 25 min at 4°C. Oligomers were re-suspended in sterile PBS. Protein concentration was measured using the bicinchoninic acid protein assay (Pierce). The samples were then centrifuged again in a microcon centrifugal filter device with a cut-off of 25 kDa at 14000 g for 25 min at 4°C. Oligomers were characterized by various methods including size-exclusion chromatography (SEC) and atomic force microscopy (AFM) as previously described (Lasagna-Reeves, Castillo-Carranza et al. 2010, Lasagna-Reeves, Castillo-Carranza et al. 2012) and stored at -80°C. Oligomers were re-suspended in PBS in order to obtain the desired concentration (0.18-1.2 mg/ml) and kept at 4°C for 15-30 min, then at room temperature for 10 min before use. Oligomers were characterized as previously described (Lasagna-Reeves, Castillo-Carranza et al. 2010).

Seeding Assay

3R and 4R tau monomer were obtained by dissolving lyophilized pellets of recombinant 3R and 4R tau at 0.3 mg/mL concentration in phosphate-buffered saline (PBS) (Lasagna-Reeves, Castillo-Carranza et al. 2010, Lasagna-Reeves, Castillo-Carranza et al. 2011) and seeded with PSP brain-derived tau oligomers isolated as described (Lasagna-Reeves, Castillo-Carranza et al. 2012). BDTO seeds were added at 1:100 (w/w) to 2.5 µM solutions of monomeric 3R and 4R tau in PBS (in duplicates) with gentle agitation at room temperature. Aliquots were taken at each time point and immediately added to ELISA plate wells containing coating buffer (0.05M sodium bicarbonate buffer, pH 9.6,

0.02% sodium azide). Other aliquots were added to mica sheets for AFM analysis. Samples were analyzed by AFM and ELISA using T22 and Tau-5.

Results

Brain Sections Present with Tau Oligomers and Classical PSP Histological Hallmarks

Sections of pons from PSP patients were labeled with T22, which is specific for tau oligomers, and with PHF13 which recognizes phosphorylated tau (Figure 2.1a-i). T22 was partially colocalized with PHF13 (Fig 2.1c,f,i), signifying the presence of both phosphorylated and unphosphorylated tau oligomers. Both extracellular and intracellular tau oligomers were recognized by T22. Tau accumulation resembling tufted astrocytes was observed to be co-labeled by T22 and PHF13 (Fig 2.1d-f). Globose-shaped NFTs comprised of phosphorylated tau and tau oligomers were also present (Fig 2.1g-i).

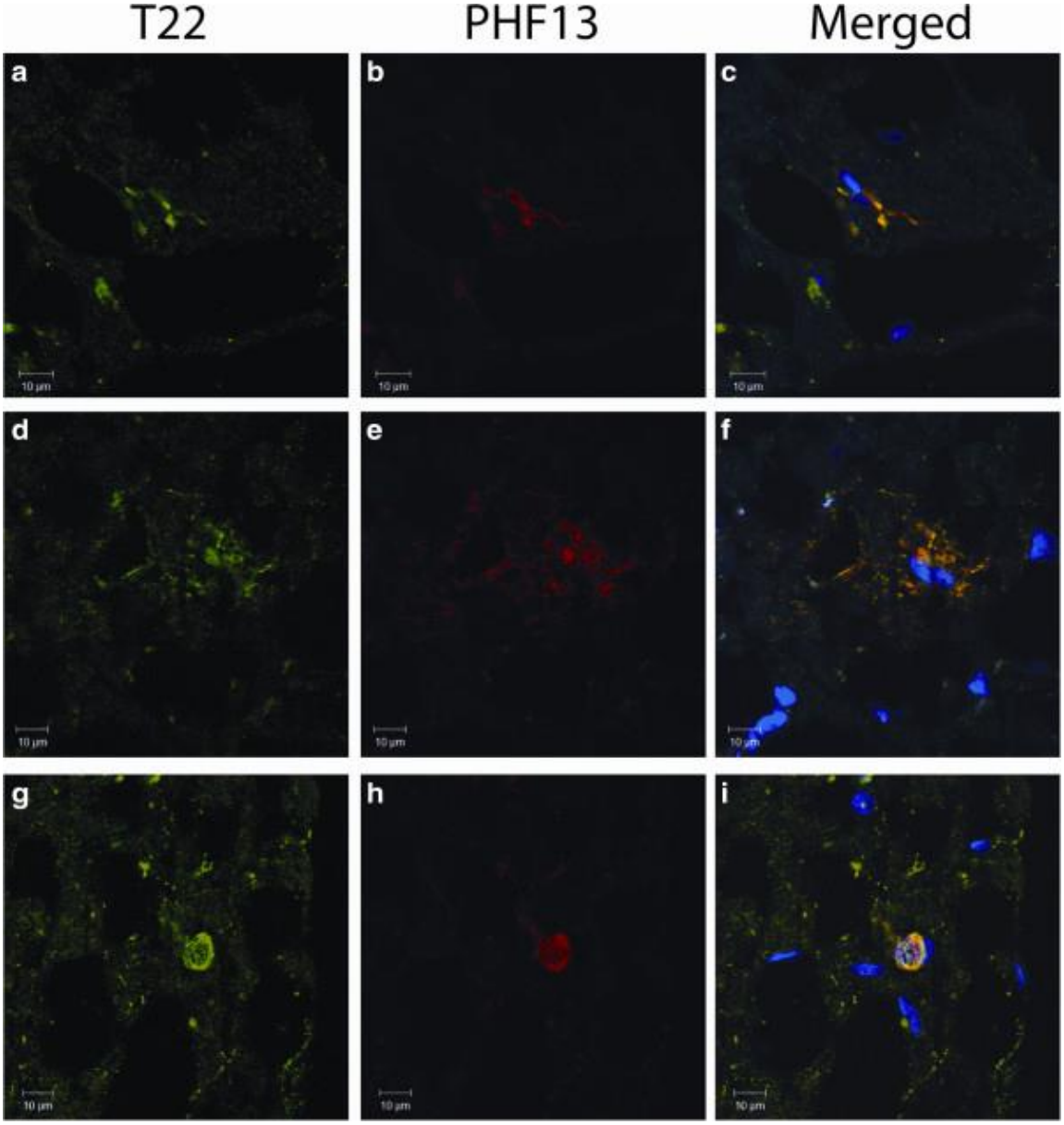

Figure 2.1 PSP brain sections labeled with T22 and PHF13 display characteristic PSP hallmarks. PSP brain sections immunofluorescently labeled with T22 (red) and PHF13 (green). Intracellular tau oligomers detected with T22 partially colocalize with PHF13 (a-c). Oligomers resembling tufted astrocytes or extracellular deposits were also detected to partially colocalize with phosphorylated tau recognized by PHF13 (d-f). Characteristic globose-type NFTs were detected by T22 and PHF13 (g-i).

Tau Oligomer and NFT Pathology is Present in PSP Brains but Not in Age-Matched Controls

Pons sections from PSP patients and age-matched controls were labeled with T22 and Tau-5 for total tau. Control brains exhibited small punctate tau staining (Figure 2.2a-c), while PSP brains displayed an increased amount of both tau oligomers and total tau, as well as the presence of large, globose NFTs surrounding many of the cells (Figure 2.2d-i). T22 showed some overlap with Tau-5, as well as some distinct oligomeric tau foci.

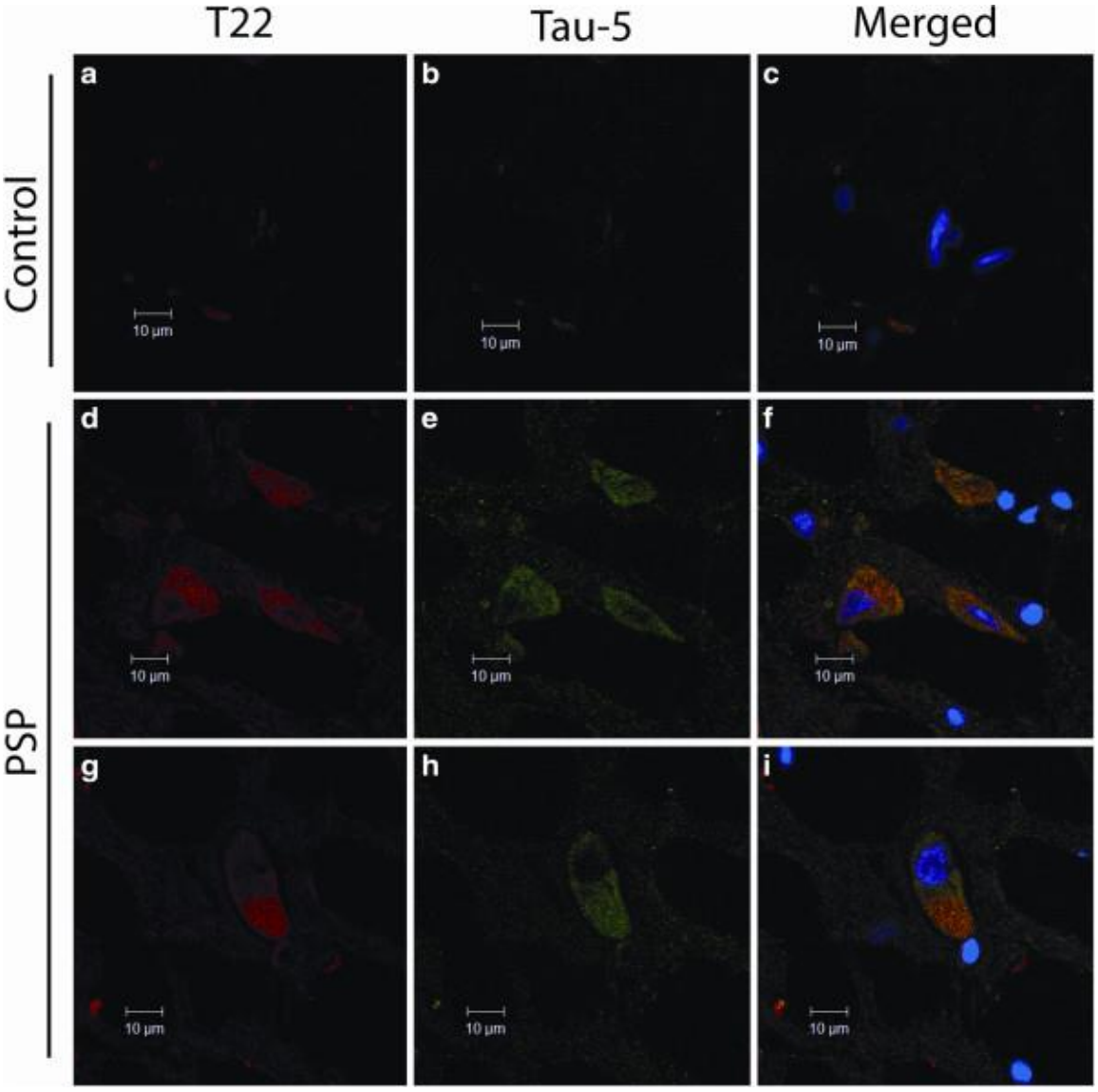

Figure 2.2 PSP brain sections express heightened levels of tau oligomers and total tau. PSP and age-matched control brain sections immunofluorescently labeled with T22 (red) and Tau-5 (green). Control brains do not exhibit NFTs labeled with Tau-5 or oligomeric tau (a-c). Globose-type NFTs are detected with Tau-5 and partially colocalize with tau oligomers (d-i).

Staining with AT8, which recognizes phosphorylated tau commonly found in NFTs, and T22 did not exhibit reactivity with control brain sections (Figure 2.3 a-c), but showed increased levels of tau oligomers in PSP brain, as well as NFTs and pre-NFTs positive for AT8 (Figure 2.3 d-i). T22 staining was largely colocalized with AT8, signifying the presence of phosphorylated tau oligomers (Figure 2.3 f&i).

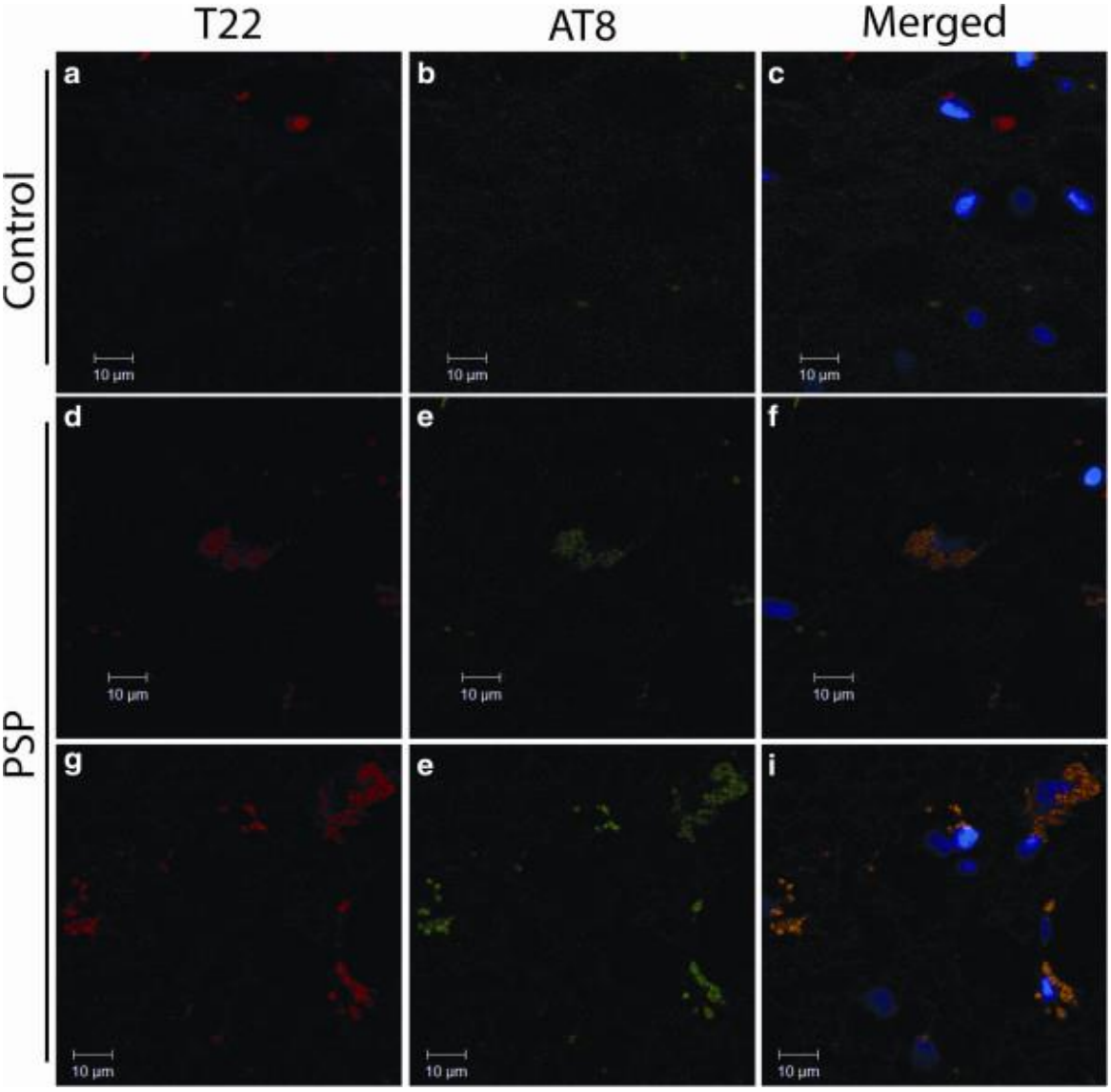

Figure 2.3 PSP brain sections display higher levels of tau oligomer and phosphorylated tau staining. PSP and age-matched control pons sections immunofluorescently labeled with T22 (red) and AT8 (green). Control brains do not exhibit phosphorylated tau or oligomeric tau deposition (a-c). Pre-NFTs and NFTs were detected with AT8, colocalizing with T22 (d-i).

Tau Oligomer Levels are Significantly Higher in PSP Versus Control Brains

Analysis of PSP brains via Western blot with T22 and Tau-5 revealed the presence of high molecular weight tau oligomers (Figure 2.4a). Additionally, analysis by direct ELISA showed a significant increase in levels of tau oligomers detected with T22 in PSP patient brains compared to control brains (Figure 2.4b). Immunofluorescent staining of individual cells with T22, Tau-5, and AT8 was corrected for cell size and background fluorescence and quantified in PSP patients and controls. PSP brain samples had significantly higher levels of tau oligomers, total tau, and phosphorylated tau (Figure 2.4c).

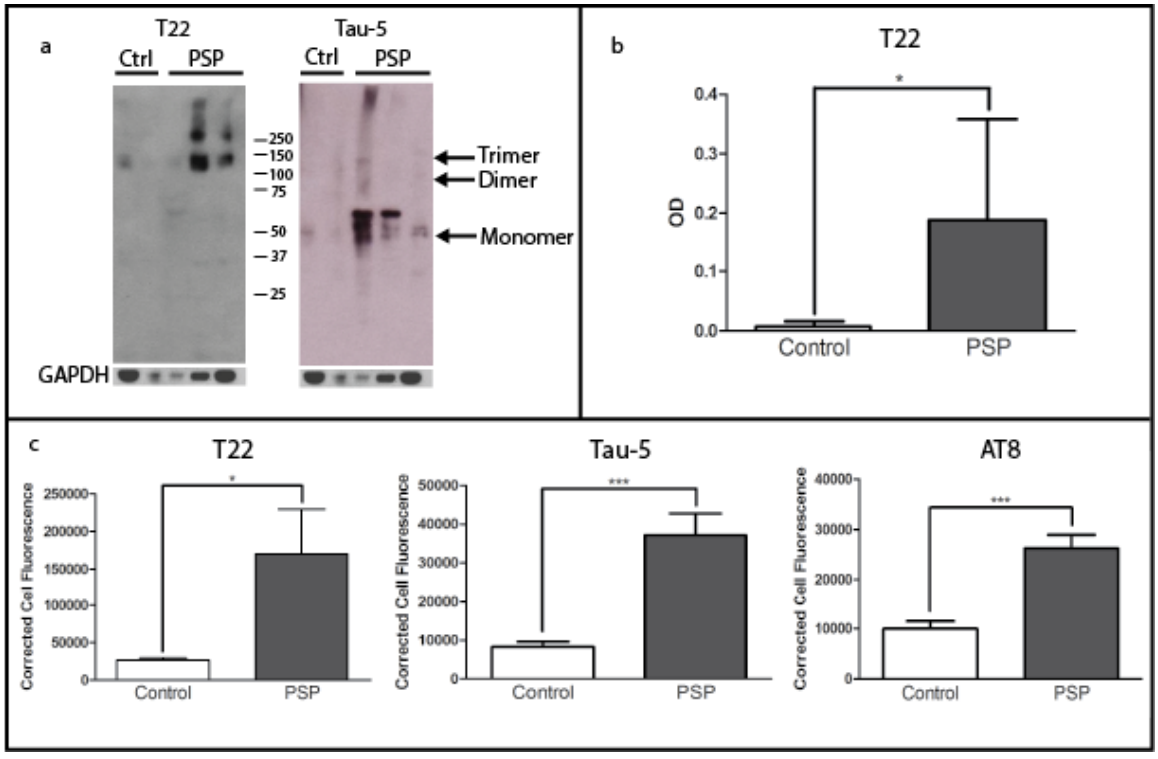

Figure 2.4 Tau oligomers are significantly increased in PSP versus control brains.

Analysis of PSP brains via Western blot with T22 and Tau-5 showed tau oligomers, while none were detected in control brains (a). Direct ELISA with T22 showed a significant increase in levels of tau oligomers in PSP patient brains compared to control brains (b). Immunofluorescent staining of individual cells with T22, Tau-5, and AT8 corrected for cell size and background fluorescence showed that PSP brain samples had significantly higher levels of tau oligomers, total tau, and phosphorylated tau. *$p<0.05$; ***$p<0.001$

PSP Brain-Derived Tau Oligomers Seed Oligomerization of Both 3R and 4R Tau

Tau oligomers were successfully immunoprecipitated from PSP brain, displaying characteristic oligomeric size and structure by analysis with AFM. Co-incubation of BDTO with 3R and 4R tau monomer induced the oligomerization of both isoforms of tau as shown by AFM (Figure 2.5a) and Western blot with Tau-5 (Figure 2.5b) Direct ELISA of 3R and 4R seeded with BDTO with Tau-5 confirmed consistent concentrations of tau in all samples, while ELISA with T22 displays an increase in levels of tau oligomers as incubation time with oligomers increases (Figure 2.5c).

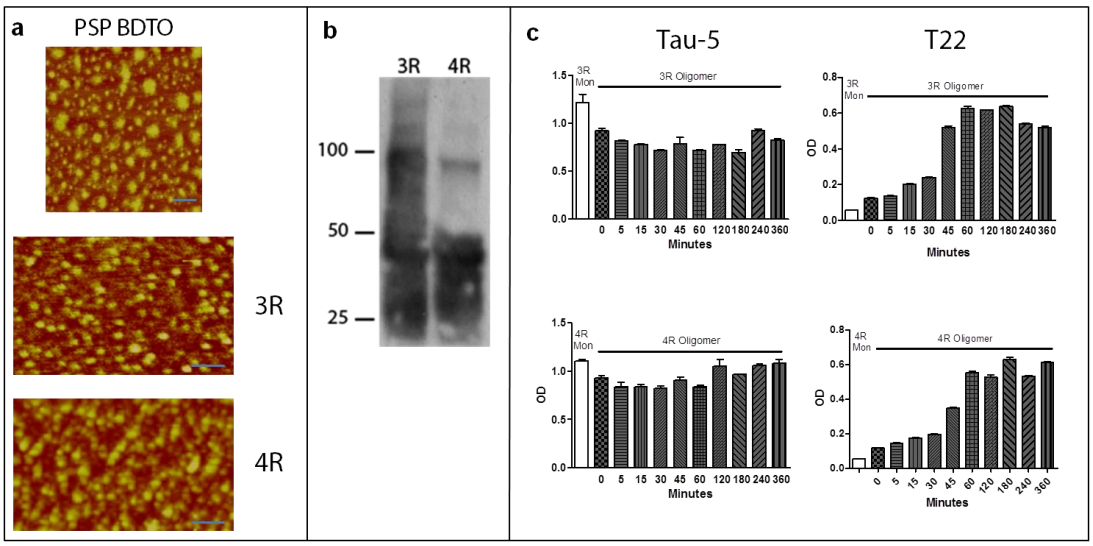

Figure 2.5 Tau oligomers derived from PSP brain seed aggregation of 3R and 4R tau. AFM image of tau oligomers immunoprecipitated from PSP brain and 3R and 4R tau seeded with PSP BDTO display characteristic oligomeric structure and size (a). Scale bar 50 nm. Western blot with Tau-5 shows oligomeric tau present in both 3R and 4R tau seeded with PSP BDTO (b). Direct ELISA with Tau-5 shows consistent tau levels in 3R and 4R tau monomer seeded with PSP BDTO and increasing levels of tau oligomers as incubation time with oligomeric tau increases (c).

Discussion

Utilizing brain samples from the pons of PSP patients and age-matched controls, we found elevated levels of tau oligomers in PSP brains compared to controls, as well as increased total tau and phosphorylated tau in the form of NFTs. Tau oligomers were found in regions exhibiting typical PSP histological hallmarks, such as globose-shaped NFTs. The results reported here indicate that tau oligomers are important components of

PSP pathology, along with NFTs. Studies supporting oligomers as the most toxic form of tau are becoming increasingly prevalent. While NFTs have been shown to correlate with disease progression in neurodegenerative tauopathies, such as AD, neuronal death and dysfunction begins to occur before the appearance of these large tau deposits (Gomez-Isla, Hollister et al. 1997, Vogt, Vogt et al. 1998, Terry 2000, van de Nes, Nafe et al. 2008). The presence of tau annular protofibrils in glial cells in PSP as well as neurons suggests that tau oligomers may play a role in tau pathology in multiple cell types (Lasagna-Reeves, Sengupta et al. 2014). Tau oligomers lead to toxicity and cognitive deficits in mice (Lasagna-Reeves, Castillo-Carranza et al. 2011) and are found to be elevated in the brains of AD patients (Maeda, Sahara et al. 2006, Patterson, Remmers et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012). However, this is the first time tau oligomers have been characterized in PSP.

Hyperphosphorylated NFTs have been known to be a main component of PSP and here we repeat results that PSP patients have increased levels of phosphorylated tau aggregates. Additionally, phosphorylated tau oligomers were detected, as well as non-phosphorylated, demonstrating the involvement of oligomeric species in the development of characteristic PSP pathology. Phosphorylation likely has important implications for toxicity, though the exact relationship between tau and phosphorylation state is not entirely certain. For one, evidence suggests that aberrant phosphorylation may increase the tendency of tau to form aggregates and that tau kinases are involved in the progression of tauopathies (Pérez 2000, Alonso 2001, Alonso 2001). Phosphorylation state also regulates cellular localization of tau, with abnormal phosphorylation leading to the release of tau from the microtubules and redistribution from the axon to the

somatodendritic compartment (Greenwood and Johnson 1995). However, other studies have highlighted the relevance of dephosphorylated tau to toxicity and have suggested an increased ability for extracellular propagation (Díaz-Hernández, Gómez-Ramos et al. 2010, Pooler 2013), implying that unphosphorylated tau oligomers detected in PSP brains may be equally important to disease progression.

The seeding of PSP brain-derived tau oligomers with both 3R and 4R tau induced the assembly of both tau isoforms into oligomers. Therefore, the mechanism by which tau pathology is induced in only 4R tau in PSP remains unknown and may not depend upon differences in tau seeding. The ability of oligomeric tau derived from PSP to seed different types of tau may underlie the frequent co-existence of other neurodegenerative tauopathies in patients with PSP (Keith-Rokosh 2008).

There are both overlaps and differences between all of the neurodegenerative diseases, hence more research is needed to understand the exact species of tau and post-translational modifications responsible for toxicity in distinct disorders, as well as to test whether the most toxic forms of tau are also those which are responsible for the spread of disease. In spite of differences in the ratio of tau isoforms, the histological hallmarks, and the localization of tau pathology in PSP, the results reported here suggest that tau oligomers, which are both phosphorylated and unphosphorylated, are involved in PSP, similarly to AD. This implies that there is likely a common mechanism for tauopathies, similarly to other well-characterized amyloids, such as amyloid beta, whereby oligomeric species underlie toxic effects. The ability of BDTO from PSP to seed the aggregation of 3R and 4R tau monomer supports other evidence for a prion-like mechanism for the spread of tau in neurodegenerative disease (Gerson and Kayed 2013). These results

combined with recent studies showing the efficacy of passive immunotherapy targeting tau oligomers (Castillo-Carranza, Gerson et al. , Castillo-Carranza, Sengupta et al. 2014) support the possibility of utilizing this strategy for therapeutics in PSP, as well as in other neurodegenerative tauopathies. The results reported here show that tau oligomers are an important component of PSP pathology, similarly to what has been seen in other tauopathies, such as Alzheimer's disease. This suggests a common mechanism for tau toxicity in pure and mixed amyloid tauopathies. Tau oligomers derived from PSP human brain tissue were capable of seeding the oligomerization of both 3R and 4R tau. As tau toxicity in PSP is primarily seen in 4R tau, rather than 3R tau, this suggests that the selection mechanism for tau toxicity may be separate from seeding. The ability of PSP-derived oligomers to seed both forms of tau also provides a potential explanation for the high prevalence of co-morbidity of additional tauopathies in patients with PSP. These results support the use of tau oligomer-directed therapeutics for the prevention of disease progression in PSP and other tauopathies. Information included in this chapter has previously been published and may be referred for further information (Gerson, Sengupta et al. 2014).

CHAPTER 3

CHARACTERIZATION AND TOXICITY OF TAU OLIGOMERS DERIVED FROM TRAUMATIC BRAIN INJURY

Introduction

Each year, there are about 1.5 million new cases of traumatic brain injury (TBI) in the United States (Thurman and Guerrero 1999). Of these patients, more than 200,000 require hospitalization, 80,000 are disabled, and 50,000 die (Thurman and Guerrero 1999). TBI causes nearly 50 billion dollars in medical expenses and lost productivity annually (Sosin, Sniezek et al. 1995), making it one of the major healthcare problems in the United States. There are more than 7.7 million (Tagliaferri, Compagnone et al. 2006) and 5.3 million (Langlois, Rutland-Brown et al. 2006) people living with disabilities from TBI in the European Union and United States, respectively (Roozenbeek, Maas et al. 2013). Thus, while there are approximately 275,000 new hospitalizations for TBI in the U.S. annually (Faul and Coronado 2015), there are millions of TBI survivors in the U.S. and worldwide. In many patients, even mild TBI is a chronic disease that contributes to neurological, neuropsychological and behavioral deficits for years post-injury (Masel and DeWitt 2010). Additionally, TBI is a significant risk factor for the subsequent development of dementia (Plassman BL 2000, Gardner, Burke et al. 2015, Perry, Sturm et al. 2015), even in patients with no posttraumatic cognitive impairments (Schofield, Tang et al. 1997).

TBI causes neuronal injury through processes that begin on impact and continue for days to months or years post-injury (Smith 1997, Bramlett 2007, Osier 2015). There have been many hypotheses about the causes of this ongoing injury (Bramlett 2015, Osier 2015), including the involvement of tau pathology (Liliang, Liang et al. 2010, Tran, Sanchez et al. 2011, Huber, Meabon et al. 2013, Cheng, Craft et al. 2014, Olivera, Lejbman et al. 2015). Recently, we proposed a possible mechanism, based on the accumulation and spreading of a toxic form of tau protein, known as tau oligomers (Hawkins, Krishnamurthy et al. 2013). A large body of research has suggested recently that the toxicity of tau protein in neurodegenerative disorders such as Alzheimer's disease (AD) does not depend on the fibrillar aggregates, neurofibrillary tangles, long known to be hallmarks of the disease. Rather, toxicity begins earlier when functional tau monomer misfolds to form soluble, oligomeric aggregates (Maeda, Sahara et al. 2006, Maeda, Sahara et al. 2007, Lasagna-Reeves, Castillo-Carranza et al. 2011, Patterson, Remmers et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012). In an earlier study, we reported rapid increases in levels of phosphorylated tau and tau oligomers that persisted in the brain for at least two weeks after experimental TBI in non-transgenic rats (Hawkins, Krishnamurthy et al. 2013). However, while we showed that TBI correlates with increased tau oligomers, thus far there have been no studies to directly investigate the impact of tau oligomers derived from TBI models on toxicity associated with long-term deficits and neurodegeneration.

Therefore, in the present study, we characterized the structure and toxicity of tau oligomers in a second rodent model of TBI using an Advanced Blast Simulator (ABS) device that produced a blast overpressure wave. Additionally, to test the hypothesis that

tau oligomers derived from TBI can induce the onset of cognitive impairment and spread of pathology, we isolated tau oligomers from the brains of rats subjected to fluid percussion injury (FPI) induced TBI, purified the proteins and then injected the tau oligomers into the brains of young, cognitively normal mice overexpressing non-mutated human tau protein (Htau mice) (Andorfer, Kress et al. 2003). Here we report that these TBI-harvested tau oligomers induced an accelerated onset of memory impairment. This finding was supported by our previous work in which tau oligomers harvested from the brains of AD patients and injected into the brains of wildtype and Htau mice produced significant memory deficits (Lasagna-Reeves, Castillo-Carranza et al. 2011, Castillo-Carranza, Gerson et al. 2014). Additionally, we saw increased levels of tau oligomers in multiple brain regions of mice that had been injected with the TBI-harvested tau oligomers. This increase in other brain regions may be due to seeding and a *prionlike* spreading effect of toxic tau oligomers (Gerson and Kayed 2013).

Methods

Animals

This study was conducted in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and all experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (UTMB). Mice were bred at UTMB free of enrichment to prevent any effect on behavioral test performance. Mice and

rats were housed at the UTMB animal care facility and maintained according to US Department of Agriculture standards (12 h light/dark cycle, food and water ad libitum).

Experimental Design

Experimental design is detailed in (Figure 3.1).

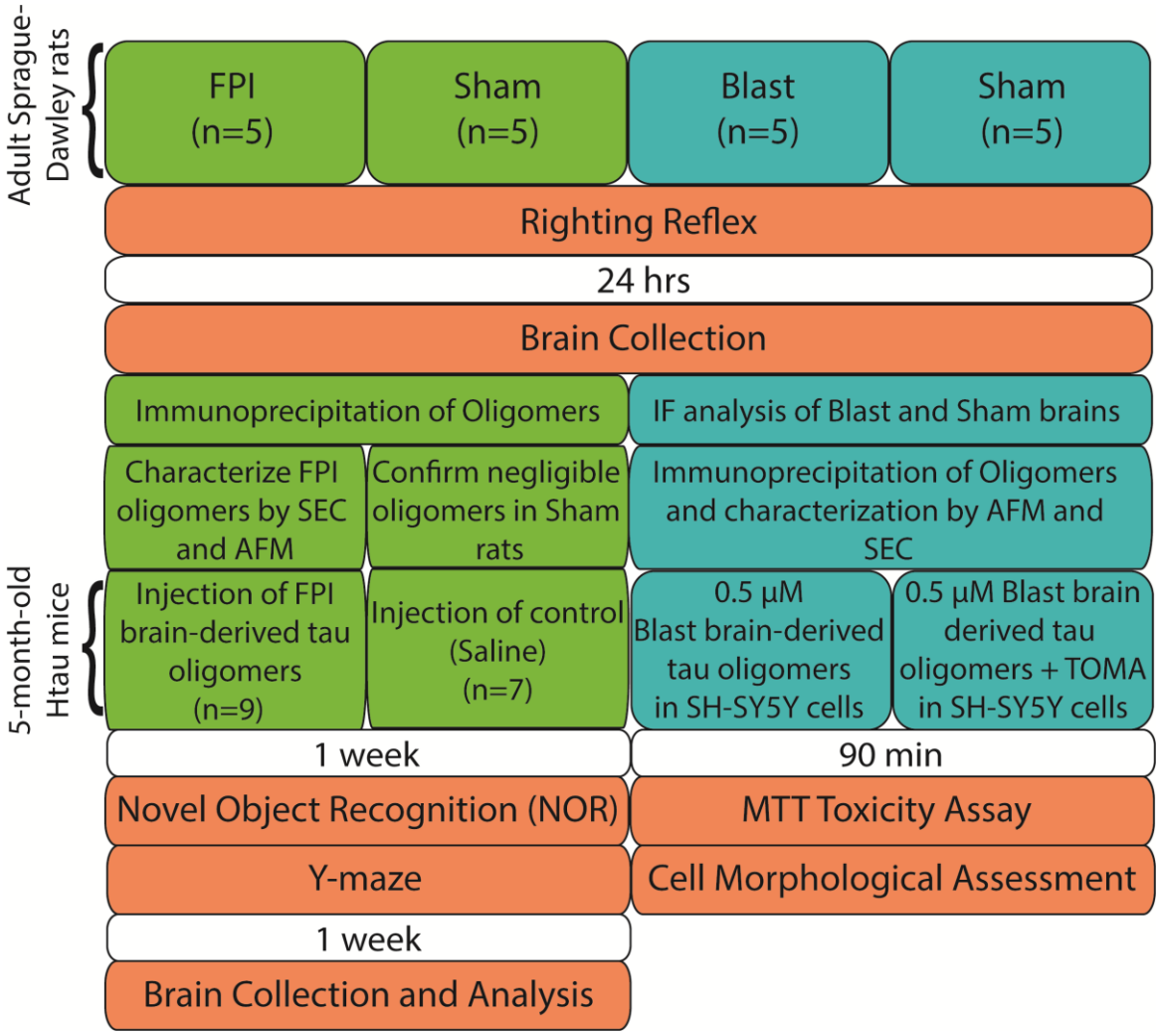

Figure 3.1 Flowchart of experimental design characterizing tau oligomers and toxicity from fluid percussion injured (FPI) and Blast injured rats.

Parasagittal Fluid Percussion Injury

Male Sprague-Dawley (Charles Rivers, Wilmington, MA) rats (400–500 grams) were anesthetized (4% isoflurane), intubated, mechanically ventilated with 1.5% isoflurane in O2:air (20:80) using a volume ventilator (NEMI Scientific, New England Medical Instruments, Medway, MA), and prepared for parasagittal FPI as described previously (McIntosh TK 1989, Hawkins, Krishnamurthy et al. 2013). Rectal and temporalis muscle temperatures were maintained within a range of 37.5 ± 0.5 °C. Rats were placed in a stereotaxic apparatus, a craniotomy was trephined 1 mm lateral (right) of the sagittal suture, midway between the lambda and bregma just prior to the FPI or sham injury. Isoflurane was briefly discontinued until the rat displayed a withdrawal response to paw pinch. Immediately after the return of the withdrawal response, the rat was subjected to moderate (2.0 atm) TBI using a FPI device (Custom Design & Fabrication, Richmond, VA). The transducer housing on our device is connected to the rat by an 18 mm nylon tube that fits into the cap (modified 20-gauge needle hub cemented into the craniotomy site with cyanoacrylic adhesive and hygienic dental acrylic), perpendicular to the craniotomy site. The sham-injured animals are prepared identically to the TBI animals with the exception of the release of the pendulum. The height of the pendulum (set at 15.0 cm) determines the intensity of the injury (between 2.0-2.2 atm) and the fluid pressure pulse was recorded on an oscilloscope (Tektronix TDS1002). The peak pressure pulse was in the range of 250-360 mV and the pressure wave duration is 25.0 ms. After TBI, the time it takes for the animal to regain three consecutive righting reflexes was recorded and then the animal was given isoflurane and the skin incision was closed and the rodent was allowed to recover. Twenty-four hours after TBI or sham injury, rats were

humanely euthanized and their brains were collected and immediately snap frozen and kept at -80C.

Blast Injury

Adult male Sprague-Dawley rats (Charles Rivers, Wilmington, MA) weighing 350 – 400 grams were anesthetized with 4% isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5-2.0% isoflurane in O_2/room air (20:80) using a volume ventilator. After intubation, the top of the scalp was shaved and foam plugs were placed into each ear. The ABS device is a shock tube designed by David Ritzel (Dyn-FX Consulting, Ltd. Ontario, Canada) and produced by Steve Parks (ORA, Inc., Fredericksburg, VA). The ABS uses a compressed air driver to produce Friedlander-type shock over/underpressure waves (Friedlander 1946) that closely replicate those recorded in open-field blasts (Cernak 2010). The ABS consists of a driver chamber, a blast wave expansion chamber, a specimen chamber and a blast wave reflection suppressor. The driving chamber is separated from the expansion chamber by a mylar membrane. The amplitudes of the shock waves are determined by the thickness of the membrane. The shock wave produced when the Mylar ruptures is propagated down the tube where it interacts with the experimental animal and then passes into a wave reflection suppressor. Just prior to the blast, each rat was disconnected from the ventilator, placed on a specimen tray with its head inside of the ABS device and secured with Velcro strips. Immediately after the return of a withdrawal response to paw pinch, the rat was subjected to ABS blast injury (17-22 psi) and removed from the specimen tray. The duration of the suppression of the righting reflex (RR) was measured as an indicator of injury severity and the average RR range for mild blast in our lab is between 3-6 minutes. After the

return of the righting reflex, rats were placed in a cage in the laboratory and monitored. Twenty-four hours later they were deeply anesthetized, decapitated and their brains were collected and immediately snap frozen and kept at -80C.

Preparation of Brain-Derived Tau Oligomers

Tau oligomers were immunoprecipitated from the brains of injured Sprague-Dawley rats with FPI (n=5) and Blast injury (n=5) as described in Chapter 2 (McIntosh TK 1989, Lasagna-Reeves, Castillo-Carranza et al. 2011, Hawkins, Krishnamurthy et al. 2013, Gerson, Castillo-Carranza et al. 2016). Immunoprecipitation protocols were also completed for sham rats from both FPI and Blast injury cohorts as a control (n=5).

Stereotaxic Injection of Brain-Derived Tau Oligomers.

Five-month-old homozygous Htau mice (The Jackson laboratory stock #005491) (Andorfer, Kress et al. 2003) were anesthetized with ketamine (80-100 mg/kg, ip) and xylazine (10 mg/kg, ip) and placed in a stereotactic apparatus (Motorized Stereotaxic StereoDrive, Neurostar)**.** The scalp of each mouse was then shaved and an incision was made through the midline to expose the skull. A craniotomy was drilled (−2.06 mm posterior, +/−1.75 mm lateral, and 2.5 mm ventral to the bregma) into the skull of each mouse. A 5.0 µl Hamilton syringe was used to inject 2 µl of either 0.3 mg/ml brain-derived tau oligomers or saline (n=10 for each group) bilaterally into the dorsal hippocampus at an infusion rate of 0.2 µl/min as described previously (Cernak 2010, Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012). The skin incision was closed using Vet-Bond, and mice were placed on a 37°C isothermal pad and continuously observed post-surgery until recovery. Attrition

following surgery left an n of 7 for the control group and an n of 9 for the oligomer-injected group.

Novel Object Recognition (NOR)

The novel object recognition task (NOR) utilizes the natural tendency of rodents to preferentially explore novel objects and environments over those that are familiar. This task has been used previously to detect memory impairment in Htau mice (Polydoro, Acker et al. 2009, Gerson and Kayed 2013). One week after brain-derived tau oligomer injection, animals were habituated to the NOR task (n = 7-9 mice per group). Mice were allowed to freely explore white open-field arena (55 cm in diameter; 60 cm in height) for 15 minutes on the first day. The next day, mice were placed in the arena for the training phase with two identical objects, either spheres or cubes, and allowed to explore for 15 minutes. On the third day, mice were placed again in the arena for 15 minutes with one familiar object previously explored in the training phase and one novel object differing in color and shape, but sharing a common size and volume. After each trial, the apparatus was thoroughly cleaned using 70% Ethanol and allowed to dry prior to placement of a new mouse. Trials were recorded and time spent exploring each object was measured using ANY-Maze software. Exploration was defined by head orientation within 2 cm of the object or physical contact with the object. The percentage of total time spent exploring the familiar object versus the novel object was measured. In order to control for any differences in exploratory behavior, the discrimination index was also calculated as the time spent exploring the familiar object subtracted from the time spent exploring the novel object, divided by the total time spent exploring both objects. Object exploration

data were analyzed using Graph Pad Prism 5.04 sofware by one-way *t*-test with a hypothesized mean of 0 for the discrimination index.

Spontaneous Alternation Y-Maze Task

This task provides a measure of spatial working memory and is based on the innate preference of mice to alternate arms when exploring a new environment. At post-injection day 11, the mice (n = 7-9 per group) were placed in a symmetrical Y-shaped maze. Arms were 40 cm long, 8 cm wide, and 12 cm high (San Diego Instruments), beige in color, non-reflective, and randomly designated A, B or C. Each mouse was placed in an arm facing the center (arm A) and allowed to explore the maze for 8 min. The number of arms entered, as well as the sequence of entries was recorded. A correct alternation occurred when the animal moved from the arm in which it began to the other two arms without retracing its steps (i.e., ABC or ACB). Spontaneous alternation, expressed as a percentage, was calculated by dividing the number of entries into all three arms on consecutive choices (correct choices) by number of arm entries subtracted by two, then multiplying the quotient by 100. A high spontaneous alternation rate is indicative of effective working memory since the animals must remember which arm was entered last in order to know not to re-enter it.

Tissue Collection and Immunofluorescence

One week following behavioral testing, mice injected with FPI brain-derived tau oligomers (n = 7-9) were anesthetized with CO_2 and brains from both FPI tau oligomer-treated mice and Blast injured rats were collected. The right hemisphere was snap-frozen and stored at -80^{0}C until processed for biochemical analysis. The other hemisphere was

embedded in OCT and sectioned on a cryostat. All sections were processed simultaneously under the same conditions. Sections (7 μm) were fixed in 4% paraformaldehyde. After blocking in bovine serum albumin for 1 hr, sections were incubated overnight with anti-tau oligomer-specific polyclonal antibody, T22 (1:250). The following day, sections were washed in PBS three times for 10 minutes each and incubated with goat anti-rabbit IgG Alexa-488 (1:500; Invitrogen) for 1 hr. Sections were then washed three times for 10 minutes each in PBS and incubated overnight with total tau antibody, Tau-5 (1:100). The next day the sections were washed in PBS three times for 10 minutes each prior to incubation with donkey anti-mouse IgG Alexa-Fluor-568 (1:500; Invitrogen). Sections were washed and mounted using Fluoromount G (Souther Biotech) mounting medium with DAPI (Invitrogen). Sections were imaged and analysed as described in Chapter 2 (Gerson, Castillo-Carranza et al. 2016). Low magnification images (20x) of the hippocampus and cerebellum were acquired for each sample. Three high magnification images (100x) were taken at the hippocampus, frontal cortex, and cerebellum of each sample and six cells were randomly selected from each image for quantification.

Cellular Toxicity Assay

Cell viability after treatment with ABS blast brain-derived tau oligomers was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) toxicity assay kit (Roche) as previously described (Lasagna-Reeves, Castillo-Carranza et al. 2012). SH-SY5Y human neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and grown to confluence in a 96-well plate. Cells (~10,000 per well) were treated with fresh media alone or containing either 0.5 μM tau oligomers or

0.5 μM tau oligomers pre-treated with an equal concentration of tau oligomer-specific monoclonal antibody (TOMA). After incubation for 90 min at 37°C, the cells were assayed using MTT colorimetric assay according to the manufacturer's directions. All measurements were made in triplicate. Results were analyzed by one-way ANOVA. Cells were also evaluated for morphological changes by microscopy.

Results

Brain Tau Oligomer Levels are Increased in Blast Injured Rats

We investigated the presence of tau oligomers in the blast injury rat model 24 h after injury. Using our novel tau oligomer-specific antibody, T22, we found that tau oligomers are present in high levels in the hippocampus of blast-injured rats compared to sham animals, which show low levels of tau oligomers (Figure 3.2A-C). Immunofluorescent staining of individual cells with T22 was corrected for cell size and background fluorescence and quantified in blast injury and sham rat samples. Blast injured rats exhibited significantly elevated levels of tau oligomers when compared to sham ($p = 0.0217$; Figure 3.2D). In order to characterize tau oligomers formed after blast TBI, we isolated tau oligomers from rat brains 24 h after injury by immunoprecipitation with tau oligomer-specific antibody, T22, and analyzed them by atomic force microscopy (AFM; Figure 3.2E-G). The presence of a heterogeneous population of oligomers, but no fibrils can be seen in samples immunoprecipitated from blast TBI brains, while negligible oligomers were found in samples isolated from sham rat brains.

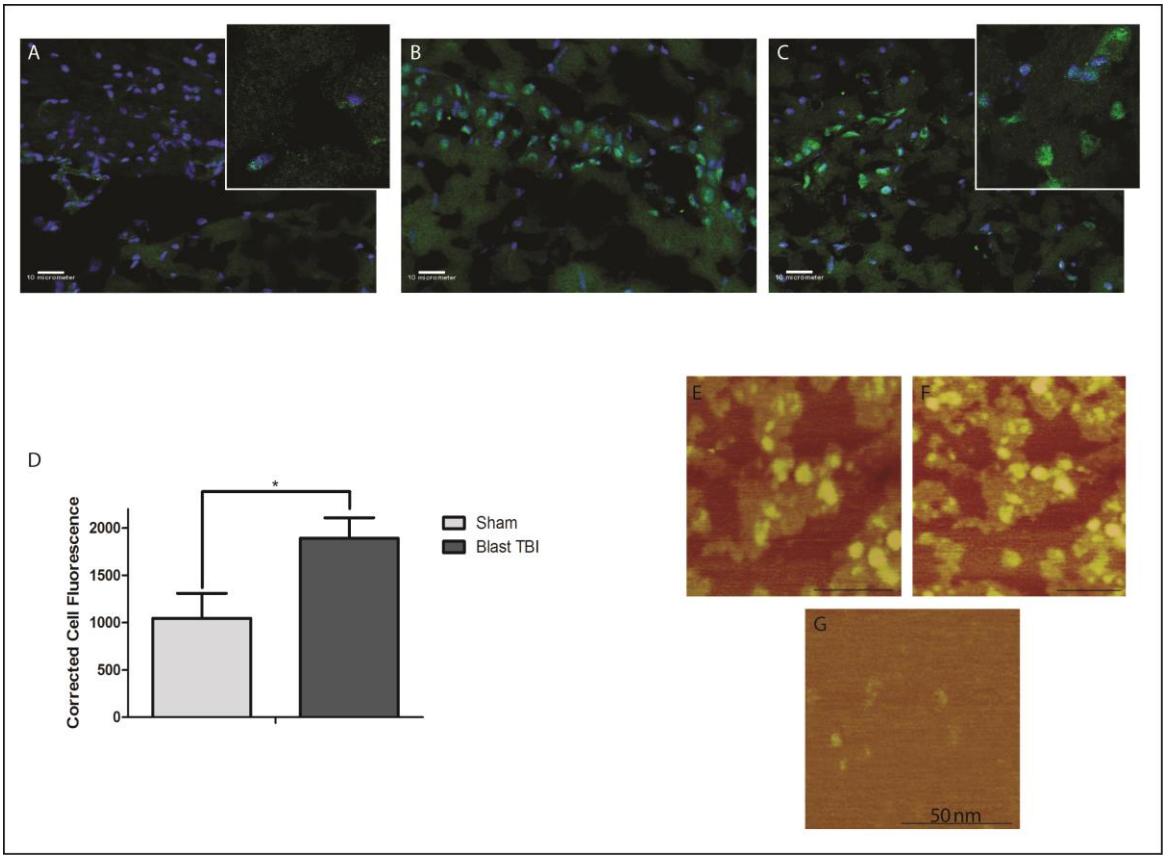

Figure 3.2 Tau Oligomers are Elevated in the Brains of Blast-Injured Rats. Immunofluorescence with tau oligomer-specific antibody, T22 in hippocampal sections at 20x (100x magnification images inset) of sham (A) and blast-injured rats (B-C). Tau oligomers are significantly elevated in the hippocampus of blast-injured rats (B-C) when compared to sham animals (A) as measured by corrected total cell fluorescence analysis, p=0.0217 (D). Tau oligomers immunoprecipitated with T22 from blast-injured rat brain homogenate can be seen by AFM (E-F) while sham animals do not exhibit characteristic oligomeric structures (G).

Single Injection with TBI-Derived Tau Oligomers Induces Cognitive Deficits in Htau Mice

In order to test the cognitive impact of tau oligomers derived from TBI rodents, we isolated tau oligomers from FPI rats and characterized them by SEC and AFM (Hawkins, Krishnamurthy et al. 2013). We then injected 0.6 μg of tau oligomers or 2 μl of PBS bilaterally in the hippocampi of 5-month-old Htau mice. One week after injection, mice were tested for cognitive deficits using the Y-maze and novel object recognition tasks. Mice injected with tau oligomers had significantly lower percent spontaneous alternation than mice injected with PBS on the spatial memory task, the Y-maze ($p = 0.0091$; Figure 3.3A), but differences in the novel object recognition task were not statistically significant (Figure 3.3B).

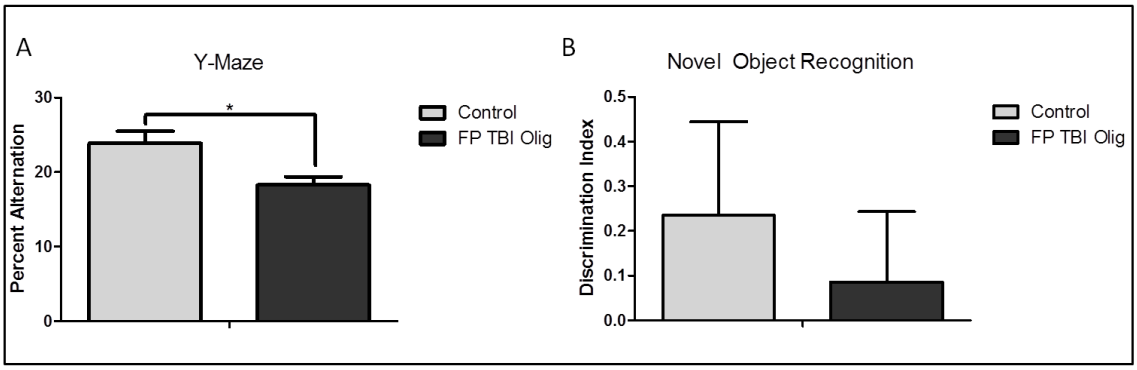

Figure 3.3 Injection with TBI-derived tau oligomers leads to cognitive impairment. Htau mice injected with TBI-derived tau oligomers exhibit significantly lower percent spontaneous alternation of the Y maze task compared to Htau mice injected with saline, p=0.0091 (A). Mice injected with tau oligomers were not significantly impaired on the novel object recognition task (B).

Tau Oligomer Levels Increase Outside the Site of Injection in Htau Mice

In order to determine whether tau oligomer levels differ between control and injected animals in specific brain regions, brain sections from Htau mice were analyzed by immunofluorescence with T22 and Tau-5. Tau oligomers were present in the hippocampus of control Htau mice, but were significantly increased in the hippocampi of mice injected with tau oligomers as measured by the corrected total cell fluorescence of randomly selected cells in each sample ($p = 0.0053$; Figure 3.4).

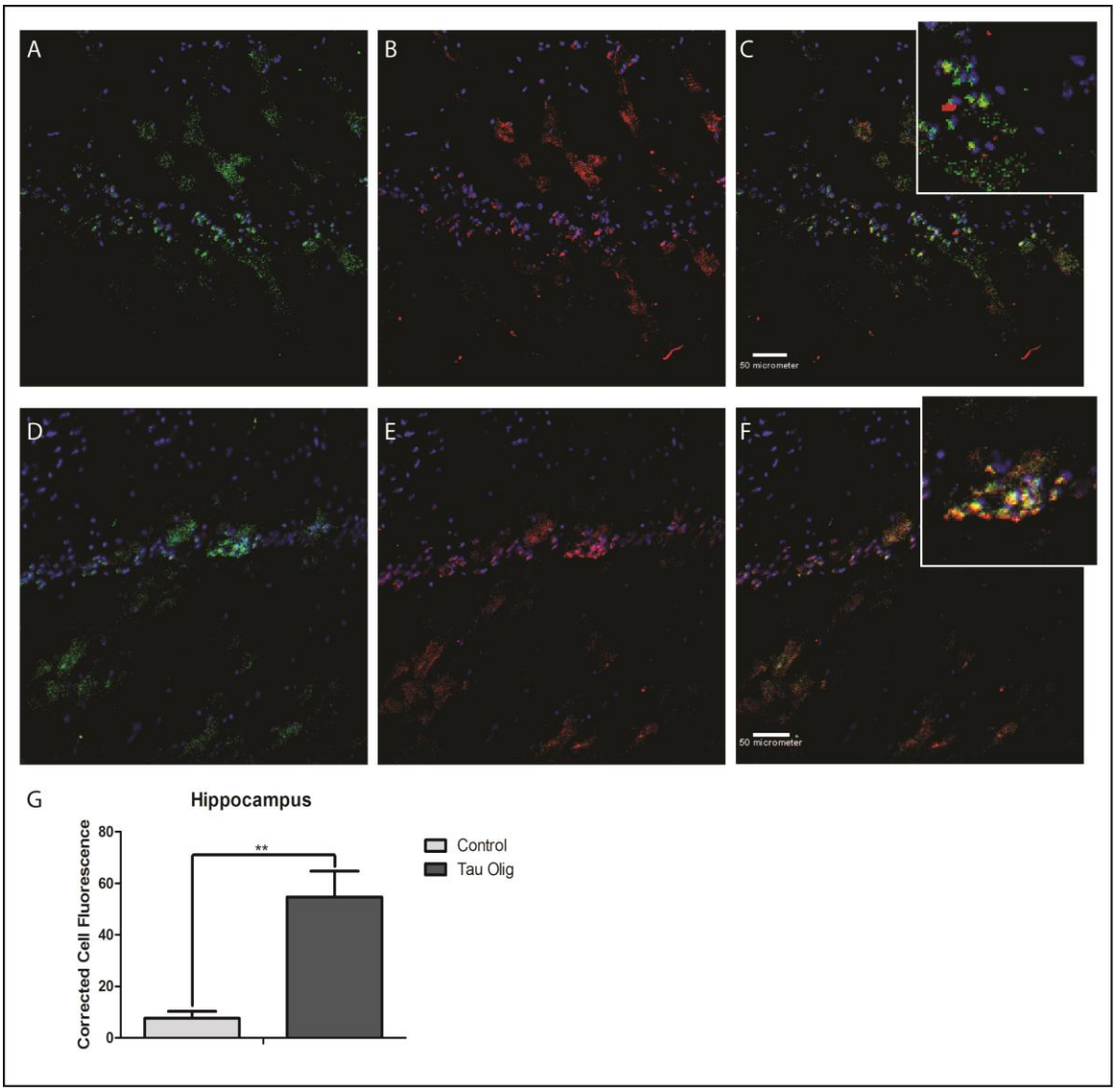

Figure 3.4 Tau Oligomer Levels Increase in the Hippocampus in TBI Oligomer-Injected Mice. Tau oligomers detected with T22 (green) and total tau levels labeled with Tau-5 (red) in the hippocampus of control Htau (A-C) and TBI oligomer-injected Htau mice (D-F). Total fluorescence of individual cells labeled with T22, corrected for cell size and background fluorescence showed that TBI tau oligomer-injected Htau brain samples had significantly higher levels of tau oligomers in the hippocampus, p=0.0053 (G).

Negligible levels of tau oligomers were detected in the cerebellum of control Htau mice, but were significantly increased in the cerebellum of mice injected with tau oligomers as measured by the corrected total cell fluorescence of randomly selected cells in each sample ($p = 0.0005$; Figure 3.5).

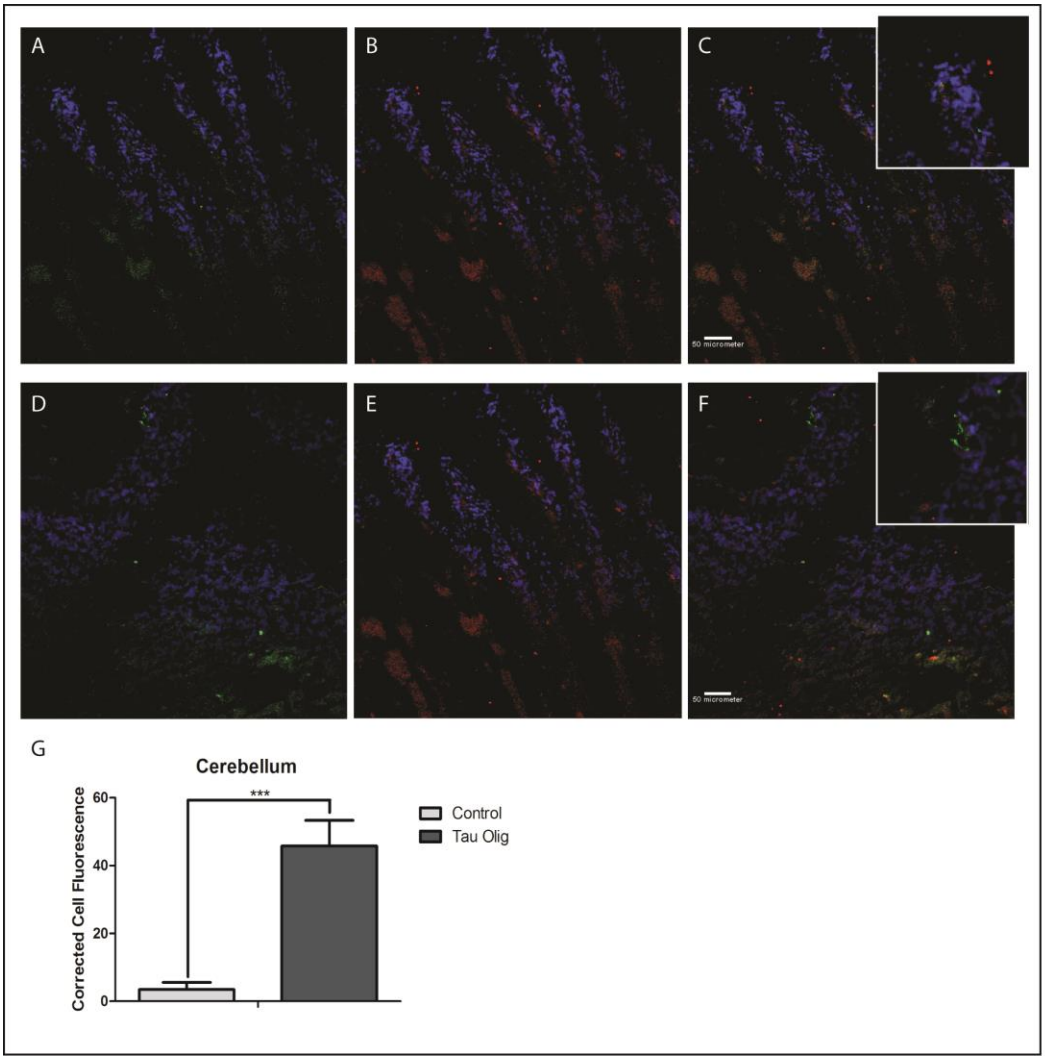

Figure 3.5 Tau Oligomer Levels Increase Outside of the Injection Site. Tau oligomers detected with T22 (green) and total tau levels labeled with Tau-5 (red) in the cerebellum of control Htau (A-C) and TBI oligomer-injected Htau mice (D-F). Total fluorescence of

individual cells labeled with T22, corrected for cell size and background fluorescence showed that TBI tau oligomer-injected Htau brain samples had significantly higher levels of tau oligomers in the cerebellum, p=0.0005 (G).

In order to test whether toxicity induced by injection with TBI brain-derived tau oligomers was dependent upon cell death, we analyzed brain sections from injected mice using Fluorojade, a dye that recognizes injured neurons. We found no effects of injection on the level on Fluorojade-positive cells (results not shown).

Blast TBI Brain-Derived Tau Oligomers Induce Toxicity Inhibited by Tau Oligomer-Specific Antibody

We treated SH-SY5Y neuroblastoma cells with tau oligomers isolated and characterized from blast TBI rats. We found that after 90 min, tau oligomer treatment led to a significant decrease in cell viability measured by the MTT cell toxicity assay when compared with cells given fresh media containing no tau oligomers ($p = 0.0005$). Moreover, we pre-incubated isolated tau oligomers with a tau oligomer-specific monoclonal antibody (TOMA) and found that antibody treatment prevented the toxic effects of tau oligomers and cell viability did not differ from control cells given fresh media. Moreover, cells treated with blast TBI-derived tau oligomers exhibited an altered cellular morphology when compared to cells treated with tau oligomers pre-incubated with TOMA (Figure 3.6).

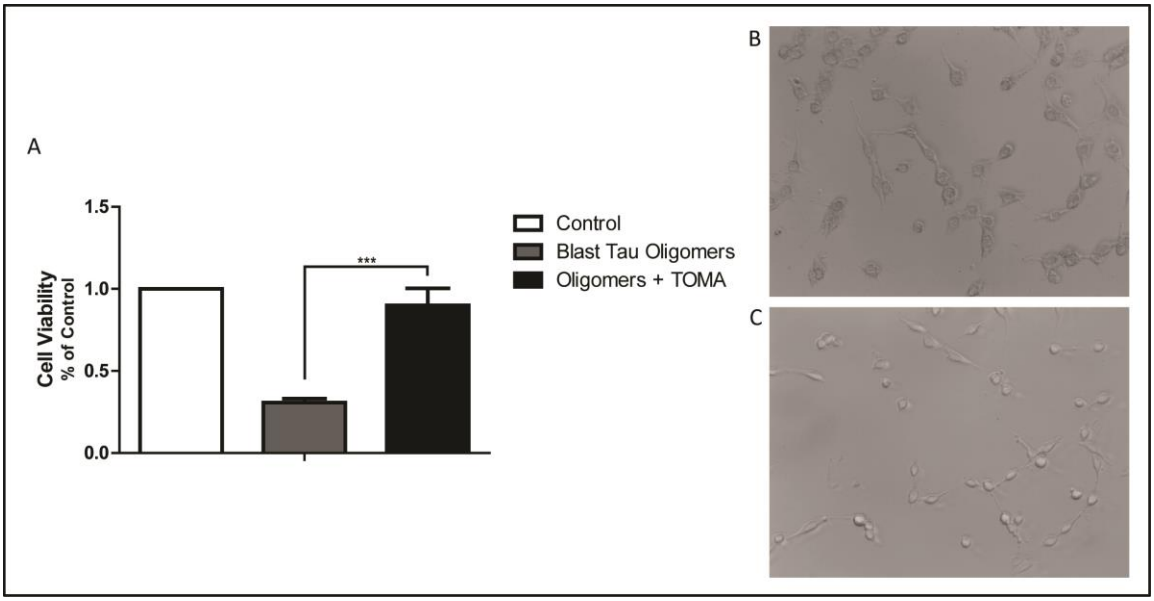

Figure 3.6 Blast-Derived Tau Oligomers Induce Toxicity in Neuroblastoma Cells Reversed by Tau Oligomer Antibody. Cell viability measured as a fraction of the absorbance detected by the MTT toxicity assay in comparison to control cells was analyzed for SH-SY5Y neuroblastoma cells treated with Blast TBI-derived tau oligomers with and without tau oligomer-specific monoclonal antibody (TOMA; A). Cells given tau oligomers pre-incubated with TOMA had significantly higher cell viability when compared with TBI-derived tau oligomers alone ($p=0.0005$). Cells administered TBI-derived tau oligomers alone (B) were compared to cells treated with tau oligomers pre-incubated with TOMA (C) and evaluated for morphological differences.

Discussion

Blast exposure is the most common cause of combat related TBIs among military personnel in the conflicts in Iraq and Afghanistan (Galarneau 2008, Wojcik 2010, Bass

2012) and blast-induced neurotrauma may be associated with the subsequent development of chronic traumatic encephalopathy (Goldstein, Fisher et al. 2012). Blast injury in humans and experimental animals is associated with increased levels of total and phosphorylated tau protein (Goldstein, Fisher et al. 2012, Huber, Meabon et al. 2013, McKee and Robinson 2014, Perez-Polo, Rea et al. 2015). We have previously shown that an increase in levels of tau oligomers results from fluid percussion TBI rats (Hawkins, Krishnamurthy et al. 2013). However, the contribution of tau oligomers to blast TBI has not yet been investigated. Moreover, whether tau oligomers in TBI directly contribute to cognitive deficits and risk for neurodegenerative disease later in life is unknown. In this study, we characterized tau oligomer pathology in blast-injured rats. Tau oligomers detected with our novel tau oligomer-specific antibody, T22, are increased in the hippocampus of rats 24 hours after blast injury. We were able to visualize tau oligomers immunoprecipitated from the brains of blast-injured rats, but not in sham animals. Therefore, tau oligomers are an important component to blast injury pathology and may be a beneficial biomarker and therapeutic target. Moreover, an independent study of TBI found that tau oligomers were detected in brain samples at 12-24 hours post injury and by decreasing the levels of phosphorylated and oligomeric tau using an antibody against cis-p-tau prevented the toxic effects of injury, further highlighting the potential role of tau oligomers in TBI (Kondo, Shahpasand et al. 2015).

Tau has been highlighted as an important protein of interest in neurodegenerative diseases such as AD and frontotemporal lobar dementia, however, it is clear that it may also be critical for the induction of long-term effects of TBI and increased incidence of neurodegeneration later in life. While the majority of tau mouse models express a

mutated form of tau, tau pathology in AD and TBI occur in humans independent of any mutations. Therefore, more useful information relevant to TBI and increased incidence of AD could be gleaned using mice that do not express mutated tau leading to drastic neurodegeneration phenotypes early in age. Previously, we isolated oligomers from brains that had AD and other tauopathies (Lasagna-Reeves, Castillo-Carranza et al. 2012, Gerson, Sengupta et al. 2014, Sengupta, Guerrero-Muñoz et al. 2015) and found that tau oligomers injected in wild-type mice exhibit toxic effects that can be protected with tau oligomer-specific antibodies, while treatment with tau monomer and fibrils does not lead to any phenotypic differences (Lasagna-Reeves, Castillo-Carranza et al. 2012, Gerson and Kayed 2013). However, wild-type mice maintain protection after antibody treatment for significantly longer periods of time after treatment than mice expressing human tau rather than mouse tau (Gerson and Kayed 2013). This difference in benefit between wildtype and Htau mice suggests that brain-derived tau oligomers may have a lower ability to seed the misfolding of endogenous mouse tau when compared to human tau. Therefore, the Htau mouse model that expresses non-mutated human tau is a particularly good model for these conditions. Htau mice begin to shown memory deficits at around 12 months of age, but do not exhibit any detriments to motor skills, sensory abilities, or overall health (Polydoro, Acker et al. 2009). Whether tau oligomers from TBI lead to similar consequences as those derived from neurodegenerative tauopathies was unclear. Therefore, we examined the hypothesis that tau oligomers derived from TBI can induce the onset of behavioral impairment and spread of pathology when injected into cognitively intact animals. We isolated tau oligomers from rats that underwent TBI and injected them into the hippocampi of young, cognitively intact Htau mice. We found that

injection of TBI-derived tau oligomers led to the rapid induction of deficits of spatial memory in mice, providing direct evidence supporting our hypothesis that tau oligomers induce many of the symptoms of TBI. We also detected tau oligomers in brain regions that were apart from the injection site, suggesting they may have spread to other areas of the brain. These results also provide a direct connection between the occurrence of TBI and the increased prevalence of acquiring neurodegenerative disease later in life. Toxicity incurred in injected mice was found to be independent of cell death, indicating that toxicity from tau oligomers is likely due to effects occurring in the absence of cell death, such as synaptic and mitochondrial toxicity, as has been shown previously in tau models of neurodegeneration (Lasagna-Reeves, Castillo-Carranza et al. 2011).

Previously, we thoroughly examined the effects of recombinant tau oligomers as well as tau oligomers derived from neurodegenerative disease on neuroblastoma cells—a cell model used extensively to examine toxicity of amyloidogenic proteins—in comparison to tau monomer and tau fibrils. We have shown in repeated experiments that tau oligomers induce significantly higher levels of toxicity in these cells when compared to control as well as other forms of tau (Lasagna-Reeves, Castillo-Carranza et al. 2010, Lasagna-Reeves, Castillo-Carranza et al. 2012, Castillo-Carranza, Gerson et al. 2014, Lasagna-Reeves, Sengupta et al. 2014). In order to determine the toxicity of tau oligomers isolated from our ABS blast TBI model and whether toxicity can be prevented by pre-treatment with tau oligomer-specific antibody (TOMA), we utilized the same procedure that we have established previously. We treated SH-SY5Y neuroblastoma cells with TBI brain-derived tau oligomers and saw a significant decrease in cell viability that was reversed when tau oligomers were pre-incubated with TOMA.

In addition to their role in toxicity in disease, tau oligomers are thought to contribute to the spread of pathology from affected regions of the brain to unaffected regions in neurodegenerative disease (Gerson and Kayed 2013). We have shown that tau oligomers derived from AD are capable of propagating pathology from the injection site to connected brain regions (Lasagna-Reeves, Castillo-Carranza et al. 2012), suggesting that oligomeric tau in TBI may similarly lead to the proliferation of toxic forms of tau from the site of injury to other areas in the brain, leading to a worsening of symptoms in the time following trauma. Here we investigated tau oligomer levels in the hippocampal injection sites, as well as the frontal cortex and the cerebellum. We found that tau oligomers begin to accumulate in the hippocampus and frontal cortex of control Htau mice, but are present in significantly higher levels throughout the hippocampus of injected mice ($p = 0.0053$). Importantly, tau oligomer levels were significantly increased in the cerebellum, but were not different in the frontal cortex (data not shown). Though more extensive evaluation of brain regions will be needed in the future, these results suggest that tau oligomers in TBI may be capable of seeding the misfolding and aggregation of endogenous tau in the brain and spreading from one region to another. Previous studies have shown that tau aggregates are capable of propagating across synaptically connected as well as neighboring brain regions (Clavaguera, Bolmont et al. 2009, de Calignon, Polydoro et al. 2012, Liu, Drouet et al. 2012). Our data support the finding in neurodegenerative disease research that tau oligomers are associated with the endosomal/exosomal pathway and can be transferred from cell to cell *in vitro* (Wu 2013), making them a likely source for the initiation of the spread of pathology.

Our results highlight the importance of tau oligomers in TBI and suggest that TBI may share a common mechanism of toxicity with neurodegenerative tauopathies. Moreover, these data suggest that the increased prevalence of acquiring AD many years after the occurrence of TBI may be due to the seeding and spread of tau oligomers released following neuronal injury. This has important implications for both the treatment of TBI and for the prevention of neurodegeneration late in life. We have previously shown that the accelerated onset of cognitive dysfunction in Htau mice induced by the injection of AD-derived tau oligomers can be prevented by treating mice with a tau oligomer-specific monoclonal antibody (TOMA)(Gerson and Kayed 2013). Further study is warranted to determine whether targeting tau oligomers in TBI may be an effective therapeutic strategy. Information included in this chapter has previously been published and may be referred for further information (Gerson, Castillo-Carranza et al. 2016).

CHAPTER 4

CHARACTERIZATION AND TOXICITY OF ALZHEIMER'S DISEASE-DERIVED TAU OLIGOMERS AND INHIBITION WITH TAU OLIGOMER-SPECIFIC ANTIBODY

Introduction

Neurodegenerative disease is one of the greatest health crises in the world today and as life expectancy rises, the number of people affected will continue to increase. The two most common neurodegenerative diseases, Alzheimer's disease (AD) and Parkinson's disease (PD) can be classified as tauopathies, which also include a number of less common diseases, such as Progressive Supranuclear Palsy (PSP) and Frontotemporal Dementia (FTD). Tauopathies are characterized by the presence of aggregated tau protein, namely in the form of neurofibrillary tangles (NFTs). In its monomeric form, tau is a critical mediator of microtubule stability. However, in disease tau becomes misfolded and forms small aggregates, known as tau oligomers, possibly en route to the formation of NFTs. Historically, NFTs have been considered the main tau species of interest in pathology, however, recently it has come to light that tau oligomers may be the most toxic form of tau and the species responsible for the spread of pathology. While NFTs have been shown to correlate with severity of disease in post-mortem human AD brains, neuron loss far exceeds NFT load (Gomez-Isla, Hollister et al. 1997, Kril, Patel et al. 2002) and begins to occur prior to tangle formation in humans and in mouse models (Spires, Orne et al. 2006, Haroutunian, Davies et al. 2007, Polydoro, Dzhala et al. 2013).

Moreover, in AD, hippocampal neurons can survive for up to 20 years with NFTs (Morsch, Simon et al. 1999). Additionally, in animal models, disease phenotypes are not associated with NFT levels (SantaCruz, Lewis et al. 2005, Berger, Roder et al. 2007, Yoshiyama, Higuchi et al. 2007, Polydoro, Acker et al. 2009, Kopeikina, Carlson et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2011, Spires-Jones, Kopeikina et al. 2011, Cowan, Quraishe et al. 2012, Sahara, DeTure et al. 2013), including in mice overexpressing human tau (Htau mice) (Andorfer, Kress et al. 2003, Andorfer, Acker et al. 2005). Therefore, it seems likely that intermediate species may underly toxicity.

Tau oligomer levels have been shown to increase in AD brains prior to NFT formation (Maeda, Sahara et al. 2006, Maeda, Sahara et al. 2007, Patterson, Remmers et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012, Ward, Himmelstein et al. 2013) and disrupt the ubiquitin proteasome system at synapses (Tai, Serrano-Pozo et al. 2012). When brain-derived tau oligomers are administered to wild-type mice, they induce both synaptic and mitochondrial toxicity (Lasagna-Reeves, Castillo-Carranza et al. 2011). Overexpression of a chaperone inducing tau oligomerization causes neuronal toxicity in mice (Blair, Nordhues et al. 2013). Furthermore, there is evidence that tau oligomers seed the misfolding of endogenous tau (Frost, Jacks et al. 2009) and are responsible for the spread of tau pathology from affected regions in the brain to unaffected areas (Gerson and Kayed 2013). Primary mouse neurons in culture internalize tau oligomers and small tau aggregates and spreading occurs both anterogradely and retrogradely, but when treated with tau fibrils and monomer, uptake does not occur (Wu, Herman et al. 2013). Tau oligomers spread from injection site to connected brain regions in wild-type mice, while

mice treated with fibrils do not show the same pattern of propagation (Lasagna-Reeves 2012).

Passive immunotherapy targeting tau oligomers may be a viable option for halting this spread of pathology, without affecting functional tau monomer (Castillo-Carranza, Lasagna-Reeves et al. 2013). Though there is a great deal of evidence for the importance of tau in Alzheimer's disease, most of the research on therapeutics thus far has targeted amyloid-β (Aβ). Passive immunotherapy in mice overexpressing mutant amyloid precursor protein (PDAPP mice) conferred cognitive benefits without decreasing amyloid plaque burden, suggesting that targeting soluble amyloid species, rather than large aggregates, is beneficial for memory (Dodart 2002). While safety concerns have been implicated in passive vaccination against Aβ, it appears as though adverse effects depend upon binding to Aβ deposits (Wilcock 2004, Racke, Boone et al. 2005). A few studies have been conducted using antibodies against tau, yielding cognitive benefits however the target has mainly been phosphorylated tau, rather than tau oligomers which confer toxicity. Additionally, these immunizations saw a decrease in NFTs, which do not seem to be toxic and may even be neuroprotective (Boutajangout, Ingadottir et al. 2011, Chai, Wu et al. 2011, d'Abramo, Acker et al. 2013). Therefore, using passive immunotherapy to target only toxic, soluble, oligomeric tau species appears to be a promising strategy.

Here we investigate the prevention of pathological tau accumulation and cognitive deficits in Htau mice immunized with anti-tau oligomer-specific monoclonal antibody (TOMA). Htau mice overexpress human tau without the expression of endogenous mouse tau. At 3 months, tau begins to relocate from microtubules in the axons to the cell body, which is important for tau oligomerization. By 9 months, tau aggregates are seen,

increasing more by 13-15 months (Duff, Knight et al. 2000, Kelleher, Garwood et al. 2007, Polydoro, Acker et al. 2009). In humans, tau is alternatively spliced to form six isoforms with either three or four microtubule binding repeats, 3R and 4R tau, both of which are affected in AD. However, while in humans 3R and 4R tau levels are approximately equal in adulthood, 4R tau is generally present in higher levels in adult mice (McMillan 2008) and only three isoforms are expressed (Gotz 1995). As there is evidence for differences in the seeding and spread of 3R and 4R tau, such as research showing that 3R tau may be more likely to be secreted (Karch, Jeng et al. 2012), Htau mice are a good model to study tau pathology in AD. Aggregation properties of 3R and 4R tau differ as well (Spillantini and Goedert 1998) and shifts in the normally equal ratio of 3R:4R tau have been found to be associated with neurodegeneration (Hutton 2001). The ratio of 3R:4R tau is also altered in Htau mice as they age (Duff, Knight et al. 2000). Additionally, Htau mice do not show any motor or sensory deficits, but do have memory deficits, impairment to long-term potentiation (LTP), and changes to dendritic spine morphology and volume in old age, making them an optimal animal model for study of cognitive impairment in human AD (Polydoro, Acker et al. 2009, Dickstein, Brautigam et al. 2010). Previous findings have shown that aged Htau mice treated with curcumin exhibit decreased tau dimer levels correlating with memory improvement (Ma, Zuo et al. 2013). Therefore, tau oligomers likely play a role in the phenotype of Htau mice.

In this study, 3-month-old Htau and wild-type mice first received intracerebroventricular injection of brain-derived tau oligomers and a single intravenous injection of TOMA or non-specific IgG and were tested for memory deficits at 3 days, 1 month, and 3 months post-injection using novel object recognition, as well as analyzed

for tau pathology (Fig. 4.3C). In a second experiment, mice were given bi-weekly injections of TOMA or control IgG following administration of tau oligomers and were tested at 3 days, 3 months, 6 months, and 9 months after treatment (Fig. 4.3D). Additionally, two groups of 3-month-old Htau mice were given bi-weekly injections of either 60 μg of TOMA or non-specific IgG without application of brain-derived tau oligomers in order to test whether TOMA provides cognitive protection against deficits arising during aging in Htau mice.

Methods

Preparation of Brain-Derived Tau Oligomers

Immunoprecipitation of tau oligomers from AD frontal cortex was performed as previously described in Chapter 2 (Lasagna-Reeves, Glabe et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012, Castillo-Carranza, Gerson et al. 2014, Gerson, Sengupta et al. 2014, Gerson, Castillo-Carranza et al. 2016).

Toxicity Assays

Alamar blue and MTS toxicity assays were completed as described previously (Lasagna-Reeves, Castillo-Carranza et al. 2010, Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves 2012). For the Alamar blue assay, SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) to confluence in 96-well plates. Cells were treated with different concentrations (0.01, 0.05, 0.1, 0.2, and 0.5 μM) of either brain-derived tau oligomers, paired helical filaments (PHF), or PBS as an untreated control for 4 h. Cell survival was measured using the AlamarBlue assay kit (Serotec). Fluorescence was measured at 590 nm using a POLARstar Omega

fluorescence microplate reader, with all treatments completed in triplicate. For the MTS assay, SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) to confluence in 96-well plates and treated with either PBS control, brain-derived tau oligomers, brain-derived PHF, or brain-derived tau fibril mixed with TOMA at different molar ratios. After four hours, cell viability was measured using the colorimetric Tetrazolium-based MTS assay (Promega) at 490 nm using a POLARstar Omega fluorescence microplate reader. Measurements were completed in triplicate and results were analyzed by student's t-test.

Animals

For experiment 1, three-month-old homozygous Htau mice (The Jackson laboratory) were given intravenous injections of either 60 μg/animal of TOMA antibody for the treatment group or non-specific IgG (Rhodamine, Genetex cat. GTX29093) for the control group (n=14 animals/group). A third group of 3-month-old C57BL/6 wild-type mice (The Jackson laboratory) received saline injection. One hour after antibody treatment, mice received stereotaxic bilateral injection of 0.6 μg/hemisphere of brain-derived tau oligomers as previously described in Chapter 3 (Lasagna-Reeves, Castillo-Carranza et al. 2012, Castillo-Carranza, Gerson et al. 2014, Gerson, Castillo-Carranza et al. 2016). All animals used were male in order to control for changes to cognition occurring in different hormonal states in female mice.

Generation of Tau Oligomer Monoclonal Antibody (TOMA)

Tau oligomers were prepared in PBS as previously described (Lasagna-Reeves, Castillo-Carranza et al. 2012) and purified by fast protein liquid chromatography (FPLC,

Superdex 200 HR 10/30 column, Amersham Biosciences). Two-month-old BALB/c mice were immunized with tau oligomers according to standard procedures using Freund's complete adjuvant (CFA). The mice received an intraperitoneal injection of 100 µl of 1:1 (antigen: adjuvant) on the ventral side (20 µg/mouse). Two weeks later, a second injection of antigen with Freund's incomplete adjuvant (IFA) was performed, followed by 5 boosts. Anti-tau oligomer antibody response was determined by screening serial dilutions of animal sera using an enzyme-linked immunosorbant assay (ELISA). ELISA plates were coated with 50 ng or 200 ng of either tau oligomers, Aβ oligomers, or α-synuclein oligomers to rule out cross-reactivity with other amyloid oligomers. Dot blot was also used to test TOMA specificity. Selected clones were tested by Western blot using *in vitro* prepared samples, and dot blot using brain homogenates. Finally, the selected clones (TOMA) were tested using human and mouse brains. Antibody isotyping and light chain composition, κ or λ, was determined using an IsoStrip™ mouse monoclonal antibody isotyping kit (Rapid Isotyping kit Plus Kappa & Lambda-mouse, Pierce). TOMA was produced from hybridoma cells grown in X-VIVO 15 (LONZA) medium following standard conditions for cell culture. The antibody was purified from the medium by standard affinity chromatography methods followed by high-performance liquid chromatography (HPLC) purification (purity > 95%). TOMA used in immunization studies was endotoxin free, as confirmed using a commercially available kit (Limulus amebocyte lysate, Chromogenic Endpoint Assay, Hycult Biotechnology). All TOMA samples were stored in appropriate endotoxin free vials at -80ºC until use.

Intravenous (IV) Injection

Htau mice were either immunized with 60 µg of TOMA/animal or 60 µg/animal of non-

specific IgG, Rhodamine. Wild-type mice were injected with 60 µl of saline solution. For IV injections, mice were placed in a restrainer (Braintree Scientific) and an inch of the tail was shaved and placed in warm water to dilate veins. Sixty µg were then injected into the lateral tail vein. Mice were returned to home cages and kept under observation.

In Vivo Imaging

Six and 18-month-old Htau and 18-month old tau knock-out mice were IV injected in the tail vein with 60 µg of TOMA labeled with a far red probe (Kodak X-Sight 640 LSS Dye, excitation 650 nm, emission 750 nm, Carestream Molecular Imaging) according to manufacturer's instructions. Briefly, 2 mg/ml of antibody was incubated with the dye for 1 hr (no light, on ice). Unlabeled antibody was removed by purifying the conjugation reaction in a column. The successful labeling of the antibody was calculated. Before injection, Htau and knock-out tau mice were shaved and anesthetized with a ketamine-xylazine cocktail. Immediately after injection, the animals were placed in the dorsal position on animal trays and imaged using the Kodak Multispectral Image Station. Mice were imaged at 30 minutes, 1, 2, 4, and 6 hrs post-injection. After imaging, mice were transcardially perfused with 1X PBS. Brains were extracted from animals and immediately imaged. Whole body optical and X-ray imaging were performed using the Kodak multispectral in vivo FX imaging system (Carestream).

Stereotaxic Injection of Brain-Derived Tau Oligomers

Htau and wild-type mice were injected with AD-derived tau oligomers bilaterally in the hippocampus as described in Chapter 3 (Lasagna-Reeves, Castillo-Carranza et al. 2011,

Lasagna-Reeves, Castillo-Carranza et al. 2012, Castillo-Carranza, Gerson et al. 2014, Gerson, Castillo-Carranza et al. 2016).

Novel Object Recognition (NOR)

Three days after receiving TOMA and brain-derived tau oligomer injection, animals from Experiment 1 were evaluated on the NOR task as described in Chapter 3 (Castillo-Carranza, Gerson et al. 2014, Gerson, Castillo-Carranza et al. 2016). In experiment one, the testing phase was repeated one month and three months after treatment. In experiment two, following testing three days post-treatment, mice were subjected to bi-weekly injections of TOMA and NOR was repeated at 3, 6, and 9 months after initial injection.

Tissue Collection and Immunohistochemistry

Following behavioral testing, brains were collected and processed as described in Chapters 2 and 3 (Castillo-Carranza, Gerson et al. 2014, Gerson, Sengupta et al. 2014, Gerson, Castillo-Carranza et al. 2016). The right hemisphere was embedded in paraffin and sectioned. Immunohistochemistry was performed on paraffin-embedded sections. All sections were processed simultaneously under the same conditions. Sections (8 μm) were deparaffinized and rehydrated. After blocking in normal goat serum for 1 hr, sections were incubated overnight with anti-tau oligomer-specific polyclonal antibody, T22 (1:300). The following day, sections were washed in PBS three times for 10 minutes each and incubated with biotinylated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch) for 1 hr. Sections were then washed three times for 10 minutes each in PBS and visualized using an ABC reagent kit (Vector Laboratories), according to the

manufacturer's recommendations. Lastly, sections were counterstained with hematoxylin (Vector Laboratories) for nuclear staining and mounted. For Gallyas silver staining, we used the FD NeuroSilverKit II (FD NeuroTechnologies, Columbia MD) and brain sections were stained according to manufacturer instructions. Immunofluorescence with anti-tau oligomer-specific polyclonal antibody, T22, Tau-5 and hyperphosphorylated tau antibody, AT8 was completed as described in Chapter 2 The sections were examined using an epifluorescence microscope (Nikon Eclipse 800) equipped with a CoolSnap-FX monochrome CCD camera (Photometrics) using standard Nikon FITC and DAPI filters, and images were acquired.

Tissue Processing

Frozen brains extracted from Htau and wild-type mice were homogenized as described in Chapter 2 (Castillo-Carranza, Gerson et al. 2014, Gerson, Sengupta et al. 2014). Pellets were collected for analyses of PBS-insoluble tau.

Western Blot

Western blot of brain homogenate with T22 for tau oligomers, Tau-5 for total tau, AT8 for phospho tau and actin was completed as described in Chapter 2 (Castillo-Carranza, Gerson et al. 2014, Gerson, Sengupta et al. 2014).

ELISA

ELISA analysis of the PBS soluble and insoluble fractions of brains was completed as described in Chapter 2 (Castillo-Carranza, Gerson et al. 2014, Gerson, Sengupta et al. 2014).

Results

Htau Mice Show Increased Tau Oligomer Levels with Age

Htau mice overexpress non-mutated human tau and do not express mouse tau. Researchers have previously shown that Htau mice begin to show evidence of tau mislocalization necessary to initiate oligomerization starting at 3 months of age and begin to see NFTs by 9 months of age, increasing significantly by 13-15 months [39, 45, 46]. In order to specifically test for the presence of oligomeric tau, immunohistochemistry with anti-tau oligomer antibody, T22 was completed on brain sections from Htau mice at different ages and tau knockout (KO) mice (Figure 4.1A-H). Tau oligomers were detected in the cerebellum and the hippocampus of Htau mice and levels increased with age. No T22 staining was seen in the cerebellum or the hippocampus of tau knockout mice, as expected. In order to quantify tau oligomer levels, brains were collected from Htau mice of various ages from 3 months to 22 months, as well as from tau knockout and wild-type (Balb/C) mice at 3 and 6 months. Immunofluorescence staining was also completed with T22 and Tau-5 in the hippocampus of 11-month-old Htau and tau knockout mice (Figure 4.1I-N). Tau-5 staining was widely distributed across the hippocampus, partially colocalizing with T22-positive tau oligomers in Htau mice, while no tau was detected in tau knockout mice, as expected. Immunofluorescence with AT8 and T22 in the hypothalamus of 11-month-old Htau mice (Figure 4.1O-Q) revealed the presence of AT8-positive hyperphosphorylated tau and T22-positive tau oligomers present in adjacent cells.

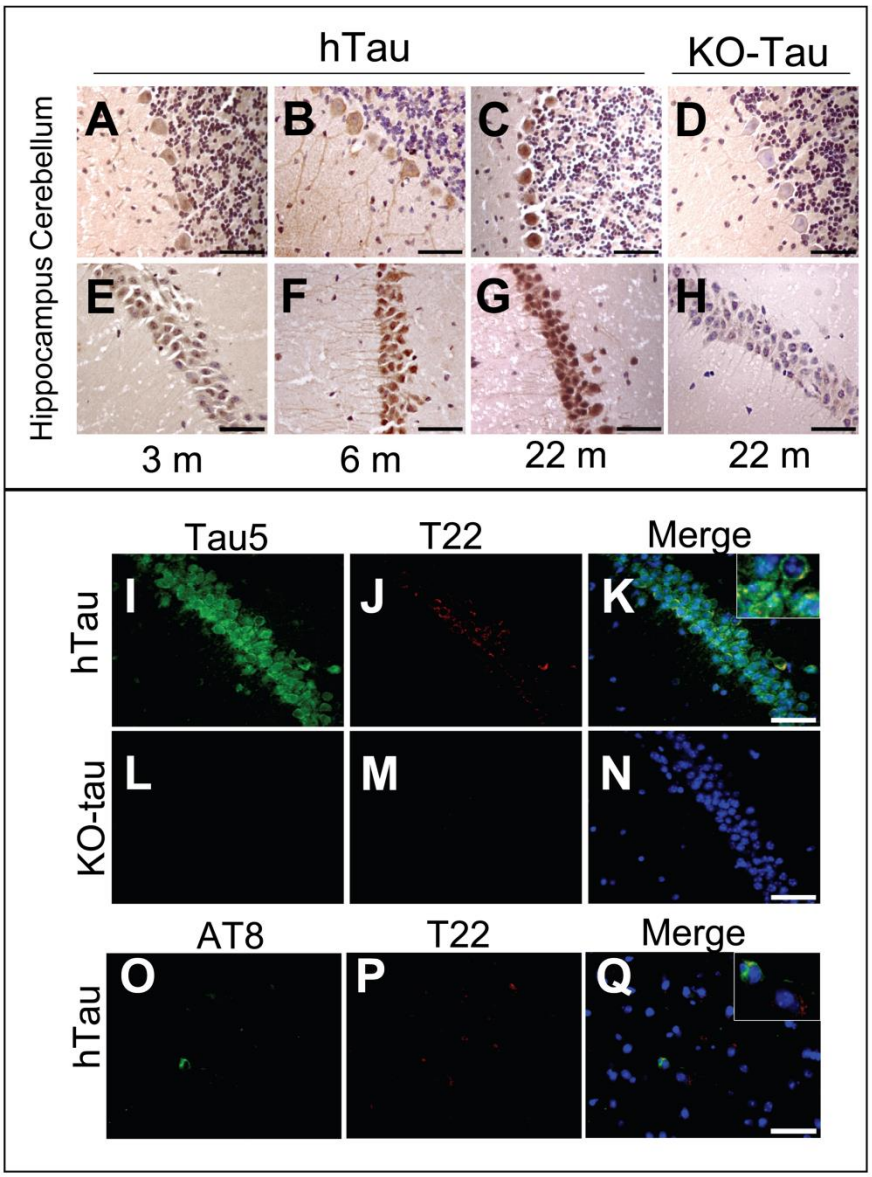

Figure 4.1 Tau Oligomer Levels in Htau Mice Increase with Age. Immunohistochemistry with T22 of Htau mice at different ages and tau knockout (KO) mice (A-H). T22-positive tau oligomers can be detected in the cerebellum (A-C) and the hippocampus (E-G) of Htau mice and levels increase with age. No T22 staining is observed in the cerebellum (D) or the hippocampus (H) in tau knockout mice. Scale bar 50 μm. Immunofluorescence with T22 and Tau-5 in the hippocampus of 11-month-old Htau and tau knockout (KO-tau) mice (I-N). Tau-5 staining was widely distributed across the

hippocampus (I), partially colocalizing with T22-positive tau oligomers in Htau mice (J-K), while no tau was detected in tau knockout mice (L-N). Immunofluorescence with AT8 and T22 in the hypothalamus of 11-month-old Htau mice (O-Q) revealed the presence of AT8-positive hyperphosphorylated tau (O) and T22-positive tau oligomers (P) present in adjacent cells. Scale bar 25 μm.

Western blot of Htau mice of different ages (6, 11-12, and 22 months) with Tau-5 and TOMA revealed an increase in higher molecular weight oligomeric tau species with age, while no tau was detected in tau knockout (tau KO) mice (Figure 4.2A-B). Direct ELISA with T22 of Htau, wildtype (WT), and tau knockout mice of different ages showed that 6-month-old Htau mice had a significantly higher level of tau oligomers than age-matched knockout and wildtype mice ($p<0.001$) (Figure 4.2C). Therefore, the presence of tau oligomers increases with age in Htau mice, precedes the established time point at which NFTs appear and may initiate toxicity prior to detection of large tau aggregates.

TOMA Reacts Specifically with Tau Oligomers

In order to verify the specificity of the anti-tau oligomer-specific monoclonal antibody (TOMA), we used western blot of recombinant 3R and 4R tau and found that TOMA specifically recognizes only oligomers from both 3R and 4R tau, with no detection of tau monomer seen with Tau-5 (Figure 4.2D). We used direct ELISA to test oligomeric species of various amyloid proteins and monomeric and fibrillar tau species (Figure 4.2E). We found that while TOMA reacts with both 3R and 4R tau oligomers, TOMA does not react with oligomers of any other amyloid proteins (synuclein, $A\beta_{40}$, $A\beta_{42}$, or

prion protein) or with tau monomer or fibril. Therefore, TOMA specifically targets only tau oligomers.

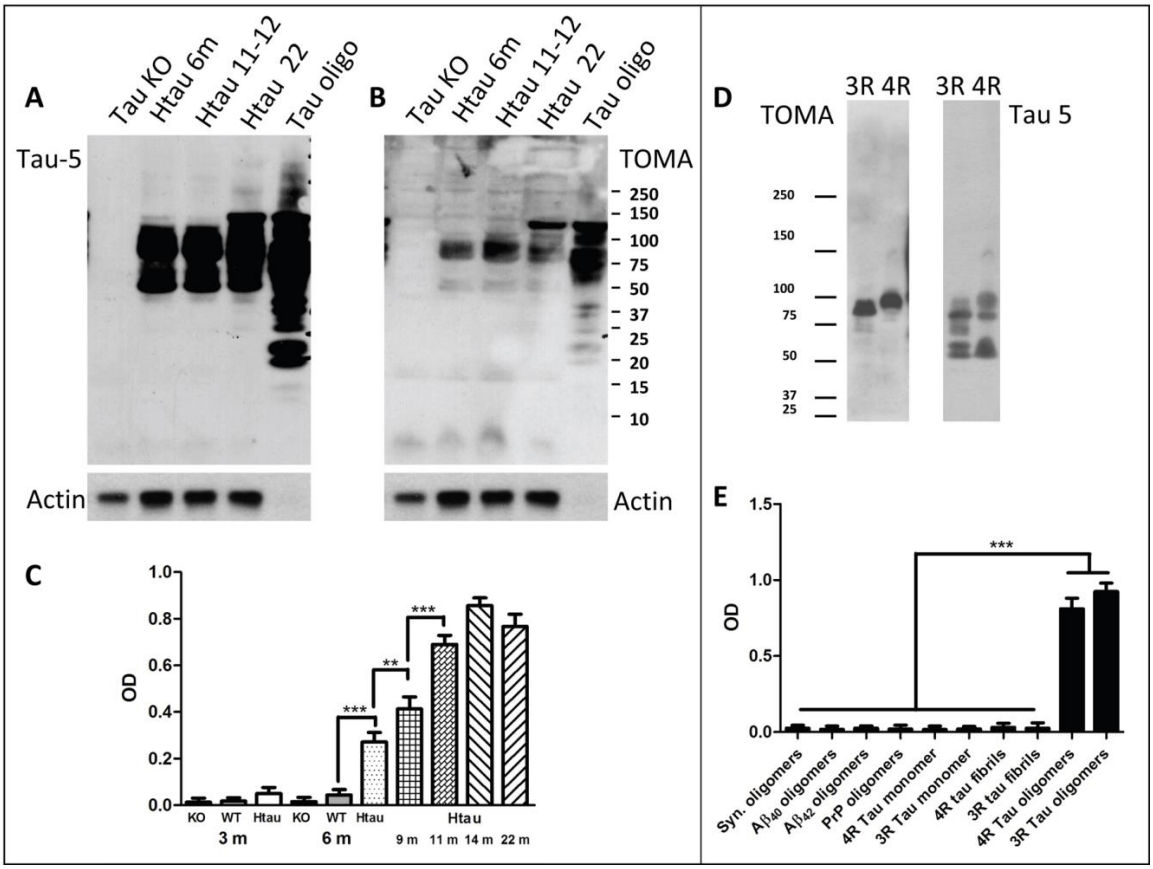

Figure 4.2 TOMA Specifically Recognizes Tau Oligomers that Increase with Age in Htau Mice. Western blot of Htau mice of different ages (6, 11-12, and 22 months) with Tau-5 (A) and TOMA (B) revealed an increase in higher molecular weight oligomeric tau species with age. No tau was detected in tau knockout (tau KO) mice. Direct ELISA with T22 of Htau, wild-type (WT), and tau knockout mice of different ages (C). 6-month-old Htau mice had a significantly higher level of tau oligomers than age-matched knockout and wildtype mice (***$p<0.001$). Oligomer levels continued to rise until 14 months of age and then did not significantly change up to 22 months. Western blot of recombinant 3R and 4R tau showed that TOMA specifically recognizes only oligomers

from both 3R and 4R tau, with no detection of tau monomer seen with Tau-5 (D). Direct ELISA with TOMA of amyloid oligomers and tau (E). TOMA reacts with both 3R and 4R tau oligomers, but not with oligomers of synuclein, $A\beta_{40}$, $A\beta_{42}$, or prion protein or with tau monomers or fibrils (***$p<0.001$).

Tau Oligomers, not Fibrils Cause Toxicity in SH-SY5Y Cells and Cognitive Impairment *In Vivo*

We have previously shown that tau oligomers induce toxicity *in vitro* and *in vivo* (Lasagna-Reeves, Castillo-Carranza et al. 2010, Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves 2012). Using the MTS toxicity assay, we show that brain-derived tau oligomers significantly decrease cell viability compared to untreated cells and cells treated with PHFs ($p<0.001$) (Figure 4.3A). When TOMA was added along with tau treatment, cells treated with tau oligomers were protected from toxicity. Using the Alamar blue assay, we replicated findings that tau oligomers can induce toxicity (Figure 4.3B).

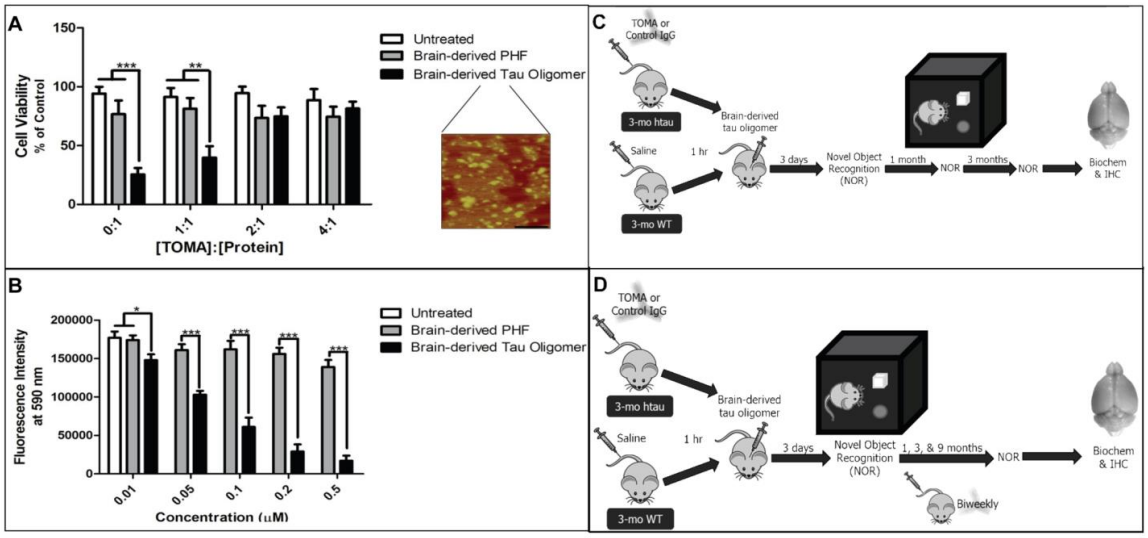

Figure 4.3 Tau Oligomers Decrease Cell Viability *In Vitro.* Toxicity measured by the MTS toxicity assay (A). Brain-derived tau oligomers significantly decrease cell viability compared to untreated cells and cells treated with PHF and tau fibrils (***$p<0.001$). When TOMA is added along with tau treatment, cells treated with tau oligomers are protected from toxicity. At a 1:1 ratio of TOMA to tau oligomers, cell viability is still significantly lowered (**$p<0.01$), while at a 2:1 ratio, tau oligomer toxicity is prevented and there is no significant difference between treatment with tau oligomers, PHF, or fibrils, or with untreated cells. Inset image shows characterization of brain-derived tau oligomers by AFM. Scale bar 100 nm. Tau oligomer toxicity detected with the Alamar Blue assay (B). Brain-derived tau oligomer treatment at the lowest concentration (0.01μM) significantly decreased cell viability compared to untreated cells and cells treated with brain-derived PHF (*$p<0.05$). Toxicity increased with increasing concentrations of oligomers (***$p<0.001$). Schematic depicting the timeline for experiment 1 (C) and experiment 2 (D).

Brain-derived tau oligomer treatment significantly decreased cell viability compared to untreated cells and cells treated with brain-derived PHF ($p<0.001$). Toxicity increased with increasing concentrations of oligomers. Wild-type (Balb/C) were injected with either PBS, tau monomers, tau fibrils, or tau oligomers ICV at 3 months of age. Using the NOR task 3 days after injection, only mice given tau oligomers were significantly impaired when compared to controls injected with PBS (data not shown).

Experiment 1

Single Injection with TOMA Antibody Prevents Object Discrimination Deficits in Htau Mice

In order to confirm that TOMA injected into the tail vein was able to cross the blood-brain-barrier and bind to tau oligomers in the Htau brain, we used *in vivo* imaging (Figure 4.4A). Binding of TOMA to tau oligomers was confirmed by imaging brain extracted from animals 6 hours after TOMA treatment (Figure 4.4B-C).

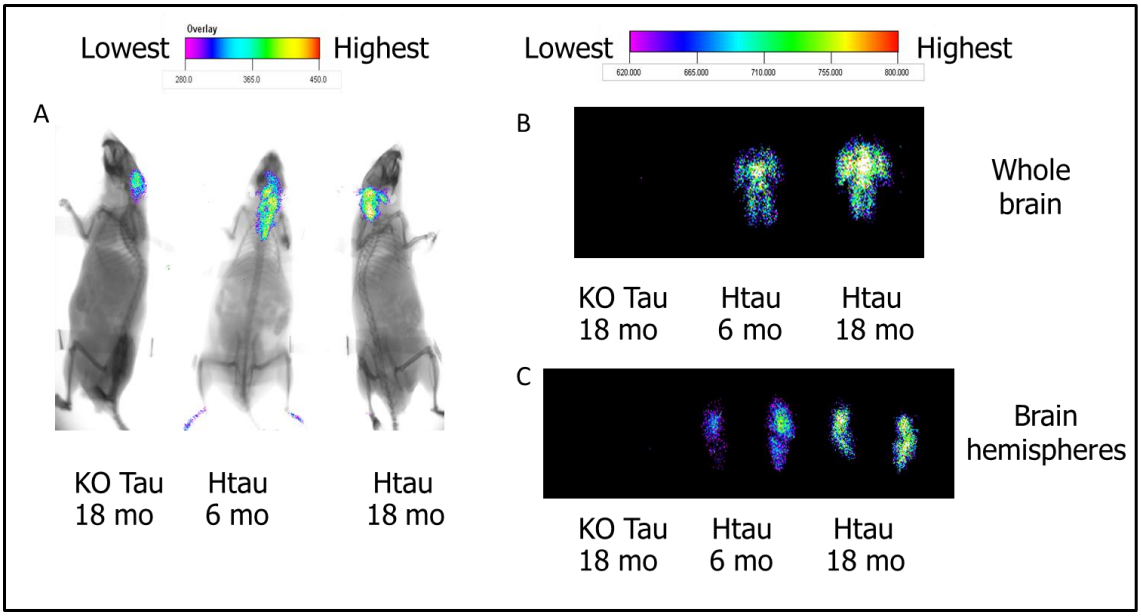

Figure 4.4 *In vivo* live imaging demonstrates that a fraction of TOMA injected into the tail vein crosses the blood-brain-barrier and binds to tau oligomers in 6 and 18-month-old htau brain (A). Whole brain (B) and brain divided by hemisphere (C) extracted from Htau mice demonstrate higher levels of fluorescence due to TOMA than brains from tau knock-out mice.

Previous study has shown that Htau mice do not show any motor, sensory, or general health deficits in comparison to age-matched wild-type mice up to 12 months of age. Additionally, young mice do not show any memory deficits as measured by the visual recognition memory task, NOR and the spatial memory task, the Morris water maze. However, 12-month-old Htau mice are cognitively impaired (Polydoro, Acker et al. 2009). In order to test whether young Htau mice which have been injected with tau oligomeric seeds show deficits and whether targeting oligomers can attenuate detrimental effects, we used the NOR task (Figure 4.3C). Once mice had been habituated to the test

environment, mice from experiment 1 were tested on the apparatus at 3 days, 1 month, and 3 months after receiving TOMA injection IV and brain-derived tau oligomer injection ICV. At 3 days and 1 month following treatment, Htau mice treated with TOMA spent a significantly higher percentage of time exploring the novel object than the familiar object ($p<0.001$). However, Htau mice treated with control IgG showed no preference for the novel object over the familiar object. After 3 months, both groups were impaired, showing no differences between time spent investigating the two objects (Figure 4.5A), while wildtype mice were still protected by TOMA after 3 months (data not shown). This difference in cognitive benefit between wildtype and Htau mice highlights the importance of using mice expressing human tau and is likely due to the heightened ability of human brain-derived tau oligomers to seed the misfolding of tau in Htau mice compared to wildtype mice. Differences were not due to exploratory behavior as there were no differences in total exploration time (data not shown). Similar results were found with the discrimination index, calculated as the time spent exploring the familiar object subtracted from the time spent exploring the novel object, divided by the total time spent exploring both objects. Htau mice receiving IgG had a discrimination index ~0, performing at chance levels and showing no preference for the novel object over the familiar object, while Htau mice receiving TOMA exhibited preference for the novel object approaching significance and performed significantly better than IgG-treated animals at 3 days ($p<0.001$) and 1 month ($p<0.05$) post-treatment (Figure 4.5B). Similarly, wild-type mice injected with tau oligomers and non-specific IgG were impaired after 3 days and TOMA injection was protective (data not shown). Therefore,

the application of oligomeric seeds induced memory deficits in young Htau mice, which were protected when treated with a single injection of TOMA for up to 1 month.

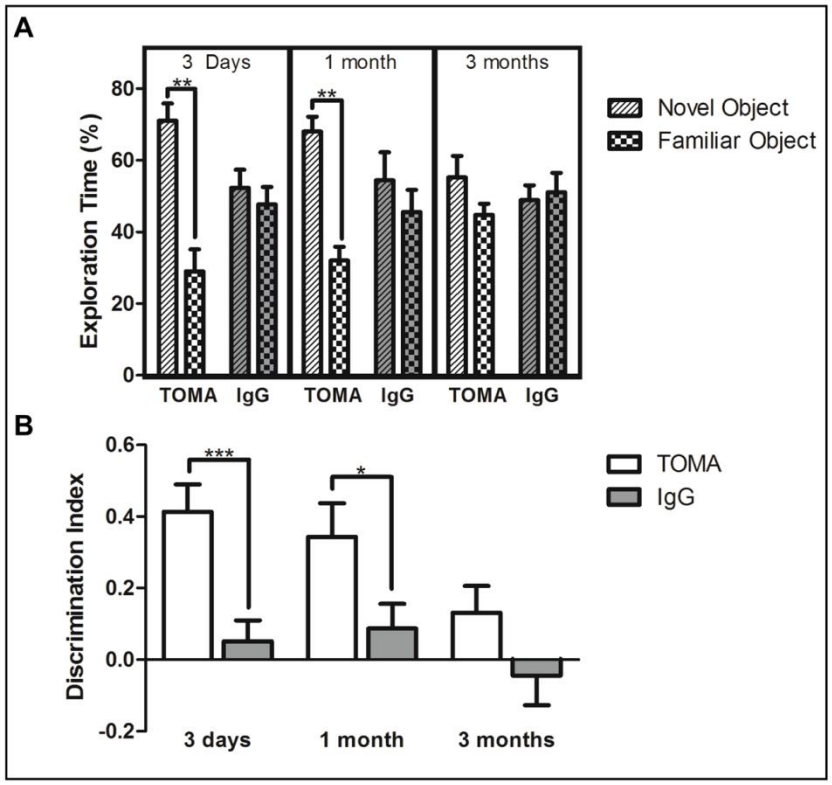

Figure 4.5 Sensory recognition memory was impaired in Htau mice injected with brain-derived tau oligomers and was protected in mice receiving TOMA treatment (white bars) rather than non-specific IgG (grey bars). Comparison of the percent exploration time spent on the novel object (striped bars) versus the familiar object (checkered bars) (A). Htau mice receiving control IgG showed no preference for the novel object over the familiar object, while mice receiving TOMA spent significantly more time exploring the novel object than the familiar object at 3 days and 1 month after injection (**$p<0.01$). Comparison of the discrimination index, calculated as the time spent exploring the familiar object subtracted from the time spent exploring the novel object divided by the

total time spent exploring both objects (B). Htau mice receiving IgG had a discrimination index ~0, performing at chance levels and showing no preference for the novel object over the familiar object, while Htau mice receiving TOMA exhibited preference for the novel object approaching significance ($p=0.05$) and performed significantly better than IgG-treated animals at 3 days (***$p<0.001$) and 1 month (*$p<0.05$) post-treatment.

Single TOMA Injection Reduces Tau Oligomers, but not Total Tau or Hyperphosphorylated Tau

Following behavioral testing, brains were collected and divided for biochemical and immunohistochemical analysis. Immunohistochemistry with T22 revealed a decrease in tau oligomer-positive cells in the CA2 and dentate gyrus regions of the hippocampus in mice treated with TOMA compared to control IgG and tau knockout mice (Figure 4.6A-F) while no difference was seen in AT8-positive NFTs (data not shown). Brains were homogenized and the PBS soluble and insoluble fractions were collected. ELISA of the PBS soluble fraction with T22 showed that mice treated with TOMA had significantly lower levels of tau oligomers than those treated with control IgG ($p<0.001$) (Figure 4.6G). However, no differences were detected by ELISA with Tau-5 which recognizes all forms of tau (Figure 4.6H). The PBS insoluble fraction was analyzed for hyperphosphorylated tau NFT levels as measured with AT8 (Ser202/Thr205 phosphorylated tau) by ELISA and no differences were seen between TOMA and non-specific IgG mice (Figure 4.6I). Therefore, a single injection with TOMA lowers levels of toxic tau oligomers, but does not affect total tau levels or NFT levels.

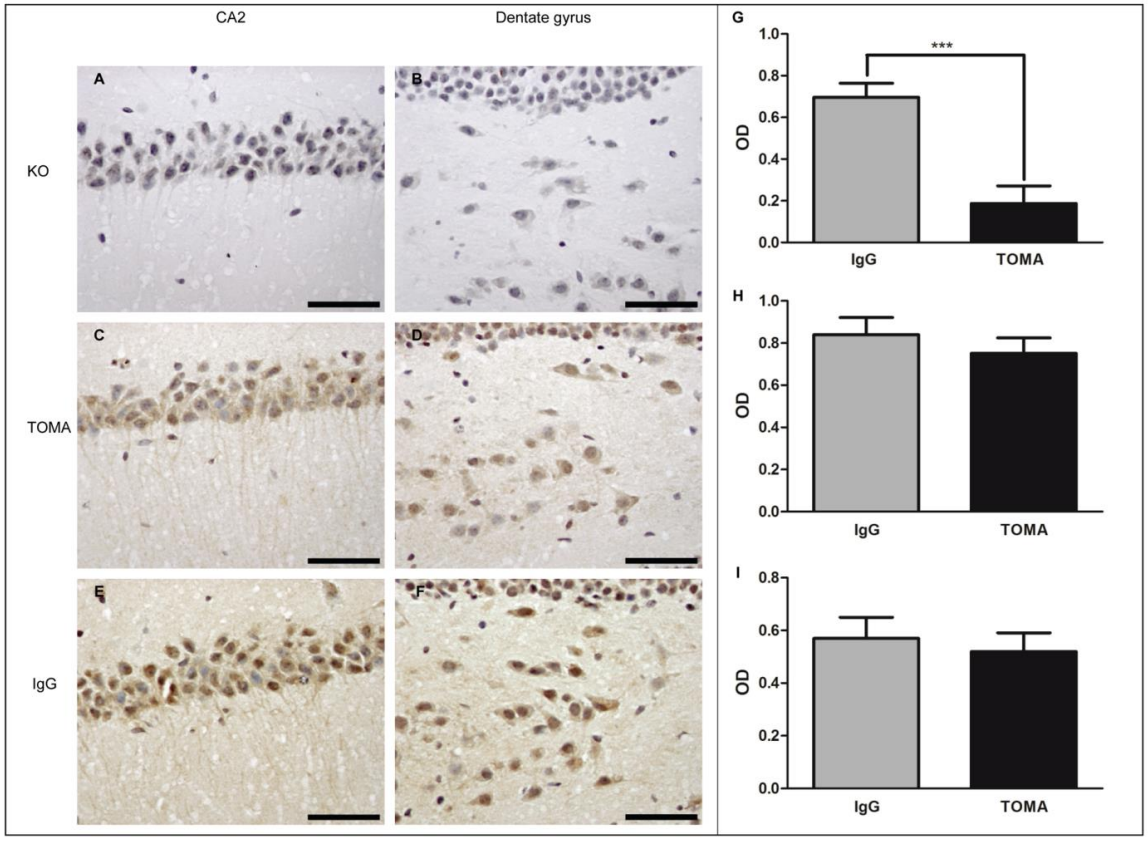

Figure 4.6 Tau oligomers are specifically decreased in the hippocampus of TOMA-treated mice. Immunohistochemistry with T22 (A-F) showed a decrease in tau oligomers in the CA2 and the dentate gyrus regions of the hippocampus in mice treated with TOMA (C-D) compared to control IgG (E-F) and tau knockout mice (A-B). Scale bar 50 µm. ELISA of the PBS soluble fraction with T22 (G) showed that mice treated with TOMA had significantly lower levels of tau oligomers than those treated with control IgG (***$p<0.001$). No differences were detected by ELISA in total tau levels as measured with Tau-5 (H) or in hyperphosphorylated tau levels as measured with AT8 (I).

Experiment 2

Biweekly Injections with TOMA Antibody Prevent Object Discrimination Deficits in Htau Mice

In order to test whether bi-weekly injections of TOMA can confer protection against cognitive deficits seen in young Htau mice injected with tau oligomeric seeds, the NOR task was again used (Figure 4.3D). In experiment 2, mice were tested at 3 days, 3 months, 6 months, and 9 months following the initial treatment and received bi-weekly TOMA injections throughout testing. Htau mice receiving TOMA injections spent significantly more time exploring the novel object than the familiar object at 3 days, 3 months, 6 months, and 9 months after initial treatment ($p<0.001$), while mice receiving control IgG were unable to discriminate between the two objects (Figure 4.7A). In order to control for overall exploratory behavior, the discrimination index was also measured in tau-oligomer treated mice receiving control IgG or TOMA. Htau mice receiving bi-weekly IgG injections had a discrimination index ~0, performing at chance levels and showing no preference for the novel object over the familiar object, while Htau mice receiving bi-weekly TOMA exhibited preference for the novel object ($p<0.001$) and performed significantly better than IgG-treated animals from 3 days to 9 months post-treatment ($p<0.05$) (Figure 4.7B). Similar results were observed in wildtype mice receiving either TOMA or control IgG (data not shown). Additionally, 3-month-old Htau mice were given bi-weekly injections of either 60 µg of TOMA or non-specific IgG without application of brain-derived tau oligomers in order to test whether TOMA provides cognitive protection against deficits due to normal aging in these mice and to control for any effects TOMA treatment may have on cognition or inflammation. Htau mice receiving bi-weekly control IgG and TOMA displayed preference for the novel

object over the familiar object up to 6 months of age ($p<0.001$) (Figure 4.7C). At 9 months of age, memory began to decline in Htau mice receiving bi-weekly non-specific IgG, while mice receiving bi-weekly TOMA still appeared cognitively normal, spending significantly more time exploring the novel object than the familiar object up to 12 months of age ($p<0.001$). No evidence of inflammation was seen in TOMA-treated mice. Based on these results, it appears that TOMA treatment is capable of counteracting cognitive detriment due to the toxicity of tau oligomers without resulting in any negative effects on general health.

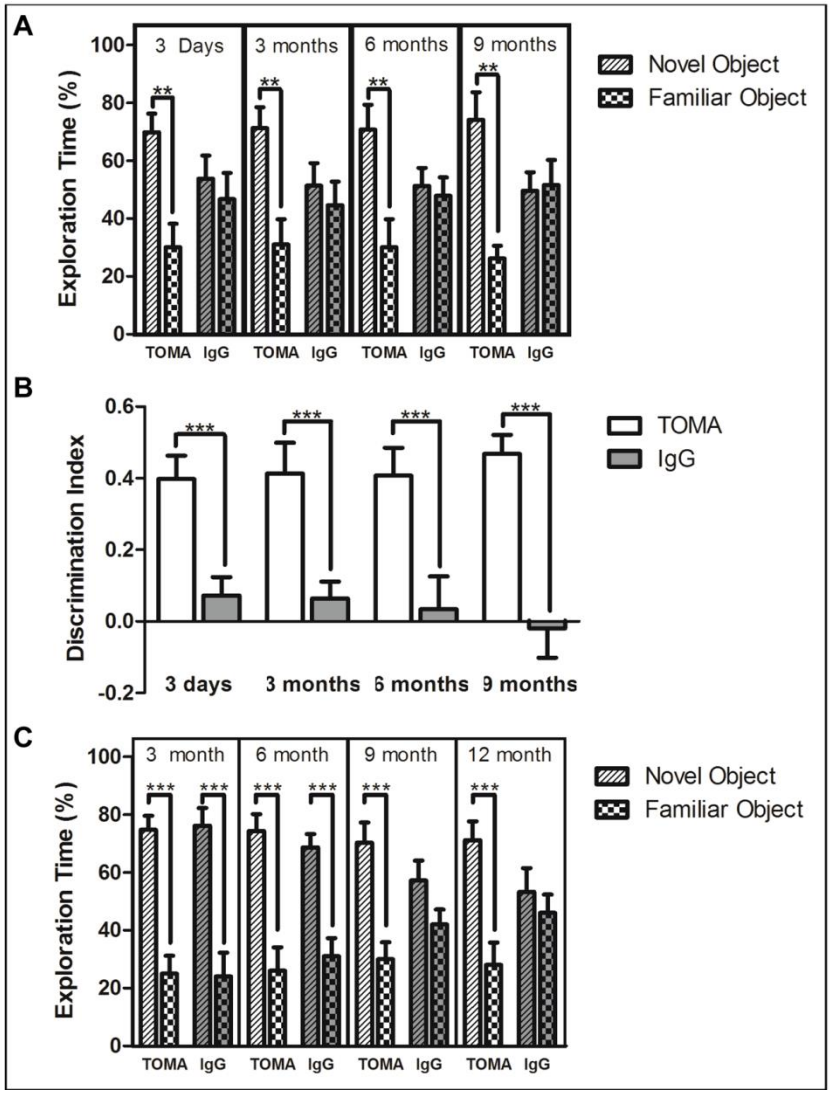

Figure 4.7 Biweekly TOMA treatment protects against sensory recognition memory deficits induced by tau oligomer injection. Sensory recognition memory was impaired in Htau mice injected with brain-derived tau oligomers and was protected in mice receiving TOMA treatment (white bars) rather than non-specific IgG (grey bars). Comparison of the percent exploration time spent on the novel object (striped bars) versus the familiar object (checkered bars) (A). Htau mice receiving bi-weekly control IgG showed no preference for the novel object over the familiar object after 3 days, while mice receiving bi-weekly TOMA spent significantly more time exploring the novel object than the

familiar object up to 9 months after initial injection (**p<0.01). Comparison of the discrimination index (B). Htau mice receiving bi-weekly non-specific IgG had a discrimination index ~0, performing at chance levels and showing no preference for the novel object over the familiar object, while Htau mice receiving TOMA exhibited preference for the novel object (***p<0.001) and performed significantly better than control IgG-treated animals from 3 days to 9 months (*p<0.05) post-treatment. Comparison of the percent exploration time spent on the novel object (striped bars) versus the familiar object (checkered bars) in control Htau mice not receiving injection of brain-derived tau oligomers (C). Htau mice receiving bi-weekly control IgG and TOMA displayed preference for the novel object over the familiar object up to 6 months of age (***p<0.001). At 9 months of age, memory began to decline in Htau mice receiving bi-weekly non-specific IgG, while mice receiving bi-weekly TOMA were protected and spent significantly more time exploring the novel object than the familiar object up to 12 months of age (***p<0.001).

Biweekly TOMA Injections Reduce Tau Oligomers, but not Total Tau or Hyperphosphorylated Tau

Brains were collected and divided for biochemical and immunohistochemical analysis. ELISA of the PBS soluble fraction with T22 showed that mice treated with TOMA had significantly lower levels of tau oligomers than those treated with control IgG (p<0.001) (Figure 4.8A). Lowered levels of tau oligomers in TOMA-treated mice were also seen by western blot with T22 of the PBS soluble fraction of Htau brains (Figure 4.8B-C). However, no differences were detected by ELISA or Western blot in total tau levels as

measured with Tau-5 (Figure 4.8D-F). No differences in hyperphosphorylated tau levels as measured with AT8 were seen by ELISA or western blot between TOMA and non-specific IgG-treated mice (Figure 4.8G-I). Similarly, immunohistochemistry with AT8 (Ser202/Thr205 phosphorylated tau) and Gallyas silver staining did not reveal any changes to levels of NFTs in Htau mice treated with TOMA versus non-specific IgG (Figure 4.8J-M). Therefore, as expected, TOMA treatment selectively targeted oligomeric tau, without lowering levels of hyperphosphorylated NFTs or tau monomer and decreasing tau oligomers alone was enough to confer benefits to memory.

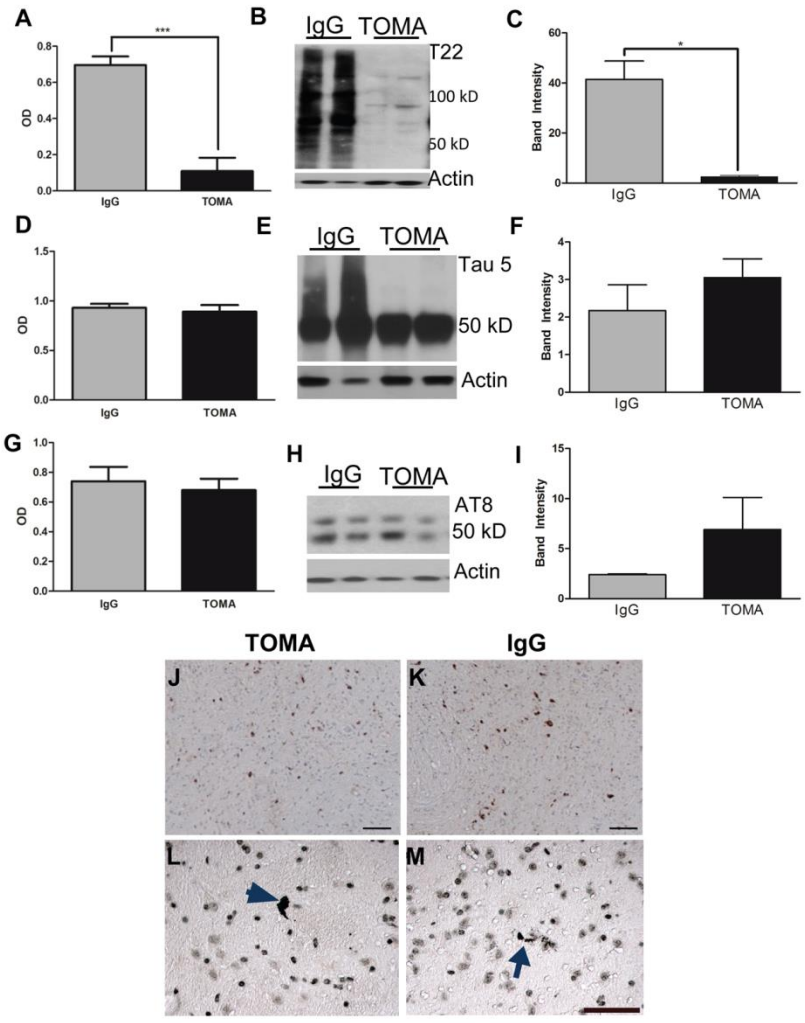

Figure 4.8 ELISA of the PBS soluble fraction with T22 showed that mice receiving bi-weekly TOMA injections had significantly lower levels of tau oligomers than those treated with control IgG (***$p<0.001$) (A). Significantly lowered levels of tau oligomers in TOMA-treated mice were also seen by western blot with T22 of the PBS soluble fraction of Htau brains (B) and densitometric quantification (C). No differences were detected in total tau levels measured with Tau-5 by ELISA (D) or western blot (E-F). No differences in hyperphosphorylated tau levels measured with AT8 were seen by ELISA (G) or western blot (H-I). Immunohistochemistry with AT8 (Ser202/Thr205 phosphorylated tau) showed no difference in levels of hyperphosphorylated tau in Htau

mice treated with TOMA (J) versus non-specific IgG (K). Scale bar 50 μm. Gallyas silver staining revealed equal NFT load (blue arrows) in Htau mice treated with TOMA (L) and non-specific IgG (M).

Discussion

Passive immunotherapy is a promising mode of treatment for neurodegenerative disease (Castillo-Carranza, Lasagna-Reeves et al. 2013). While studies have traditionally focused on targeting amyloid-β to prevent and treat AD, more research is now being dedicated to tau. However, most of the studies conducted using tau passive immunotherapy have targeted phosphorylated tau species known to be present in the late stages of aggregation and have resulted in a decrease of NFTs (Boutajangout, Ingadottir et al. 2011, Chai, Wu et al. 2011, d'Abramo, Acker et al. 2013). While NFTs have historically been the main tau species of interest, more results are emerging to suggest that toxicity and neuronal loss exceeds and occurs prior to NFT accumulation (Gomez-Isla, Hollister et al. 1997, Morsch, Simon et al. 1999, Kril, Patel et al. 2002, Spires, Orne et al. 2006, Haroutunian, Davies et al. 2007, Polydoro, Dzhala et al. 2013), thereby implicating the importance of intermediate tau species in toxicity. Evidence is growing suggesting that tau oligomers are the most toxic tau species in neurodegenerative tauopathies and that they may also seed the spread of tau pathology from cell to cell (Maeda, Sahara et al. 2006, Yoshiyama, Higuchi et al. 2007, Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012, Lasagna-Reeves, Castillo-Carranza et al. 2012, Gerson and Kayed 2013, Wu, Herman et al. 2013).

Therefore, in order to stop the initial toxicity and the spread of disease, tau oligomers may be the best target. Moreover, it was recently shown that antibodies confer beneficial effects by targeting tau found extracellularly, implying that antibodies do not need to enter cells in order to reduce the toxic effects of tau, but may instead target tau which may be involved in the propagation of pathology (d'Abramo, Acker et al. 2013, Yanamandra 2013). Additionally, studies of tau therapeutics for neurodegenerative disease have primarily used transgenic mouse models expressing mutant tau. While these models are comparable to diseases such as frontal temporal lobe dementia, AD and PD do not arise due to mutations to tau, making the Htau mouse a good model for investigating immunotherapy.

TOMA is a monoclonal antibody specific to tau oligomers, but not recognizing tau monomers or fibrils. Here we show that tau oligomer levels increase with age in Htau mice and are reduced by passive immunotherapy with TOMA in Htau mice injected with brain-derived tau oligomers from AD cortex, as measured by ELISA, Western blot, and immunohistochemistry. A single injection of TOMA antibody protected 3-month-old Htau mice injected intracerebrally with brain-derived tau oligomers from cognitive deficit for up to 1 month, while bi-weekly TOMA treatments prevented memory decline for 9 months when compared to animals injected with non-specific IgG. However, NFTs and total tau levels were not affected by treatment, implying that reducing oligomeric species of tau alone are enough to confer benefits to cognition. TOMA successfully halted the induction of cognitive impairment occurring naturally with age in Htau mice which were not injected with tau oligomers. There was no evidence of negative effects to general health observed due to TOMA treatment. These results are in support of a study using

curcumin treatment in Htau mice which found that a decrease in tau dimers ameliorated memory impairment as defined by improved performance in NOR, as well as a spatial memory task, without affecting insoluble tau (Ma, Zuo et al. 2013). The ability to target only pathogenic tau prevents any negative side effects which may occur from inhibiting the endogenous function of functional tau monomer.

We have shown previously that tau oligomers induce toxicity and seed the aggregation of endogenous tau in wild-type mice when injected intracerebrally (Lasagna-Reeves, Castillo-Carranza et al. 2012) and show here that tau oligomers, but not monomers or fibrils induce toxicity in neuroblastoma cells and impair memory in wild-type mice. The prevention of cognitive deficit due to tau oligomer injection by TOMA is consistent with these results. As Htau mice express all six isoforms of human tau, rather than mouse tau and do not express any mutations, these results are particularly applicable to sporadic AD. Furthermore, Htau mice do not have any motor, sensory, or general health impairments, but do acquire memory deficits with old age (Polydoro, Acker et al. 2009). As the only tau species affected in these mice was tau oligomers, which induced premature memory deficits, it appears as though oligomeric tau alone may underlie cognitive changes in Htau mice. In addition, studies have implicated tau pathology in traumatic brain injury (TBI), another growing health concern worldwide affecting millions, the repetitive occurrence of which has also been shown to increase the likelihood of acquiring neurodegenerative disease later in life (Goldman, Tanner et al. 2006, Chen, Richard et al. 2007, Lehman 2012). The sudden onslaught of tau oligomers induced by intracerebral injection is comparative to the increase in pathological tau seen in humans following TBI (Ost 2006). Aged Htau mice subjected to repeated closed head

mild TBI exhibit increased tau pathology compared to sham Htau mice (Ojo, Mouzon et al. 2013). Moreover, we have shown that in rats subjected to fluid percussion injury, levels of tau oligomers are significantly increased in comparison to sham animals, while levels of total tau remain the same, indicating an induction of tau oligomerization after injury (Hawkins, Krishnamurthy et al. 2013). Therefore these results are relevant for neurodegenerative tauopathies, such as Alzheimer's disease which are not dependent upon mutations to tau, as well as TBI. Targeting oligomeric tau seeds with anti-tau oligomer-specific antibodies, such as TOMA, may be an effective means of prevention and reversal of cognitive deficits associated with tau pathology. Information included in this chapter has previously been published and may be referred for further information (Castillo-Carranza, Gerson et al. 2014).

CHAPTER 5

CHARACTERIZATION OF TAU OLIGOMERS IN A SYNUCLEINOPATHY MODEL AND PROTECTION AGAINST DEFICITS WITH TAU OLIGOMER-SPECIFIC ANTIBODY

Introduction

Parkinson's Disease (PD) and Lewy Body Dementia (DLB), behind Alzheimer's disease, are the most common neurodegenerative disorders and affect hundreds of millions of people, both those afflicted as well as their caretakers. In the United States alone, the estimated cost to treat PD is around $25 billion a year (Parkinson's Disease Foundation, 2014). There are currently no effective treatments and as life expectancy continues to increase, the immense financial toll these diseases have on our health care system will continue to rise. It is essential that we find a way to prevent and treat these and other neurodegenerative tauopathies—characterized by the presence of aggregated tau protein.

The pathological hallmark of PD is the presence of cytoplasmic inclusions called Lewy bodies (LB), comprised primarily of α-synuclein and hyperphosphorylated tau (Piao, Hayashi et al. 2001, Zhukareva, Vogelsberg-Ragaglia et al. 2001, Galpern and Lang 2006, Goris, Williams-Gray et al. 2007) in surviving dopaminergic neurons. Synuclein-mediated neurotoxicity is thought to arise from a number of factors, such as its ability to form LB aggregates (Chandra, Gallardo et al. 2005, Periquet, Fulga et al. 2007), however the importance of LB to the disease is questionable (Parkkinen, Kauppinen et al. 2005, Parkkinen, Pirttila et al. 2005, Weisman, Cho et al. 2007, Jellinger 2008,

Parkkinen, Pirttila et al. 2008). Phosphorylated tau aggregates have been reported in all synucleinopathy mouse models (Frasier, Walzer et al. 2005, Duka, Rusnak et al. 2006, Duka, Duka et al. 2009, Emmer, Waxman et al. 2011, Haggerty, Credle et al. 2011, Kaul, Credle et al. 2011), suggesting a possible synergistic interaction between α-synuclein and tau in mediating neurodegeneration in PD, as α-synuclein may increase tau aggregation (Giasson, Forman et al. 2003, Duka, Duka et al. 2009, Lasagna-Reeves, Castillo-Carranza et al. 2010, Jellinger 2011, Nubling, Bader et al. 2012) and tau may have a similar effect on α-syn.

In its native form, tau is a mediator of microtubule dynamics and is important for cellular health. However, in neurodegenerative tauopathies, tau undergoes misfolding which causes it to aggregate and form neurofibrillary tangles (NFTs). NFTs have long been considered the main pathological hallmark for tauopathies, however, recent studies suggest that intermediate forms of tau—tau oligomers—between the monomeric form and NFTs, are the true toxic species in disease and the best targets for anti-tau therapies (Spires, Orne et al. 2006, Berger, Roder et al. 2007, Maeda, Sahara et al. 2007, Kopeikina, Carlson et al. 2011, Cowan, Quraishe et al. 2012, Sahara, DeTure et al. 2013). While α-synuclein is commonly viewed as the main pathogenic protein in Parkinson's disease, tau also plays an important role. Tau and α-synuclein have been shown to interact in disease by multiple different groups. Large genome-wide association studies have revealed a genetic interaction between the genes encoding the two proteins (Colom-Cadena, Gelpi et al. , Simon-Sanchez, Schulte et al. 2009). Additionally, the proteins have been found to co-occur in disease and α-synuclein aggregation has been demonstrated to increase levels of tau fibrils (Giasson, Forman et al. 2003, Guo, Covell et

al. 2013). However, no thorough investigation has been completed to study the interaction between the most toxic form of the two proteins, the oligomers. Unlike fibrils, we have shown that oligomeric α-synuclein can seed the oligomerization of tau protein (Lasagna-Reeves, Castillo-Carranza et al. 2010), suggesting that a direct interaction between the oligomeric states of both proteins may exist. We hypothesize that tau and α-synuclein have a synergistic function, whereby the presence of the misfolded, aggregated form of one protein can seed the aggregation of the other (Figure 5.1).

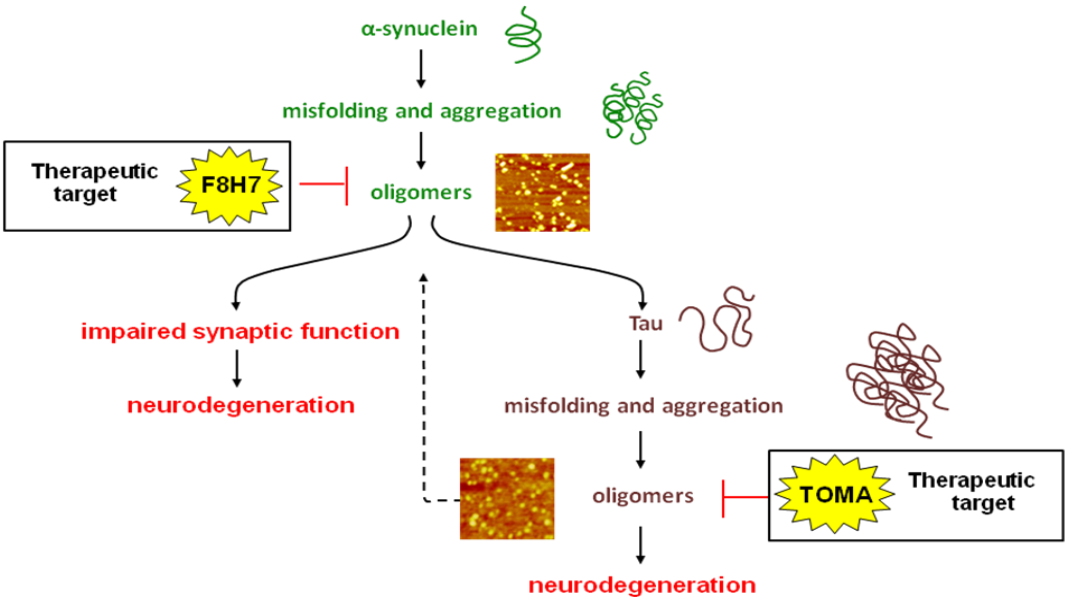

Figure 5.1 Schematic for the toxic synergism of α-synuclein and tau oligomers (Lasagna-Reeves, Castillo-Carranza et al. 2010, Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves 2012, Sengupta, Guerrero-Muñoz et al. 2015).

We have shown that tau oligomers form in PD and DLB human brains, highlighting their importance in synucleinopathies (Sengupta, Guerrero-Muñoz et al.

2015). Moreover, we directly studied the interaction between the oligomeric states of both proteins by creating antibodies specific to α-synuclein oligomers (F8H7) and tau oligomers (T22 and TOMA). We found that in PD and DLB human brains, we could detect independent oligomers from both proteins, as well as the presence of colocalized oligomers, suggesting that the two proteins coexist and potentially interact in disease. Furthermore, we immunoprecipitated tau oligomers from PD and DLB and analyzed them by Western blot with antibodies for tau and α-synuclein. We found that both tau and α-synuclein oligomers were present in immunoprecipitated samples. We also performed the experiment in reverse, immunoprecipitating oligomers with an α-synuclein oligomer-specific antibody and by Western blot we saw again the presence of not only α-synuclein oligomers, but also oligomeric tau. Our results suggest that tau and α-synuclein may coaggregate in disease. Therefore, targeting tau in synucleinopathies may not only improve function due to pathological tau protein, it may also decrease the formation of pathogenic α-synuclein aggregates and co-aggregates with tau. A53T mice overexpress mutant alpha-synuclein and have been shown to have both tau and α-synuclein (Lewy body) aggregates (Wills, Credle et al. 2011), resulting in motor and cognitive deficits (Oaks, Frankfurt et al. 2013, Paumier, Sukoff Rizzo et al. 2013). In addition to the brain, α-synuclein pathology is seen in the spinal cord in A53T mice (Mendritzki, Schmidt et al. 2010), which corresponds with evidence that PD may initiate in the spinal cord and travel towards the brain later in disease (Braak, Tredici et al. 2003). Therefore, we chose to specifically target oligomeric tau by passive immunotherapy to test their efficacy as a novel drug target for the treatment of PD.

Tau oligomers may exert their effects by not only causing toxicity in cells where they are present, but also by spreading from affected parts of the brain to unaffected regions. Therefore, targeting extracellular tau oligomers may effectively eliminate the spread of disease. We have previously shown that TOMA targets tau oligomers specifically in mouse models of tauopathy and Alzheimer's disease and improves disease phenotype without entering the cell (Castillo-Carranza, Gerson et al. 2014, Castillo-Carranza, Sengupta et al. 2014, Castillo-Carranza, Guerrero-Muñoz et al. 2015). Passive immunotherapy is a promising mode of treatment against neurodegenerative disease as antibody dose can be controlled and targeted for the toxic species alone, without impacting functional protein. Importantly, at the point most patients are diagnosed with a neurodegenerative disorder, the condition is already very advanced, making a protein vaccine approach very appealing for prevention of disease as well. However, while evidence suggests that tau oligomers initiate a cascade of toxic effects likely downstream of α-synuclein, thus far they have not been the primary focus of therapeutic efforts. Furthermore, most studies that have considered tau have used therapeutics directed for either large aggregates, which may be non-toxic or even protective, or unfolded tau monomer, which has an important function in the cell, for review (Castillo-Carranza DL 2013, Gerson, Castillo-Carranza et al. 2014). Moreover, we found that in a mouse model expressing mutated amyloid-β (Aβ) protein, targeting tau oligomers not only decreased tau toxicity, but also was able to lower levels of oligomeric Aβ (Castillo-Carranza, Guerrero-Muñoz et al. 2015). As Aβ and tau have been shown to interact in disease similarly to α-synuclein and tau, these results support the hypothesis that tau oligomer-specific antibodies may be capable of mediating both tau and α-synuclein toxicity in

disease. In order to assess the efficacy of targeting tau oligomers against tauopathies with α-synuclein pathology, we have used a passive immunotherapy strategy with a novel anti-tau oligomer-specific monoclonal antibody (TOMA) in A53T mice.

Methods

Animals

Animals were housed as described in Chapter 3 (Gerson, Castillo-Carranza et al. 2016). Both male and female animals were balanced in each group in order to determine whether sex differences were present. A53T mice (The Jackson laboratory; B6;C3-Tg(Prnp-SNCA*A53T)83Vle/J) and nontransgenic littermate (WT) animals were bred at UTMB and were raised free of enrichment following weaning to prevent any effect on behavioral test performance.

Immunization

Seven-month-old homozygous A53T mice were given intravenous injections of 120 μg/animal of either TOMA (n=17), a total tau antibody, Tau-13 (catalog #MMS-520-R; Covance; n=7), or anti-Rhodamine antibody (IgG, nonspecific; catalog #GTX29093; Genetex; n=17) for the control treatment group (Ctrl IgG) as described in Chapter 4 (Castillo-Carranza, Gerson et al. 2014). Wildtype littermates were given 120 μL of saline injected intravenously (n=16).

In Vivo Imaging

In vivo imaging following TOMA IV injection was completed in three and nine-month-old A53T and 3-month old wild-type mice as described in Chapter 4 (Castillo-Carranza, Gerson et al. 2014).

Behavioral Analyses

To determine the effect of immunotherapy on behavioral deficits, the cognition and motor phenotype of A53T mice was evaluated using the novel object recognition (NOR), rotarod, footprint, and nesting tests. Prior to performing behavioral tasks, mice were habituated to frequent experimenter handling. The NOR test was performed 2 weeks after immunization, followed by the three motor tasks.

Novel Object Recognition (NOR)

A53T mice have been shown to exhibit memory deficits that may be detected by NOR starting at six months of age (Lim, Kehm et al. 2011, Paumier, Sukoff Rizzo et al. 2013). Two weeks after injection, mice were evaluated on the NOR task as described in Chapter 3 (Castillo-Carranza, Gerson et al. 2014, Gerson, Castillo-Carranza et al. 2016).

Rotarod Test

At early ages, A53T mice do not demonstrate gross motor deficits, but frequently show a hyperactive phenotype. Researchers have previously shown that A53T mice tested by rotarod and other motor tasks have significantly higher activity levels than wildtype mice at around six months of age, giving way to motor dysfunction at around nine to twelve months of age (Graham and Sidhu 2010, Paumier, Sukoff Rizzo et al. 2013). Rotarod measures the ability of a rodent to maintain balance on a rotating rod (diameter 3.2 cm) and may be used to evaluate both motor coordination and hyperactivity. Mice were first

habituated in one session of four trials to reach a baseline level of performance. The next day, mice were tested in one session of four trials (Rotarod meter; Stoelting). Five mice were placed onto the rod, one per testing station. The speed started at 4 rpm and accelerated at 0.1 rpm/s. The latency to fall from the rotating rod was determined. Latency significantly higher than that of wildtype was determined to be indicative of a hyperactivity phenotype.

Footprint Test

A53T mice have been shown to have gait alterations similarly to what is seen in PD disease patients clinically (Gispert, Turco et al. 2003, Paumier, Sukoff Rizzo et al. 2013). The footprint test was used to compare the gait of A53T transgenic mice in each treatment group with that of wild-type control mice. Mice were first trained to walk along a 50-cm-long, 10-cm-wide runway (with 10-cm-high walls) into an enclosed box containing a fruit loop. All mice had four training trials. To obtain footprints, hindfeet and forefeet of each mouse were coated with blue and red nontoxic paints, respectively. One day following training, mice were allowed to complete a testing trial. Any mouse that did not walk continuously along path was given another trial. A fresh sheet of white paper was placed on the floor of the runway for each run. Footprints were analyzed for different gait parameters measured in centimeters as has been previously described (Carter, Lione et al. 1999), including stride length (average forward distance between each stride), hindpaw width and forepaw width (average distance between left and right hind and front footprints, respectively), and front footprint /hind footprint overlap for uniformity of steps (a value of zero is recording when the center of the hind and front footprints overlap and the distance between the two centers was recorded for those which

did not overlap). For each step parameter, six to eight values were measured from each run, excluding footprints made at the beginning and the end of the run to avoid inaccurate measurements during initiation and completion of movement. The mean value of each set of measurements was compared between groups by one-way ANOVA and Tukey's post-hoc test using Graph Pad Prism 5.04 software.

Nesting Test

The nesting task is designed to utilize the innate ability of both male and female mice to build nests for heat, shelter, and reproduction and has been shown to be affected by very minor sensorimotor deficits (Deacon 2006). A53T mice begin to show minor deficits which can be detected by nesting behavior as young as two to three months of age, prior to any gross locomotor phenotype (Giasson, Duda et al. 2002, Paumier, Sukoff Rizzo et al. 2013). The nesting task was completed as previously described (Deacon 2006, Paumier, Sukoff Rizzo et al. 2013). Two hours prior to the dark phase, each mouse was placed in an individual testing cage identical to home cage and given a single cotton nestlet. At one hour and 24 hours after placement, nesting quality was determined by two independent researchers blinded to treatment group based on previously defined criteria described as follows; 1: nestlet not visibly touched; 2: nestlet partially torn but at least 50% remaining untouched; 3: 50-90% of nestlet shredded but scattered distribution throughout the cage; 4: at least 90% of nestlet is shredded and a compact, flat nest can be identified; 5: a nearly perfect nest with over 90% shredded and walls of the nest higher than the mouse's body. Nesting scores were averaged and analyzed using Graph Pad Prism 5.04 software by the Kruskal-Wallis non-parametric ANOVA and Tukey's post-hoc test.

Tissue Collection and Immunohistochemistry

To evaluate the pathological presence of oligomeric tau in synucleinopathy mouse models, brain samples were collected from A53T (Giasson, Duda et al. 2002), Thy1-α-synuclein (Masliah, Rockenstein et al. 2000, Rockenstein, Mallory et al. 2002) and E46K-human-synuclein (Emmer, Waxman et al. 2011) mice at different ages. Following behavioral testing, half of the animals in each group were randomly selected and anesthetized with CO2 and brains, spinal cord, eye, and blood were collected for analysis. Brains were processed for immunohistochemistry as described in Chapter 3 (Gerson, Castillo-Carranza et al. 2016). Immunohistochemistry with tyrosine hydroxylase antibody for dopamine (1:200) was completed. Immunofluorescence with tau oligomer-specific polyclonal antibody T22, α-synuclein oligomer-specific antibody Syn33 (1:100), glial fibrillary acid protein (GFAP) antibody for activated astrocytes (1:300, Covance), LB509 (Abcam) and 4D6 (Covance) was completed. Eyes and spinal cords were embedded in OCT and sectioned for future immunohistochemistry analysis.

Preparation of Brain Homogenate

Brains were homogenized as described in Chapter 2 (Castillo-Carranza, Gerson et al. 2014, Gerson, Sengupta et al. 2014) For insoluble tau fractions, pellets were resuspended in 10% Sarkosyl in PBS, vortexed, and sonicated, then centrifuged 30 mins at 100,000 g at 4ºC.

Western Blot Analysis

PBS soluble and insoluble fractions of brain extracts were analyzed by Western blot with Syn33, T22, Tau5, LB509, 4D6, tyrosine hydroxylase, synapsin 1, synaptophysin, and β-

actin as described in Chapter 2 (Castillo-Carranza, Gerson et al. 2014, Gerson, Sengupta et al. 2014).

ELISA

ELISA of the PBS soluble and insoluble fractions of brain homogenate with Syn33, 4D6, and LB509 was completed as described in Chapter 4 (Castillo-Carranza, Gerson et al. 2014).

Results

Elevated Levels of Tau Oligomers in α-synuclein Mouse Models

Similarly to previous studies in human brains, we found toxic tau oligomeric species in synucleinopathy mouse models, Prnp-SNCA*A53T (Giasson, Duda et al. 2002)(Figure 5.2), the Thy1-α-synuclein (Masliah, Rockenstein et al. 2000, Rockenstein, Mallory et al. 2002) and the E46K-human-synuclein (Emmer, Waxman et al. 2011)(data not shown). It is important to note that these animals are not designed to overexpress tau, yet, using T22, we found high levels of tau oligomers in the brains of these animals. The molecular weight of these SDS stable oligomers is similar to the ones found in mouse models of tauopathy (SantaCruz, Lewis et al. 2005, Spires, Orne et al. 2006, Berger, Roder et al. 2007).

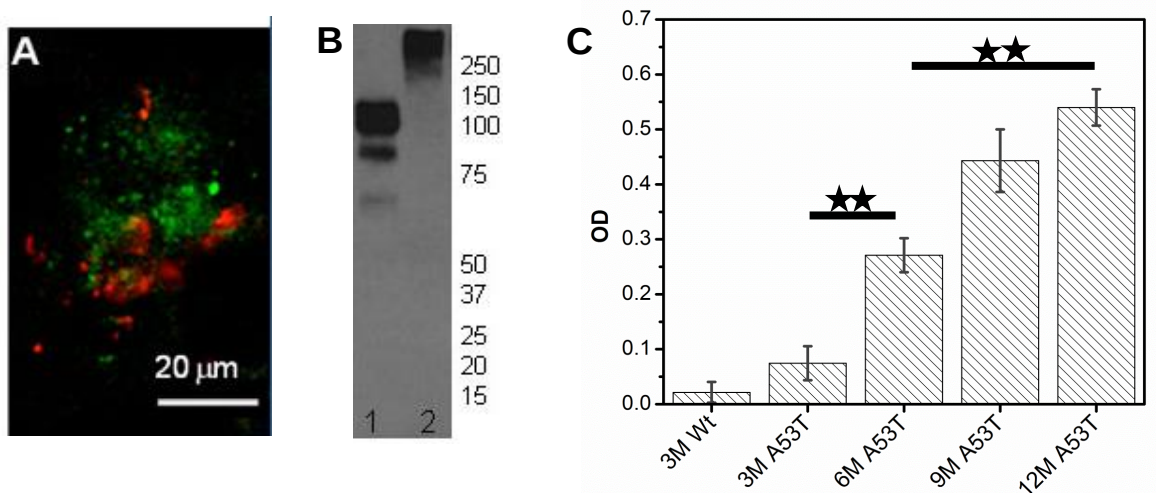

Figure 5.2 Tau oligomers in A53T mice (A) tau oligomers with T22 (red) are found in the vicinity of α-synuclein oligomers (green). (B) WB with TOMA shows detection of tau oligomers in A53T mice; lane 1 PBS soluble A53T mouse brain, lane 2 Sarkosyl soluble A53T mouse brain. (C) ELISA analysis of the PBS soluble fraction from whole brain shows that tau oligomer levels in the A53T mouse model are elevated when compared to wild-type mice and increase with age.

TOMA Binds to Tau Oligomers in the Brains of A53T Mice

We have previously shown that TOMA can bind to oligomeric tau in the brains of tauopathy model mice (Castillo-Carranza, Sengupta et al. 2014). However, this was the first study investigating the specific targeting of tau oligomers in a synucleinopathy mouse model by passive immunotherapy. In order to confirm that TOMA injected into the tail vein was able to cross the blood-brain-barrier and bind to tau oligomers in the A53T mouse brain, we labeled TOMA with a far red probe and used *in vivo* imaging (Figure 5.3). We found that levels of TOMA were heightened in aged A53T transgenic

mice over time when compared to young A53T mice and wild-type controls. Binding of TOMA to tau oligomers was confirmed by imaging brain extracted from animals 4 hours after TOMA treatment.

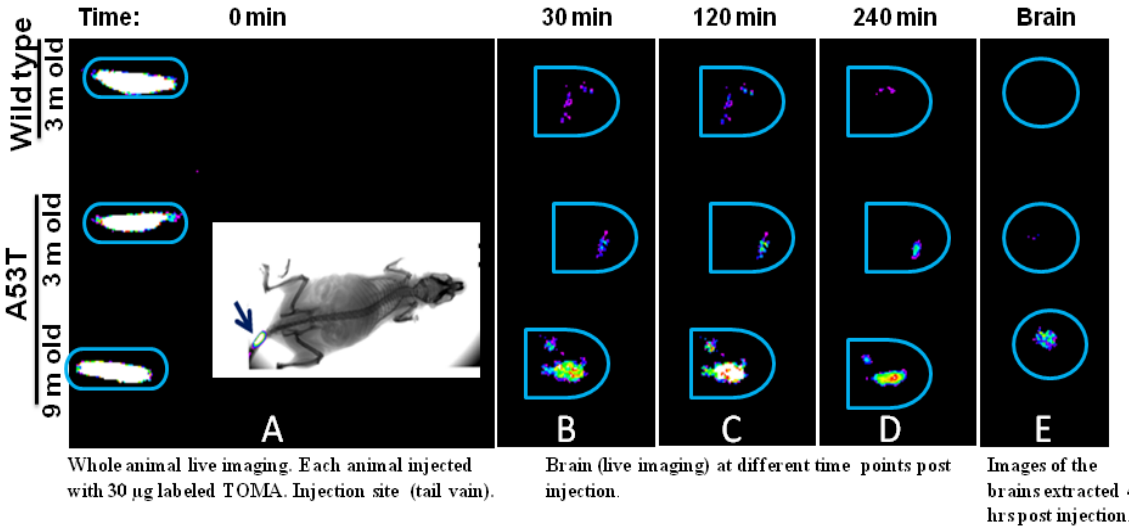

Figure 5.3 TOMA crosses the BBB in A53T mice. *In vivo* live imaging demonstrates that a fraction of TOMA injected into the tail vein crosses the BBB and binds to tau oligomers in 9-month-old Prnp-SNCA*A53T brain. TOMA labeled with the far red probe (Kodak X-Sight 640 LSS Dye, excitation 650 nm, emission 750 nm from Carestream.

TOMA Protects Against Behavioral Deficits in A53T Mice

TOMA injected IV has been shown to specifically modulate tau oligomers and reverse the phenotypes in three different animal models of tauopathy and Alzheimer's disease without any side effects (Castillo-Carranza, Gerson et al. 2014, Castillo-Carranza,

Sengupta et al. 2014, Castillo-Carranza, Guerrero-Muñoz et al. 2015). In order to test whether tau oligomer immunotherapy can protect against deficits in a synucleinopathy mouse model, we injected 7-month-old A53T mice with either TOMA, total tau antibody Tau-13, or a control IgG and compared them to wild-type littermate controls injected with saline. Two weeks after injection, we conducted behavioral tasks for cognition and motor behavior, as summarized (Figure 5.4).

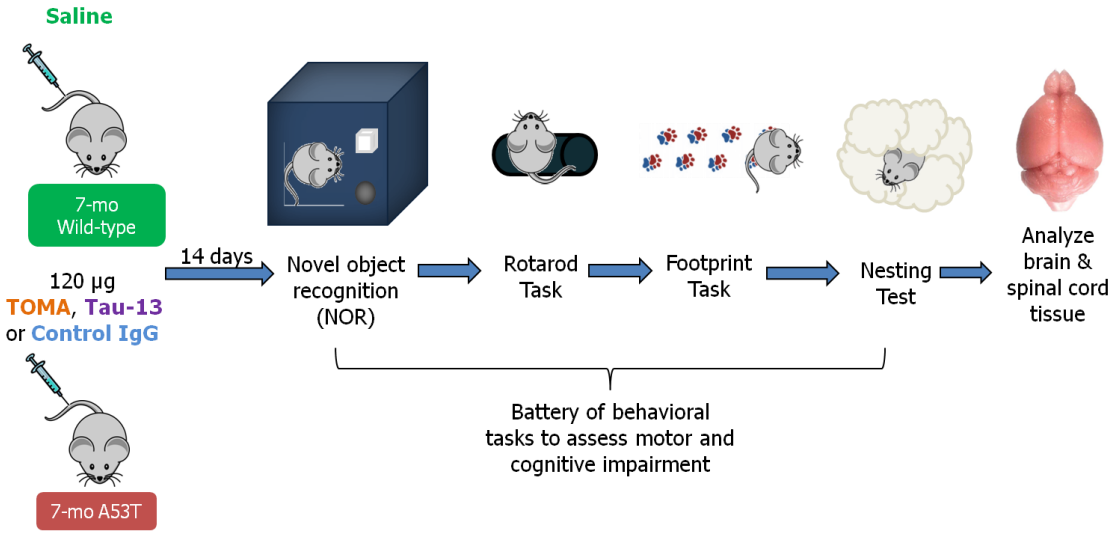

Figure 5.4 Passive immunotherapy with TOMA in A53T mice experimental design.

A53T mice begin to show memory deficits beginning at six months of age (Lim, Kehm et al. 2011, Paumier, Sukoff Rizzo et al. 2013). Using the NOR task, we found that A53T mice injected with Control IgG antibody had significantly lower discrimination index scores when compared to wild-type animals. Moreover, mice injected with a total tau antibody, Tau-13 appeared to perform even worse than those injected with Control IgG,

having significantly lower scores than both wild-type and TOMA-injected A53T mice. A53T mice showed no significant differences from wildtype (Figure 5.5A). In order to evaluate the effect on the motor phenotype of A53T mice, we first used the rotarod task. While no significant differences were observed between groups, the Tau-13 treated A53T mice showed a trend towards higher latency to fall off of the rotating rod when compared to wild-type mice, suggesting that these mice may have an exacerbated hyperactivity phenotype compared to Control IgG and TOMA-treated mice (Figure 5.5B). In order to detect more sensitive differences in motor behavior which can be seen starting around two months of age (Paumier, Sukoff Rizzo et al. 2013), we performed the nesting test. Representative images for each score on the task were recorded (Figure 5.5C). One hour after receiving cotton nestlet, A53T mice treated with Control IgG and with Tau-13 antibodies has significantly lower scores for nesting quality when compared to Wild-type controls, while TOMA-treated mice showed no differences from Wild-type (Figure 5.5C). Lastly, we performed the footprint task in order to measure differences in gait seen in the different treatment groups, similarly to what is evaluated clinically in PD patients. We found no significant differences on all but one of the parameters, as can be expected at this age as gross motor deficits do not begin until around 9 months. In the measure of front and hind paw overlap, however, only the Control IgG-treated A53T mice showed greater distance between the front and hind paws, while TOMA and Tau-13-treated mice were no different than Wild-type controls (Figure 5.5D).

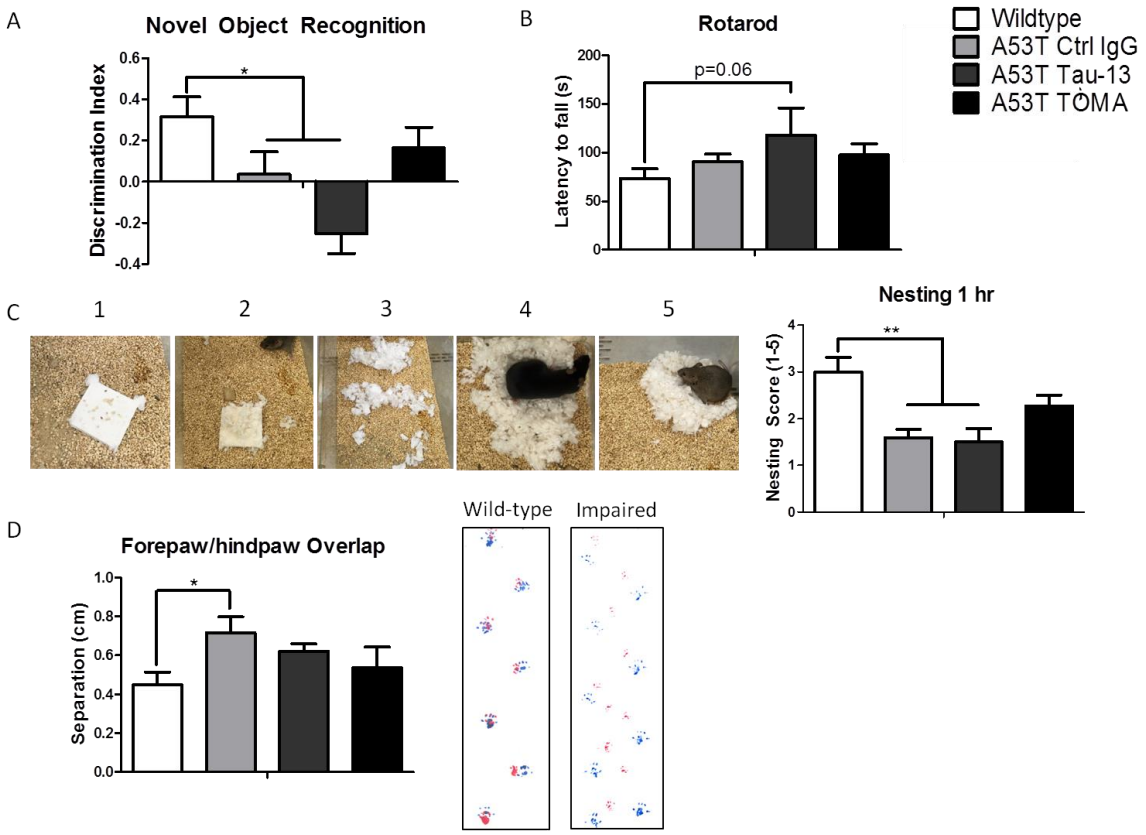

Figure 5.5 TOMA treatment ameliorates A53T phenotype. (A) TOMA-treated animals are protected against memory deficits in the NOR. (B) Tau-13 treated mice have exacerbated hyperactivity phenotype measured by increased latency in the Rotarod. (C) Tau-13 and Ctrl IgG treated mice have impaired fine motor behavior, while TOMA treatment is protective, as shown by lower quality scores on the nesting task. (D) TOMA treatment protects against gait abnormalities shown by greater separation between front and hind footprints in the footprint task.

Tau Oligomers are Specifically Depleted in TOMA-Treated Mice

In order to determine the effects of antibody treatment on tau oligomer levels, the PBS soluble fraction of total brain homogenate was analyzed by Western blot with T22 and

Tau-5. TOMA-treated mice had significantly lower levels of a 37 kD fragment of tau detected with T22 when compared to Control IgG (p=0.003; Figure 5.6A) and oligomeric tau when compared to Control IgG-injected mice (p=0.014 ;Figure 5.6B). However, levels of tau monomer detected with Tau-5 were unaffected by treatment (Figure 5.6C). Levels of tau dimers and tetramers measured with Tau-5 were also significantly decreased in TOMA-treated mice when compared to Control IgG and Tau-13 (Figure 5.6 D and E).

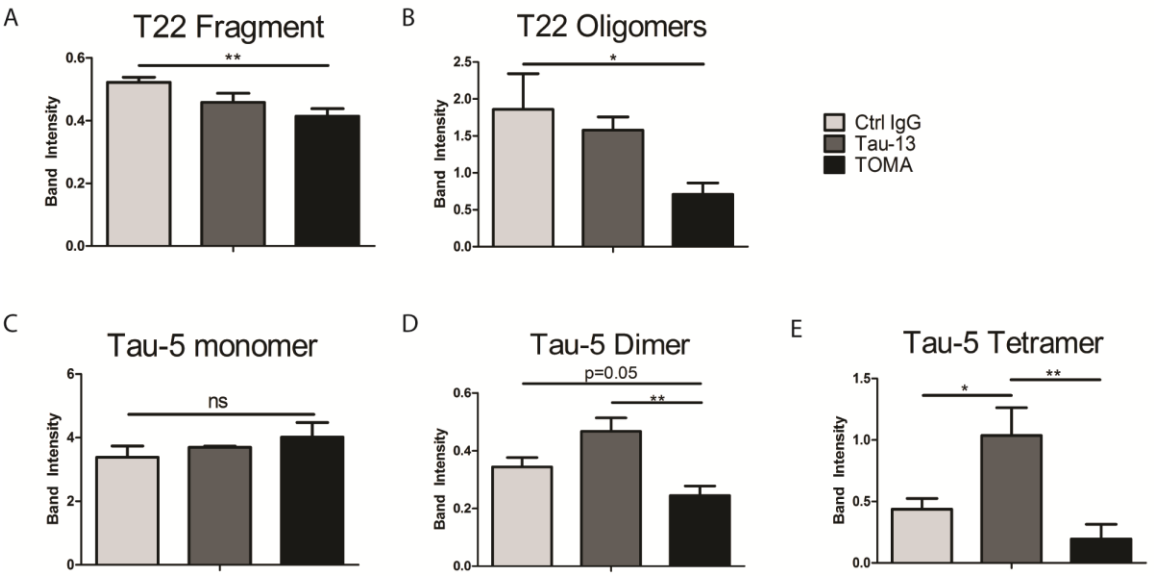

Figure 5.6 Levels of tau oligomers specifically are reduced in A53T mice treated with TOMA. (A) Western blot of the PBS soluble fraction of whole brain homogenate with T22 for tau oligomers showed that TOMA-treated mice had significantly lower levels of a 37 kD fragment when compared to Control IgG (p=0.003). (B) Levels of tau oligomers were also significantly lowered when compared to Control IgG-injected mice (p=0.014). (C) Tau monomer levels measured by Western blot with Tau-5 were unaffected by

treatment. (D) Levels of tau dimer measured by Tau-5 were significantly elevated in Tau-13 treated mice when compared to TOMA ($p=0.005$) and approached significance in Control IgG-treated mice ($p=0.05$). (E) Tau-13 treated mice also had significantly increased levels of tau tetramers measured with Tau-5 when compared to Control IgG-treated mice ($p=0.02$) and TOMA-treated mice ($p=0.002$).

Immunofluorescence analysis with T22 for tau oligomers showed significantly decreased tau oligomers in TOMA-treated mice when compared to Control IgG ($p=0.0089$). Wildtype mice had significantly lower levels of tau oligomers when compared to Control IgG ($p=0.0041$), but did not show any differences with TOMA-treated A53T mice (Figure 5.7A-B).

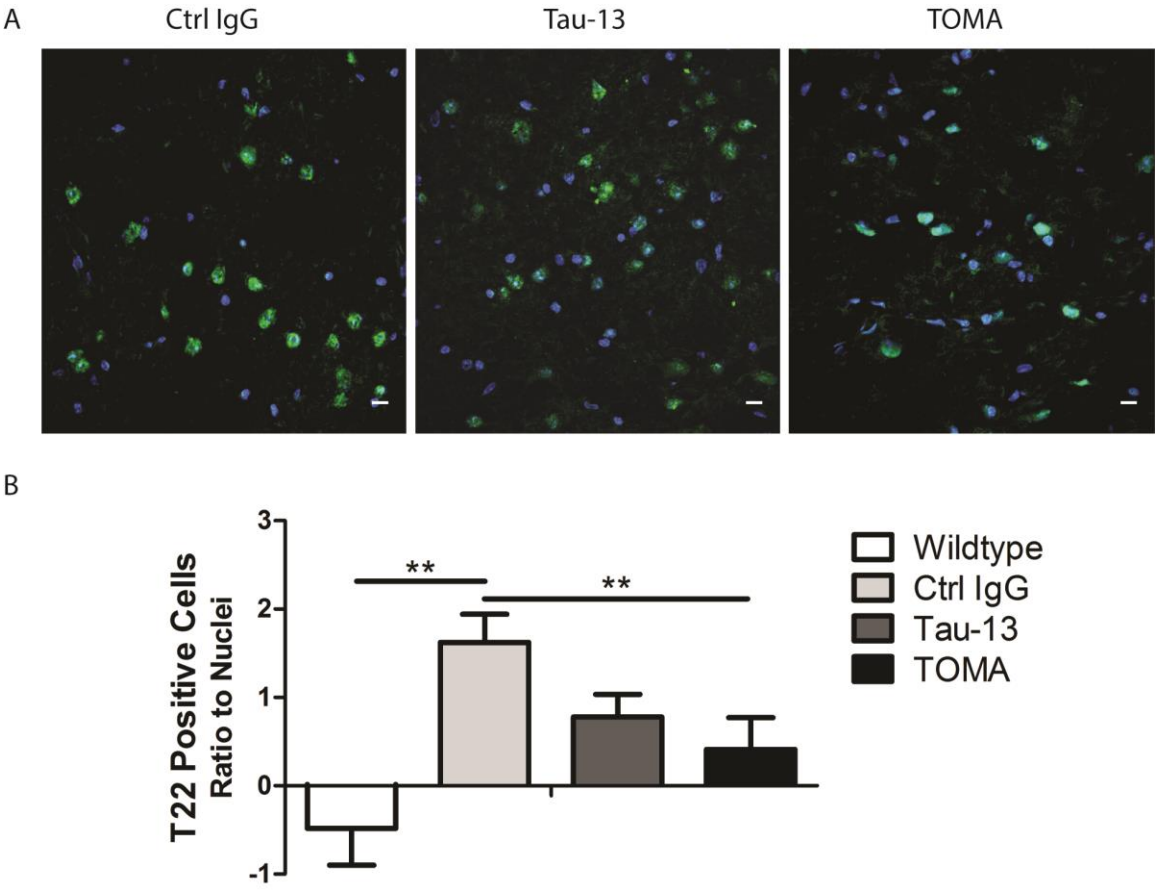

Figure 5.7 Immunofluorescence imaging detects significantly lower levels of tau oligomers in TOMA-treated mice. (A) Brain sections were labeled with T22 (green) and DAPI for cell nuclei (blue). (B) Control IgG-treated mice had significantly higher levels of T22 positive cells as a ratio to nuclei when compared to wildtype mice (p=0.004) and TOMA-treated A53T mice (p=0.009). Scale bar 10 μm.

TOMA-treated A53T Mice Show Decreased Signs of Inflammation

In order to evaluate the effect of treatment on inflammation in A53T mice, brain sections were co-labeled with T22 for tau oligomers and GFAP and astrocytes. Total numbers of astrocytes did not differ between any of the treatment groups (data not shown). However,

while wildtype brains and TOMA-treated A53T mice presented with primarily non-reactive astrocyte morphology, both Tau-13 and Control IgG treatment was associated with larger, reactive astrocytes (Figure 5.8). Additionally, tau oligomers were found to be associated with cerebrovasculature in Control IgG and Tau-13 treated mice, with surrounding astrocytic endfeet, while TOMA-treated mice had lower levels of tau oligomers.

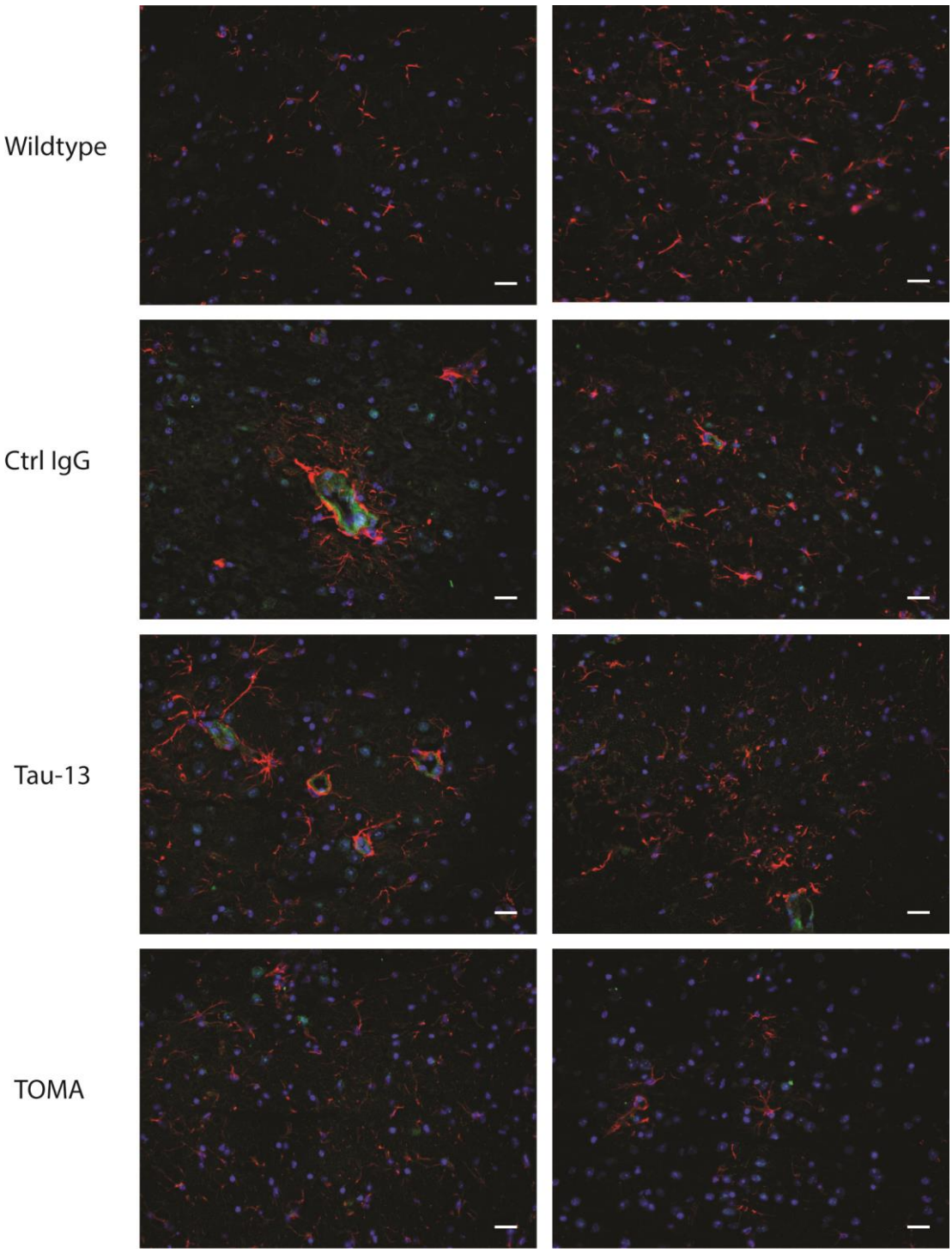

Figure 5.8 Increased signs of inflammation are present in Control IgG and Tau-13 treated A53T mice. Astrocytes were labeled with GFAP (red) and tau oligomers were detected with T22 (green). Control IgG and Tau-13-treated mice do not have significantly

different numbers of astrocytes when compared to Wildtype or TOMA-treated mice, but show increased reactivity and astrocytic endfeet associated with T22-positive vasculature. Scale bar 20 μm.

TOMA Treatment is Associated with Alterations in α-synuclein Aggregation

In order to determine the effects of TOMA treatment on the potential interaction between tau and α-synuclein oligomers, PBS soluble and insoluble total brain homogenate was evaluated for levels of α-synuclein oligomers with Syn33 by ELISA. Soluble brain homogenate analysis exhibited increased α-synuclein oligomers in TOMA-treated mice when compared to Control IgG (Figure 5.9A). However, ELISA with Syn33 on the PBS insoluble brain homogenate showed significantly increased levels of α-synuclein oligomers in Tau-13-treated mice when compared to Control IgG and TOMA ($p<0.0001$; Figure 5.9B). Immunofluorescence of brain sections with Syn33 was completed with and without Proteinase K (PK) treatment (Figure 5.9C). TOMA-treated mice again showed elevated levels of α-synuclein oligomers over Control IgG-treated mice approaching significance ($p=0.05$) in sections that were not treated with PK (Figure 5.9D). However, proteinase treatment showed that PK-resistant α-synuclein oligomers were significantly increased in Control IgG-treated mice compared to TOMA treatment ($p=0.036$). Further analysis is thus being completed to come to a full understanding of the effect of TOMA treatment on α-synuclein oligomers.

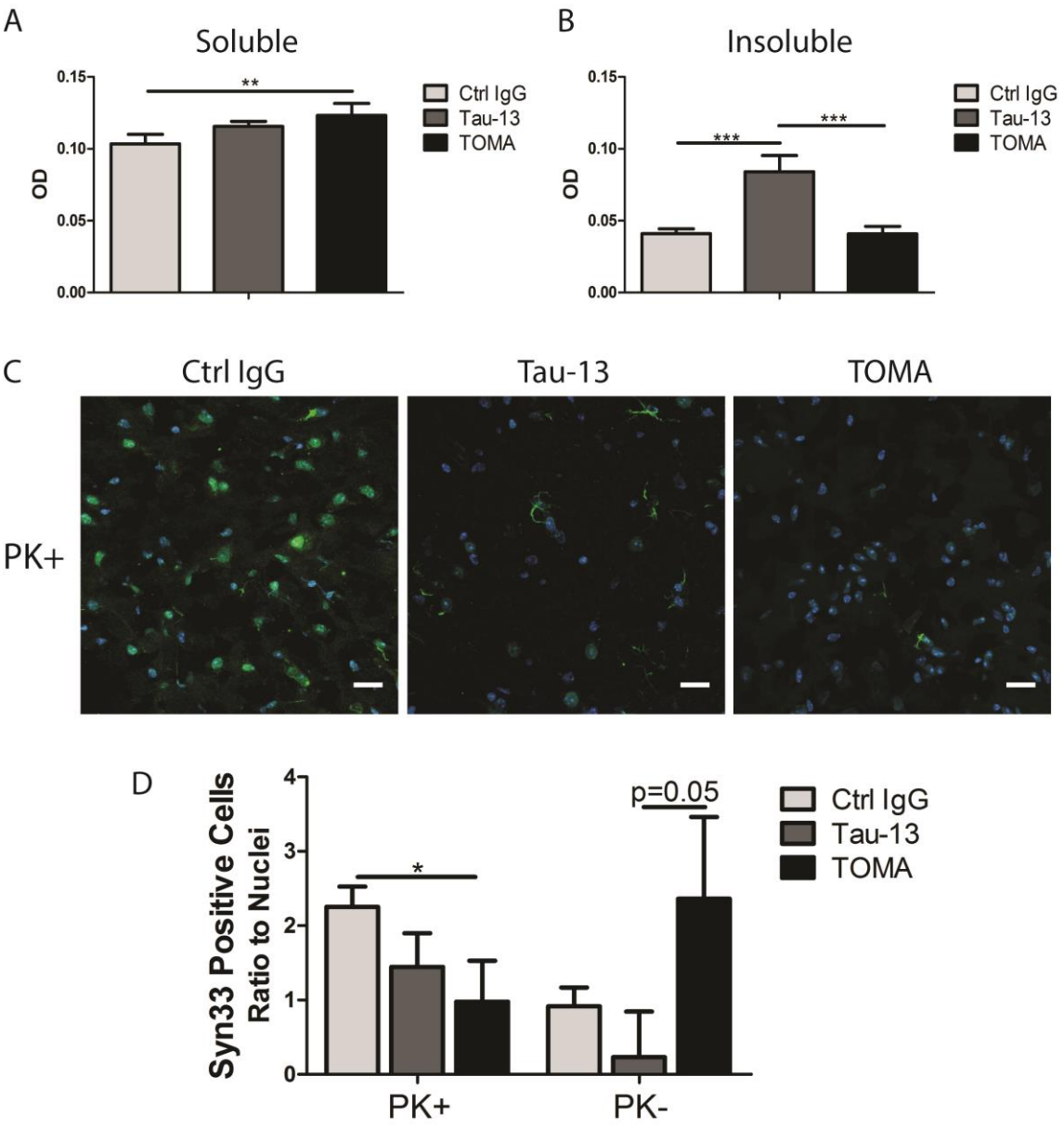

Figure 5.9 TOMA treatment alters α-synuclein oligomerization. (A) ELISA with Syn33 on soluble brain homogenate shows significantly increased α-synuclein oligomers in TOMA mice over Control IgG ($p=0.002$), however Syn33 ELISA with the PBS insoluble brain homogenate (B) showed significantly increased levels of α-synuclein oligomers in Tau-13 mice over Control IgG and TOMA ($p<0.0001$). (C) Immunofluorescence with Syn33 (green) and DAPI for nuclei (blue) was completed both with and without

Proteinase K (PK) treatment. (D) In sections that were not treated with PK, TOMA-treated mice showed elevated levels of α-synuclein oligomers over Control IgG approaching significance ($p=0.05$), but PK-resistant α-synuclein oligomers were found to be increased in Control IgG mice compared to TOMA ($p=0.036$). Scale bar 20 μm.

In order to determine whether increases in soluble, PK-sensitive α-synuclein oligomers were reflective of a converse alteration to levels of insoluble, fibrillar Lewy body structures, brain sections and homogenate were analyzed with LB509. The ratio of Lewy bodies to total nuclei was quantified from immunofluorescence imaging (Figure 5.10A). Control IgG-treated mice had a significantly higher ratio of Lewy bodies when compared to TOMA-treated mice ($p=0.039$; Figure 5.10B). ELISA of the PBS insoluble fraction of total brain homogenate revealed increased levels of LB509 approaching significance in Control IgG-treated mice when compared to TOMA ($p=0.05$; Figure 5.10 C). However, ELISA with LB509 of the PBS soluble fraction showed significantly elevated reactivity in Tau-13 treated mice compared to both Control IgG ($p=0.0012$) and TOMA ($p=0.003$; Figure 5.10D). In order to confirm that total α-synuclein levels were not different in any of the groups, ELISA with 4D6 in both the PBS soluble and insoluble fractions of total brain homogenate was completed and no differences were found between groups.

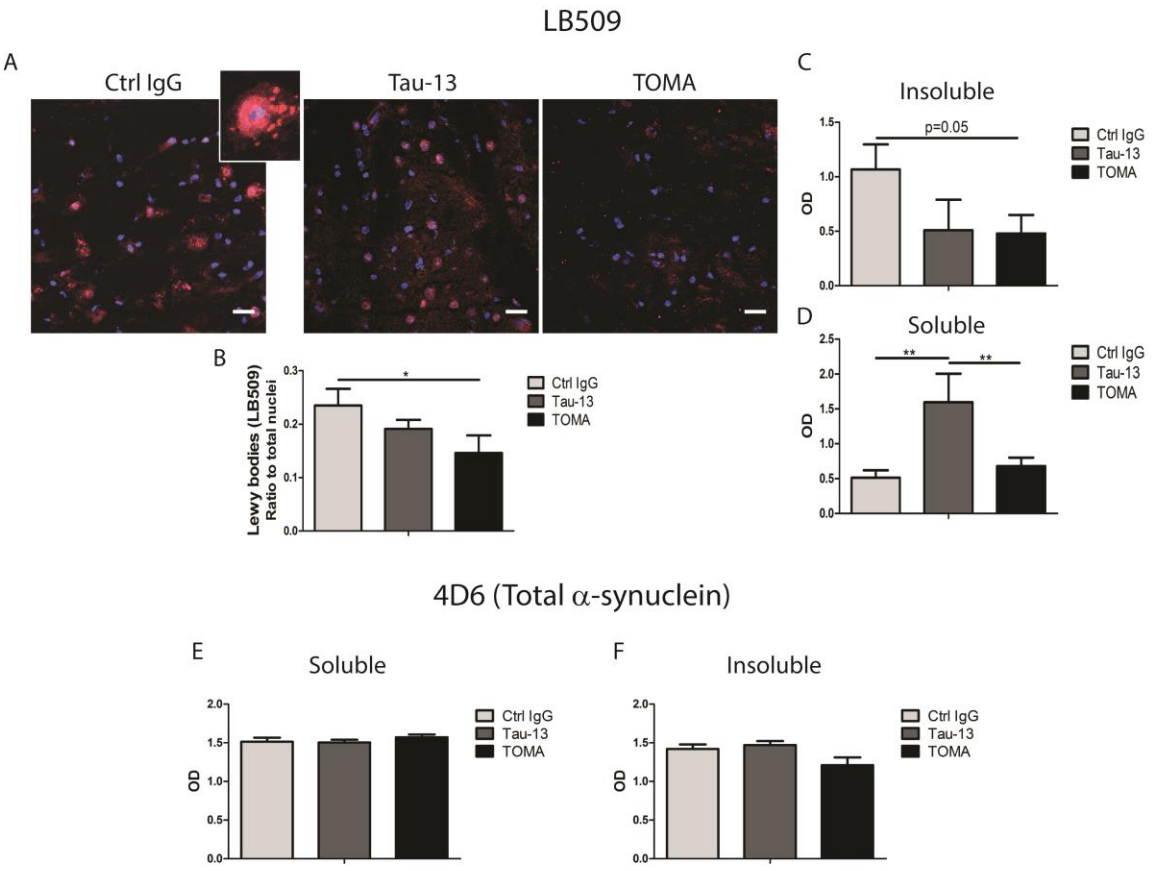

Figure 5.10 Lewy bodies are significantly decreased in TOMA-treated A53T mice. (A) Lewy bodies were detected by immunofluorescence with LB509 (red) and nuclei were labeled with DAPI (blue). (B) Control IgG-treated mice had significantly higher ratio of Lewy bodies to total nuclei when compared to TOMA-treated mice (p=0.039). (C) Additionally, ELISA of the PBS insoluble fraction of total brain homogenate showed that Control IgG mice have increased levels of LB509 reactivity approaching significance when compared to TOMA mice (p=0.05). (D) ELISA with LB509 with the PBS soluble fraction showed significantly elevated reactivity in Tau-13 treated mice compared to both Control IgG (p=0.0012) and TOMA (p=0.003). (E-F) Levels of total α-synuclein detected by ELISA with 4D6 were unchanged in both the PBS soluble and insoluble fractions of total brain homogenate between all three treatment groups. Scale bar 20 μm.

TOMA Treatment Increases Dopamine and Synaptic Proteins

In order to evaluate effects of antibody treatment on Parkinson's disease-specific toxicity, levels of catecholamines in the olfactory bulb were measured by immunohistochemistry with Tyrosine Hydroxylase (TH; Figure 5.11A). Number of TH-positive cells were significantly decreased in both Control IgG and Tau-13 treated mice when compared to Wildtype ($p=0.001$ and $p=0.011$ respectively). TOMA-treated mice did not have significantly different levels of TH compared to Wildtype, but were significantly increased compared to Control IgG mice ($p=0.028$; Figure 5.11B). Levels of TH in soluble total brain homogenate were elevated in TOMA-treated mice approaching significance when compared to Control IgG ($p=0.08$; Figure 5.11C).

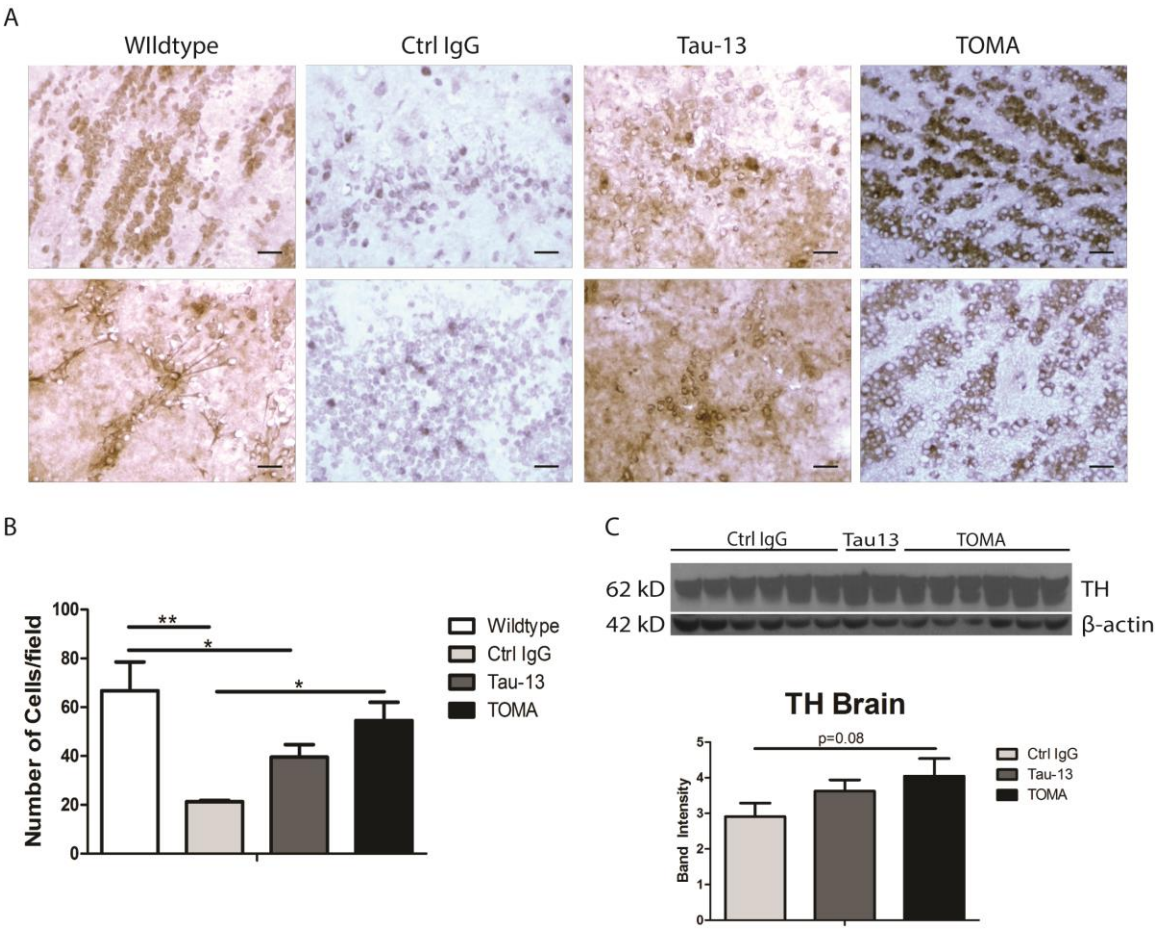

Figure 5.11 TOMA-treated mice are protected against dopamine loss in the olfactory bulb. (A) Sections of olfactory bulb were labeled with Tyrosine Hydroxylase (TH). (B) Number of TH-positive cells were significantly decreased in both Control IgG and Tau-13 treated mice when compared to Wildtype (p=0.001 and p=0.011 respectively). TOMA-treated mice did not have significantly different levels of TH compared to Wildtype, but were significantly increased compared to Control IgG mice (p=0.028). (C) Levels of TH in soluble total brain homogenate were elevated in TOMA-treated mice approaching significance when compared to Control IgG (p=0.08). Scale bar 20 μm.

Rescue of synaptic toxicity was measured by Western blot with antibodies specific to synaptic proteins. TOMA treatment was associated with significantly elevated levels of synapsin 1 in the PBS soluble fraction of total brain homogenate when compared to Control IgG (p=0.003; Figure 5.12A). Synaptophysin levels were also found to be significantly higher in TOMA-treated mice compared to Tau-13 (p=0.036; Figure 5.12B).

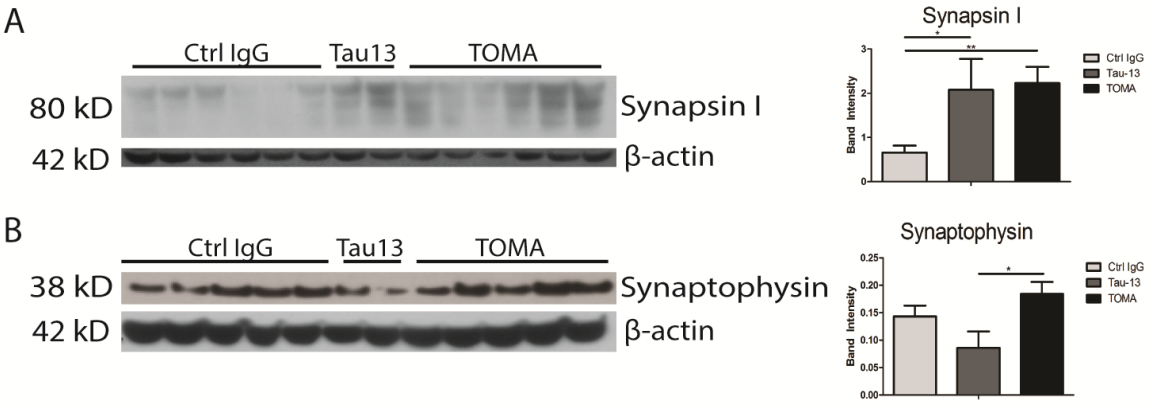

Figure 5.12 TOMA treatment is associated with elevation in synaptic protein. (A) Levels of synapsin 1 detected in the PBS soluble fraction of total brain homogenate by Western blot are significantly increased in TOMA and Tau-13-treated mice when compared to Control IgG (p=0.003 and p=0.035 respectively). (B) Synaptophysin levels are significantly elevated in TOMA-treated mice compared to Tau-13 (p=0.036).

Discussion

Synucleinopathies, including PD and DLB have no effective treatment and affect millions of people worldwide. Most therapeutic approaches thus far have focused on targeting the symptoms or decreasing α-synuclein protein. However, research from our lab and others suggest that tau protein is also a critical pathological component in synucleinopathies. We recently showed that the most toxic form of both tau and α-synuclein, the oligomers, coexist and coaggregate in PD and DLB (Sengupta, Guerrero-Muñoz et al. 2015). These results combined with studies showing a genetic and molecular interaction between tau and α-synuclein (Colom-Cadena, Gelpi et al. , Giasson, Forman et al. 2003, Simon-Sanchez, Schulte et al. 2009, Guo, Covell et al. 2013) and the ability of α-synuclein oligomers to seed the aggregation of tau (Lasagna-Reeves, Castillo-Carranza et al. 2010) suggest that the two proteins may have a synergistic interaction in disease. Therefore, targeting tau oligomers may prevent toxicity induced by toxic tau, as well as affect co-aggregation with α-synuclein.

In order to fight tau oligomers, we previously created a tau oligomer-specific antibody, TOMA, which we found to be effective in attenuating disease phenotype in three different mouse models of tauopathy and Alzheimer's disease (Castillo-Carranza, Gerson et al. 2014, Castillo-Carranza, Sengupta et al. 2014, Castillo-Carranza, Guerrero-Muñoz et al. 2015). Importantly, in a model of Alzheimer's with Aβ and tau pathology, decreasing tau oligomers was not only effective in altering cognitive dysfunction and synaptic function, but also effectively lowered levels of toxic oligomeric Aβ (Castillo-Carranza, Guerrero-Muñoz et al. 2015). These results suggest that targeting toxic tau, may also rid the brain of toxic upstream targets, such as α-synuclein. Thus far, no studies have investigated the therapeutic potential of lowering levels of tau oligomers in a

synucleinopathy model. Here we passively immunized a well-characterized synucleinopathy mouse model, the Prnp-SNCA*A53T model (Giasson, Duda et al. 2002). This mouse model has been studied extensively and shown to develop insoluble synuclein aggregates, synuclein oligomers, and hyperphosphorylated tau (Giasson, Forman et al. 2003, Kotzbauer, Giasson et al. 2004, Mazzulli, Mishizen et al. 2006, Tsika, Moysidou et al. 2010, Waxman and Giasson 2011). We demonstrated that the A53T mouse accumulates neurotoxic tau oligomers as early as 3 months old and levels continue to increase with age. Colabeling with markers for α-synuclein and tau oligomers show that similarly to human synucleinopathies, A53T mice show the co-existence of the oligomeric form of both proteins. Moreover, we performed live *in vivo* imaging that clearly showed that TOMA crosses the blood brain barrier (BBB**)** in this mouse model, suggesting that it may be a viable therapeutic.

We evaluated the cognitive and motor function of A53T mice administered either TOMA or a total tau antibody (Tau-13) when compared to wild-type controls and A53T mice given a control antibody. Surprisingly, we found that A53T mice treated with a total tau antibody were not protected against cognitive dysfunction, hyperactivity, or sensorimotor deficits. However, TOMA treatment produced a behavioral phenotype that did not differ significantly from wildtype controls on any measure evaluated for memory or motor dysfunction. These results support recent findings suggesting that targeting all forms of tau protein may actually lead to negative effects in mouse models without tau overexpression (Mably, Kanmert et al.). The lack of positive results from targeting all forms of tau and in some instances, induction of increased toxicity may be due to various factors. For one, the antibody may simply target tau oligomers at a very low rate at the

same concentration as TOMA since it is also targeting all other forms of tau, which are present at much higher concentrations than tau oligomers. Another possibility is that the antibody is targeting the monomeric form of tau, leading to functional deficits in axonal transport. Lastly, the mechanism of antibody clearance may be inducing some negative side effects, such as inflammation.

The cognitive and motor benefit seen in mice treated with TOMA suggested that lowering levels of tau oligomers may be sufficient to reduce toxicity due to tau and possible α-synuclein toxicity in synucleinopathies. In order to investigate the mechanism of protection exerted by TOMA and likewise, the effects of Tau-13 treatment that prevent similar benefits, brains of A53T mice were collected for analysis of tau and α-synuclein levels. As expected, TOMA-treated mice had significantly lowered tau oligomers when compared to A53T mice injected with a control antibody, as well as lowered levels of a 37 kD fragment that was detected by tau oligomer-specific antibody, T22. However, Tau-13 treated mice did not show the same level of decrease and actually had significantly higher levels of low molecular weight tau oligomers when compared to TOMA. Thus, the difference in behavioral effects between TOMA and Tau-13 may be partially explained by ineffective targeting of the most toxic tau species by Tau-13. Levels of functional tau monomer, on the other hand, were not found to differ between groups.

In the testing of any potential therapeutic, it is important to consider possible side effects. Anti-amyloid immunotherapy approaches and some reports have shown that inflammation may be associated with the clearance of amyloid deposits (Bard, Cannon et al. 2000, Wilcock, DiCarlo et al. 2003, Wilcock, Rojiani et al. 2004, Masliah, Rockenstein et al. 2011). In order to evaluate the role of inflammation in each treatment,

we examined astrocytes as an inflammatory marker with GFAP by immunofluorescence microscopy. We did not find any evidence of increased inflammation with TOMA treatment, as the number was astrocytes was unchanged with treatment (results not shown). However, Tau-13 and Control IgG treated mice did show changes to astrocyte morphology, with increased reactive astrocytes, as well as astrocytic endfeet associated with tau oligomer-positive vasculature, suggesting that inflammation may partially underlie some of the toxicity in control and Tau-13-treated A53T mice.

The co-aggregation and interaction of tau and α-synuclein led to the hypothesis that decreasing tau oligomers will also deplete oligomeric α-synuclein. Surprisingly, evaluation with α-synuclein oligomer-specific antibody, Syn33, showed that in the PBS soluble brain fraction, oligomers may actually be increased in TOMA-treated mice compared to control mice and insoluble brain oligomers were elevated with Tau-13 treatment. In order to investigate the potential differences in toxicity between α-synuclein oligomers in each treatment group, immunohistochemistry was also completed with and without Proteinase K (PK) treatment as a recent study showed that the most toxic form of oligomeric α-synuclein is likely partially resistant to PK (Roberts, Wade-Martins et al. 2015). In the proteinase treated sections, TOMA treatment was associated with opposite effect, significantly lowered levels of α-synuclein oligomers. We then sought to determine whether changes to oligomer levels were associated with a converse outcome on Lewy bodies. Indeed, we found that the number of Lewy bodies detected by immunohistochemistry was significantly higher in the control antibody group when compared to TOMA and similar results were seen when LB509 levels were measured in the PBS soluble brain fraction. However, using LB509 in insoluble brain revealed

significantly elevated levels in Tau-13 treated mice. Levels of total α-synuclein protein were consistent across all three treatment groups, as expected. Thus, the relationship between tau oligomer depletion and α-synuclein aggregation appears to be multi-faceted and more research will be needed to fully understand the effects of treatment with TOMA.

Following analysis of tau and α-synuclein alterations with treatment, we examined the functional outcome of treatment on neurotransmitter and synaptic changes common to synucleinopathies. While the effect of A53T expression in mice on dopamine levels has not been conclusively determined, researchers have shown that A53T expression has no effect on levels of tyrosine hydroxylase (TH) in the substantia nigra, suggesting that it is more of a model of synucleinopathy than PD specifically (Sotiriou, Vassilatis et al. 2010). However, TH levels in the spinal cord, olfactory bulb and the locus coeruleus are decreased in A53T mice (Sotiriou, Vassilatis et al. 2010, Farrell, Krishnamachari et al. 2014). Olfactory dysfunction and decreased dopamine has also been shown to be an important factor in both Alzheimer's and PD (Mazzulli, Mishizen et al. 2006, Ubeda-Bañon, Saiz-Sanchez et al. 2010, Kim, Park et al. 2014). Changes to TH levels may actually reflect a combination of both dopaminergic and noradrenergic effects, both of which may induce behavioral deficits in mouse models of synucleinopathy. We found that treatment with tau oligomer-specific antibody (TOMA) in A53T mice led to significant increases in levels of TH in the olfactory bulb when compared to control A53T mice, suggesting that tau oligomer toxicity may impact dopamine and other catecholamine levels. Moreover, investigation of synaptic proteins revealed benefits of

TOMA treatment as well. Both Synapsin 1 and Synaptophysin were increased in TOMA-treated mice when compared to control groups.

Our results show for the first time, a benefit of a tau oligomer therapeutic for the prevention of cognitive and motor deficits, as well as synaptic dysfunction in a synucleinopathy model, suggesting that tau may be heavily involved in these negative consequences of synuclein-related neurodegeneration. Moreover, negative effects with a total tau antibody seem to be due to both ineffective lowering of tau oligomers, as well as potential inflammatory side effects. Surprisingly, treatment with a tau oligomer-specific antibody did not appear to decrease soluble α-synuclein oligomers in preliminary analyses, though proteinase-resistant oligomers and Lewy bodies were lowered. Further research will be necessary to conclusively determine the effects of TOMA on α-synuclein aggregation. However, these findings suggest that a combination therapeutic approach using an antibody specific for oligomeric α-synuclein as well as tau, rather than either alone, may be the most effective strategy at reversing toxicity from both tau and α-synuclein oligomers.

CHAPTER 6

TAU OLIGOMERIC STRAINS: GENERATION, CHARACTERIZATION AND IMPLICATIONS FOR DISEASE PHENOTYPES

Introduction

In the search for an effective therapeutic strategy against Alzheimer's disease (AD), the most common cause of dementia worldwide, aggregated amyloid-β (Aβ) emerged as the main target of interest and has remained so for many years. However, as more and more clinical trials against the protein failed, researchers searched for a new and hopefully more effective route to treatment (Godyń, Jończyk et al. 2016). The pathological aggregation of the microtubule-associated protein tau and its subsequent deposition in neurofibrillary tangles (NFTs) are also defining histopathological features of AD that correlate better with symptoms than Aβ (Braak and Braak 1995). Therefore, the study of tau has become an active field of study with a lot of promise (Šimić, Babić Leko et al. 2016). Tau aggregation also plays a role in many other neurodegenerative disorders, collectively known as tauopathies, including Pick's disease (PiD), progressive supranuclear palsy (PSP), dementia with Lewy bodies (DLB), Parkinson's disease (PD), frontotemporal dementia (FTD), and corticobasal degeneration (CBD) (Ballatore, Lee et al. 2007, Gendron and Petrucelli 2009). Although this large group of related disorders shares the common pathological hallmark of accumulated tau protein aggregates in the brain, they are diversified with a vast array of pathologies and symptom progressions.

NFTs may play a role in the pathophysiology of tauopathies, but little is known about the mechanisms underlying their formation and the role of soluble tau intermediates between tau monomers and filaments. Hyperphosphorylation contributes to the formation of NFTs, which until recently were believed to trigger processes that lead to neuronal cell death (Andrade-Moraes, Oliveira-Pinto et al. 2013). However, NFT accumulation alone may be insufficient to cause cell death, as neuropathological studies in AD patients suggest that neuronal loss and cognitive deficits precede NFT formation (Haroutunian, Davies et al. 2007). Supporting this, animal models show that tau oligomers appear prior to NFTs and contribute to learning and memory deficits and neuronal cell death, while NFTs are not associated with neuronal death (Lasagna-Reeves, Castillo-Carranza et al. 2011, Cowan, Quraishe et al. 2012, Cook, Kang et al. 2015, Kim, Choi et al. 2016).

Despite increasing research on this topic, the mechanism by which tau induces toxicity and disease progression remains unknown. One of the largest barriers to conclusive mechanistic investigations of amyloidogenic and tau proteins is inconsistency in the protein species studied (Cowan and Mudher 2013), an importance consideration asthe aggregation state of tau is critical to its function. Within the same aggregation state, tau exhibits conformational differences that could exert diverse downstream effects (Hyman 2014, Sanders, Kaufman et al. 2014). Many parallels exist between prions and amyloidogenic proteins present in neurodegenerative disease, suggesting that tau and other aggregate-prone proteins may form prion-like strains that affect disease progression. Therefore, understanding the characteristics of different tau aggregates may

be critical for the investigation of tau's toxicity and may be responsible for the pathological and symptomatic differences underlying various tauopathies (Hyman 2014).

Prion Structural Polymorphisms and Strains

One of the most puzzling phenomena in prion biology is the existence of prion strains, defined as infectious protein isolates that, when transmitted to identical hosts, induce distinct prion-disease phenotypes(Bessen 1992, Bartz 2000, Klimova, Makarava et al. 2015). The phenotypic traits include incubation times, histopathological lesion profiles, and specific neuronal target areas. A common measure of prion strain differences is stability of the stable core of the aggregated protein, which is measured by resistance to proteinase K and other degradation methods. Truncation of different prion conformations leads to detection of variable patterns of fragments. Moreover, detection in tissue can be completed to evaluate the morphology and location of proteinase-resistant forms of the protein. These traits typically persist following serial transmissions, thereby stabilizing strain conformation(Bartz 2000). The strain phenotypes might be encoded by the same aggregate assuming different conformations that have distinct properties. These conformations can also be manipulated(Legname, Nguyen et al. 2005, Tanaka, Collins et al. 2006). It has been demonstrated that multiple strains may exist in different brain regions in the same patient, and this is hypothesized to underlie the individual variability in the clinical manifestations of patients with Creutzfeldt-Jakob disease(Schoch 2006). Prion strains can compete with one another, suggesting that less pathogenic prions could be used as a therapeutic agent against the most toxic strains(Morales, Abid et al. 2007, Klimova, Makarava et al. 2015). A recent study in the prion field showed that a single mutation leading to a particular prion strain led to complete protection against toxic

misfolded prion protein due to an inability to propagate the toxic prion conformation(Asante, Smidak et al. 2015). The strain phenomenon has recently been hypothesized to extend to many amyloidogenic proteins that form distinct conformations and can be classified as strains, including amyloid-beta (Aβ)(Heilbronner G 2013, Lu JX 2013) and α-synuclein(Guo, Covell et al. 2013).

Amyloidogenic Protein Oligomeric Strains

Similarly to tau, many groups have reported that fibrils, once thought to be the most toxic species, are actually less toxic than intermediate Aβ aggregates (spherical oligomers and protofibrils) (Glabe and Kayed 2006). There are a variety of Aβ oligomer conformations (Glabe 2008) that can be produced by several pathways. Simple manipulation of conditions in which Aβ aggregates can alter conformation, highlighting the complexity of oligomer formation (Kayed, Head et al. 2003, Glabe and Kayed 2006, Kayed and Glabe 2006, Kayed, Head et al. 2007, Necula, Kayed et al. 2007, Glabe 2008, Mina, Lasagna-Reeves et al. 2009). Several studies suggest that oligomeric species form via different mechanisms and exhibit different toxicities (Cizas, Budvytyte et al. 2010, Zako 2010, Kayed and Lasagna-Reeves 2013). A recent study found that two different Aβ oligomers that differ in toxicity can be differentiated using conformation-specific antibodies (Liu, Reed et al.). Aβ conformational differences have also been specifically correlated with more rapidly progressive AD (Cohen, Kim et al. 2015). Moreover, structurally distinct α-synuclein strains can lead to different behavioral phenotypes and levels of toxicity (Peelaerts, Bousset et al. 2015) and may be differentiated using similar techniques used in the prion field, such as resistance to proteases (Roberts, Wade-Martins et al. 2015). Strains of α-synuclein derived from multiple systems atrophy (MSA) were recently

shown to be capable of serial transmission in cell culture as well as to induce neuronal dysfunction following successive inoculations in mice (Prusiner, Woerman et al. 2015, Woerman, Stöhr et al. 2015). Thus a variety of oligomeric species may be common to many proteinopathies and underpin evolution of disease, as well as distinct sub-types of disease.

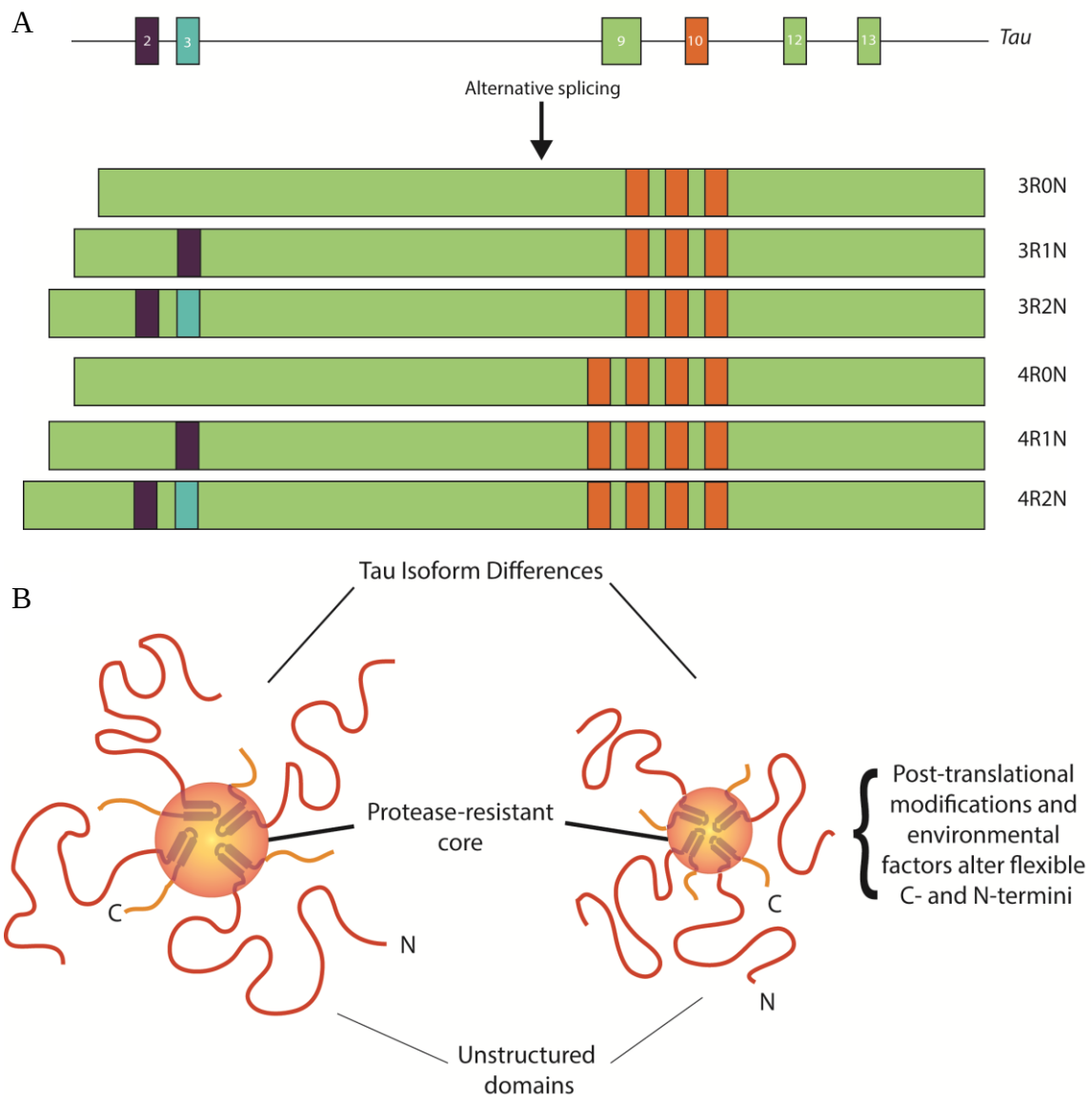

Figure 6.1 *Alternative splicing leads to the formation of six different tau protein isoforms. (A)* Alternative splicing of tau and tau isoform makeup may alter protein folding and aggregate conformation. Tau forms six different isoforms, three of which have three repeats in the microtubule binding domain (3R0N, 3R1N, 3R2N) and three of which have four repeats (4R0N, 4R1N, 4R2N). *(B)* Tau isoforms may lead to differential stability and structure in tau oligomers depending upon the rigidity of the structure.

Modifications and environmental conditions can alter the unstructured C- and N-termini surrounding the protease-resistant core, which is stabilized by its β-sheet conformation.

Classes of Aggregated Tau

Due to the variety of diseases involving aggregated tau with distinct pathological features and symptomology, conformational differences in tau may be a crucial factor distinguishing tauopathies. Different conformers may be present in one or many of the forms of aggregated tau.

Oligomers

Tau in its native state exists as an unfolded monomer which has an important function stabilizing microtubules. *Tau* undergoes alternative splicing to form six different protein isoforms (Figure 6.1A) present at varying levels during development, normal adulthood, and disease states. The isoform of tau has important implications for its structure as the number of microtubule binding repeats in the stable core of affects its aggregation stability and the unstructured N- and C-terminal domains may underlie much of the conformational diversity as they provide environmentally-dependent flexibility to the protein (Wegmann, Medalsy et al. 2013, Xu, Zheng et al. 2016) (Figure 6.1B). The unfolded state of monomeric tau, when released from microtubules allows intermolecular contacts and exposure of hydrophobic regions, which can lead to the misfolding and aggregation of tau. Multimeric tau species which form, known as tau oligomers, have repeatedly been found to induce neurotoxicity and cell death in neurodegenerative tauopathies, rather than the large fibrillar structures known as neurofibrillary tangles

(NFTs) (Spires, Orne et al. 2006, Berger, Roder et al. 2007, Maeda, Sahara et al. 2007, Kopeikina, Carlson et al. 2011, Cowan and Mudher 2013, Sahara, DeTure et al. 2013, Cowan, Quraishe et al. 2015). We have shown that these dynamic tau aggregates are detected in human disease and can propagate through the brain, causing synaptic and mitochondrial dysfunction associated with memory deficits when administered to cognitively normal mice (Lasagna-Reeves, Castillo-Carranza et al. 2011, Lasagna-Reeves, Castillo-Carranza et al. 2012). Importantly, reducing levels of tau oligomers with oligomer-specific antibodies leads to protection against behavioral deficits and tau pathology in multiple mouse models (Castillo-Carranza, Gerson et al. 2014, Castillo-Carranza, Sengupta et al. 2014, Castillo-Carranza, Guerrero-Muñoz et al. 2015).

Protofibrils

Protofibrils are intermediate, metastable species that form en route to fibrillization. Protofibrils share some structural similarities with mature fibrils, including a rope-like fibrillar structure detectable by EM and AFM and stable hydrogen bonding. However, protofibrils are inherently more dynamic and unstable and do not show high response to thioflavin T (Williams, Sega et al. 2005), which recognizes the β-sheet structure characteristic of amyloid fibrils. Protofibrils can be detected with oligomer-specific conformational antibodies, indicating their similar structure (Kayed, Head et al. 2003). Similarly to oligomers, these soluble intermediates have been shown to be more toxic and relevant to disease pathogenesis than the larger, more stable fibrillar structures and neurofibrillary tangles (Harper, Wong et al. 1997, Walsh, Lomakin et al. 1997, Habicht, Haupt et al. 2007, 2008, Rijal Upadhaya, Capetillo-Zarate et al. 2012)

Annular Protofibrils

The formation of pore-like structures, known as annular protofibrils, has been known for amyloid proteins, including amyloid-β and α-synuclein for years (Hafner, Cheung et al. 2001, Kril, Patel et al. 2002, Lasagna-Reeves, Glabe et al. 2011). Recently, the formation of tau annular protofibrils was shown *in vitro* and in human disease tissue (Lasagna-Reeves, Sengupta et al. 2014). Results suggest that tau oligomers can go on to form annular protofibrils by a specific off-pathway mechanism from fibril formation. These pore-like structures are believed to disrupt permeability of the cell membrane, leading to non-specific ion leakage.

Tau Fibrils

Paired helical filaments (PHF) and straight filaments (SF) that make up NFTs are comprised of hyperphosphorylated tau (Grundke-Iqbal, Iqbal et al. 1986, Lee, Balin et al. 1991) in a β-sheet conformation (von Bergen, Barghorn et al. 2005). Differences in NFT location and structure exist among various tauopathies. In a study by Clavaguera et al., brain homogenate derived from various tauopathies was injected into the brains of ALZ17 mice, which express non-mutated human tau, and this induced the deposition and spread of characteristic tau aggregates from each disease (Clavaguera, Akatsu et al. 2013). Recently, Boluda et al. extended these results, showing that at much earlier time points than the original study, CBD and AD-specific pathology propagates in ALZ17 mice following brain homogenate injection, with distinct cell specificity (Boluda, Iba et al. 2015).

While this is strong evidence for the existence of tau strains, it is critically important to accurately identify the species in total brain homogenate responsible for

seeding the spread of unique tau pathologies. In addition to isoform composition, research suggests that disulfide linkages impact fibril structure (Furukawa, Kaneko et al. 2011). Sanders et al. (2014) reported that conformationally distinct tau fibrils generated from the tau repeat domain propagate in cells over multiple passages. Additionally, when injected bilaterally in the hippocampi of P301S mice, different strains are propagated over multiple generations and can be differentiated by their biochemical characteristics (Sanders, Kaufman et al. 2014). However, fibrillar strains were derived from tau fragments, which may not accurately reflect natural conditions in human disease and as no behavior was completed, toxicity *in vivo* is difficult to assess from this study (Sanders, Kaufman et al. 2014). Nonetheless this study provided good evidence for the idea that the conformational diversity of tau strains dictates its toxic potential and transmissibility. In addition, expressing specific point mutations in truncated tau also leads to diversification of fibrillar structure (Meyer, Dinkel et al. 2014).

Thus it is of great interest to determine whether toxic tau oligomers form distinct strains that differ by disease. Similarities between tau and other amyloidogenic proteins with unique conformational strains suggest that this is likely the case (Figure 6.2).

Other Potentially Toxic Species

Misfolded tau monomer is another possibly toxic species in tauopathies. While most studies show that tau monomer is non-toxic and incapable of propagating tau pathology (Lasagna-Reeves, Castillo-Carranza et al. 2012, Wu, Herman et al. 2013), an abnormal, higher molecular weight form of tau monomer known as PHF-tau or A68 has been shown to exhibit some potentially toxic characteristics (Liu 1993). The 68 kD tau is found in higher proportions in tau aggregates in disease brains and is more

highly phosphorylated than native tau monomer (Bramblett, Goedert et al. 1993, Shin, Bramblett et al. 1993, Miller 1999). Truncated tau may also be a toxic species of interest. Fragmentation of tau has also been shown to be affected in disease states, led to increased aggregation *in vitro*, and increased toxicity (Zilka, Filipcik et al. 2006, De Strooper 2010, Reifert, Hartung-Cranston et al. 2011).

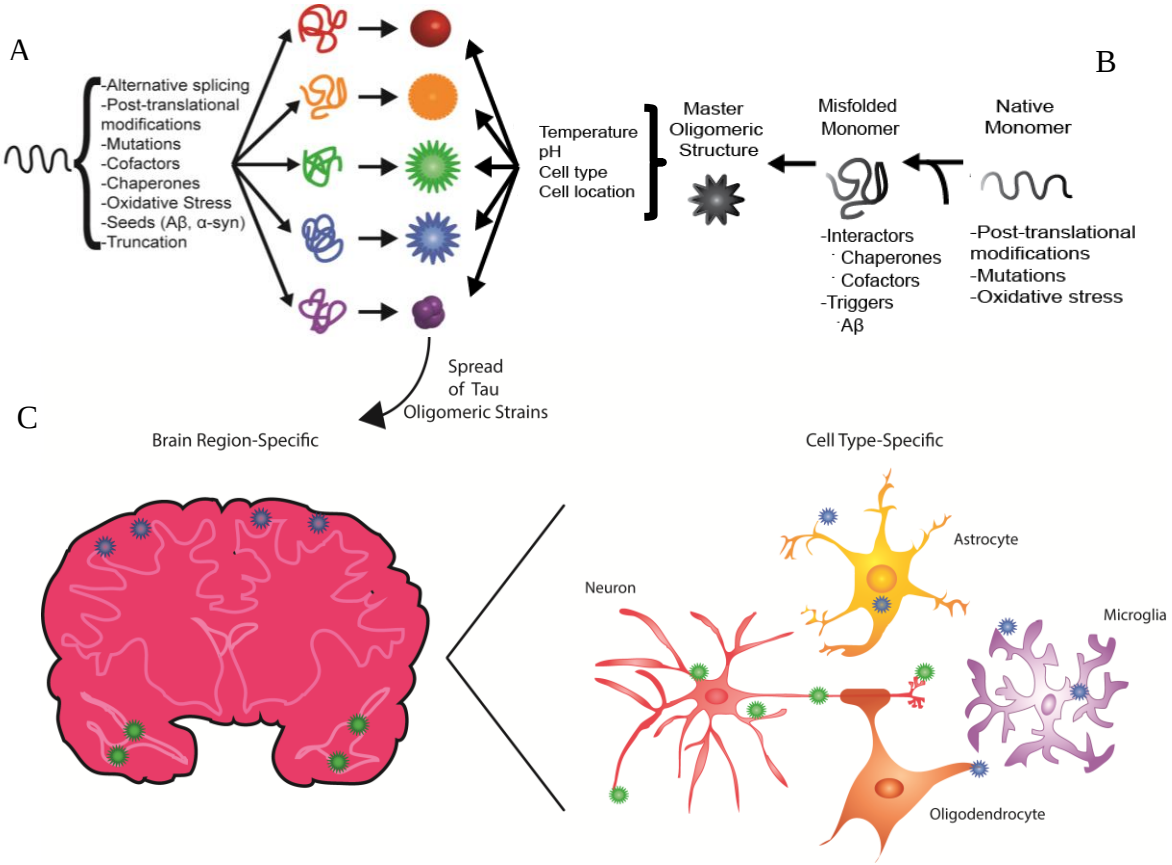

Figure 6.2 *Hypothetical models for the formation of tau oligomeric conformers and different factors that may lead to the formation of different tau strains.* (A) Risk factors cause tau misfolding and lead to the formations of conformationally distinct misfolded monomers that aggregate into different strains. (B) Misfolded tau induces the formation of a flexible, dynamic, master oligomeric strain that transforms into different oligomeric strains depending on the less defined risk factors.

Tau Oligomeric Strains

Collectively the evidence suggests that dynamic tau intermediate soluble aggregates (tau oligomers) are the toxic tau entities and initiators of tau pathology and propagation. The formation of other amyloidogenic protein oligomeric strains and evidence for tau fibrillar strains suggests that tau oligomers may exist in different conformationally distinct conformers (strains) that play a critical role in disease phenotypes. One pre-fibrillar tau oligomer species that has been characterized and appears to have differential structure and toxicity is the granular tau oligomer, reviewed by Cowan and Mudher (Cowan and Mudher 2013). This is a 40mer structure identified in AD brains believed to be a precursor of tangles. However, it does not appear to be as clearly toxic as other oligomeric tau species(Cowan, Quraishe et al. 2015), similarly to the fibrillar Aβ oligomer strain that was found to be less toxic than other conformational states(Liu, Reed et al. 2015). The reason for the lowered toxicity could be due to a lack of beta pleated sheet conformation in granular tau oligomers or a larger and therefore less dynamic structure. This illustrates the fact that tau aggregates vary in constituent tau composition and conformation, which may play a role in determining their toxicity. Thus, the elucidation of the different tau oligomeric conformers may reveal new targets for novel therapeutic strategies tailored for different neurodegenerative tauopathies and perhaps could be used to screen the best candidates and experimental drugs.

Tau Aggregation as a Secondary Amyloidosis

The critical role of amyloidogenic oligomers has been revealed for multiple neurodegenerative diseases (Walsh and Selkoe 2004). Recently published works support the idea that prefibrillar soluble oligomers of amyloidogenic proteins, including α-

synuclein and amyloid-beta (Aβ) are more toxic than insoluble Lewy bodies and senile plaques (Klein, Krafft et al. 2001, Walsh, Klyubin et al. 2002, Gispert, Del Turco et al. 2003, Kayed, Head et al. 2003, Lesne, Koh et al. 2006, Walsh and Selkoe 2007, Danzer, Krebs et al. 2009, Tsika, Moysidou et al. 2010). Pathogenic proteins involved in different neurological disorders often interact with one another and modulate downstream activities that disrupt their normal function. Although Alzheimer's disease and Parkinson's disease have unique pathological features, there is considerable overlap between these two disorders. The effects of Aβ aggregation on tau misfolding and aggregation are well-established (Hardy 2002, Oddo, Vasilevko et al. 2006, Clinton, Blurton-Jones et al. 2010, Bloom 2014). It is also well-established that aggregated Aβ is an important contributor to tau phosphorylation and aggregation in animal models and cell cultures. In primary neuronal culture, Aβ is capable of inducing tau phosphorylation (Busciglio, Lorenzo et al. 1995). Aβ42 fibrils induced formation of neurofibrillary tangles in P301L tau transgenic mice (Gotz, Chen et al. 2001), and pre-aggregated Aβ42 induced tau paired helical filament (PHF) formation in cells over-expressing human tau (Ferrari, Hoerndli et al. 2003, Pennanen and Gotz 2005). These experiments were performed with aggregated Aβ, which implies that they contain different prefibrillar and fibrillar Aβ aggregates. Recently, it has been shown that soluble Aβ oligomers trigger tau aggregation *in vitro* and *in vivo* (Chabrier, Blurton-Jones et al. 2012). We previously demonstrated that Aβ and α-synuclein oligomers prepared *in vitro* are capable of inducing toxic tau oligomer formation (Lasagna-Reeves, Castillo-Carranza et al. 2010), targeting tau oligomers in an Aβ precursor protein (APP) overexpression mouse model also leads to a decrease in toxic Aβ aggregates and a genome-wide association study reported genetic

interactions between tau and α-synuclein in Parkinson's disease pathogenesis (Simon-Sanchez, Schulte et al. 2009). Moreover, fibrillar α-synuclein has been shown to induce tau tangle formation *in vitro* (Waxman and Giasson 2011) and accelerate tau phosphorylation, further enhancing its likelihood to form aggregates (Jensen, Hager et al. 1999). Recently two different α-synuclein fibrillar seeds have been identified with varying capacities of causing tau aggregation both in neuronal cultures and *in vivo* (Guo, Covell et al. 2013). A study also provided evidence that α-synuclein induces fibrillar tau formation and found that both tau and α-synuclein exert synergistic effects on each other leading to the formation of fibrillar amyloid structure (Giasson, Forman et al. 2003). Although insoluble forms of α-synuclein and tau proteins have been observed to co-exist in DLB patients (Iseki, Takayama et al. 2002), our recent report was the first published evidence that these proteins occur together within oligomeric organizations that further perpetuate their aggregation (Sengupta, Guerrero-Muñoz et al. 2015).

Collectively this illustrates that the complexity of neurodegenerative tauopathies is likely to be partially dependent upon interactions between other proteins, particularly other amyloids. Cross-seeding between aggregant proteins in synucleinopathies (Chapter 5, Figure 5.1) and Alzheimer's disease (Figure 6.3) may induce the formation of diverse tau conformers differing between disease. In order to evaluate differences in tau oligomeric strains that interact with other amyloidogenic proteins, Aβ and α-synuclein, in this study tau oligomeric strains were characterized in both pure tauopathies, PSP and FTLD, and mixed protein pathology tauopathies, AD, PD and DLB.

While tau is a key hallmark in the common mixed pathology disorders, AD, PD, and DLB, phenotypes and pathologies also differ between tauopathies lacking other

amyloidogenic proteins in addition to tau. Therefore, other mechanisms that are independent of other aggregant proteins must be at play.

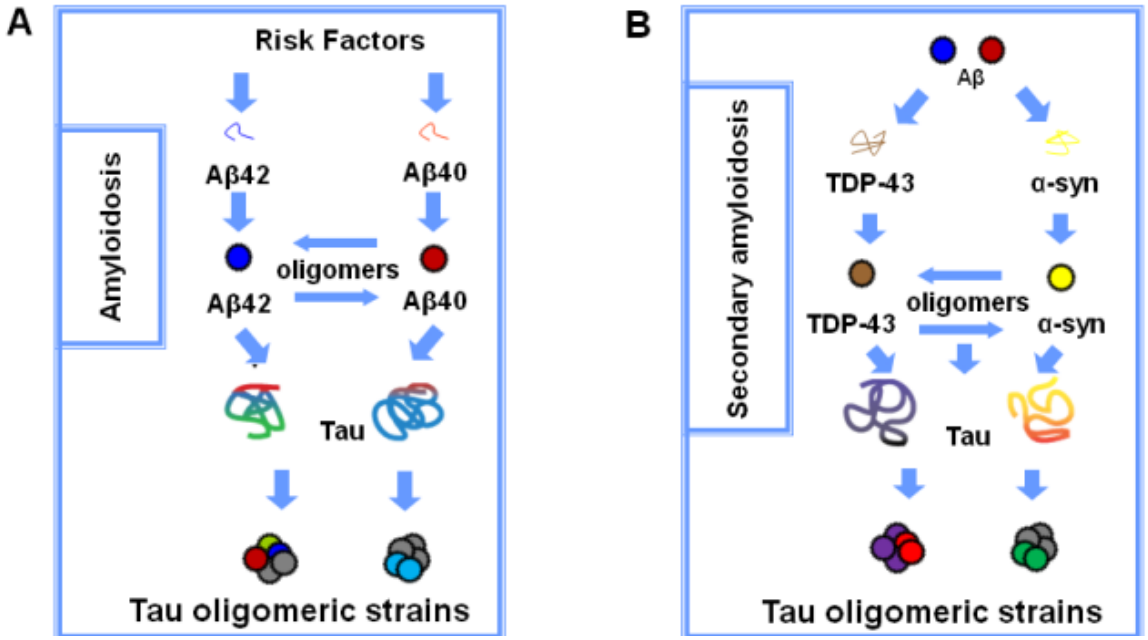

Figure 6.3 Cross-seeding of tau oligomers with other amyloidogenic proteins leads to the formation of tau oligomeric strains.

Tau Modifications

Modifications to tau take place in the form of alternative splicing, mutations, post-translational modifications, and truncation. These changes could alter the folding of the protein and lead to the formation of different conformations. In support of this idea is the fact that tauopathies are associated with different tau isoform compositions. For example, PSP and CBD display 4R tau pathology and 3R tau aggregation, respectively, while PiD and AD display both 3R and 4R aggregates. Studies have found that 3R tau forms mainly

twisted PHFs, while 4R tau fibrils are mainly SFs *in vitro* (Goedert 1996, Barghorn and Mandelkow 2002). However, the *in vivo* fibrillar conformations of isoform-specific aggregates in PiD and CBD vary (Munoz-Garcia 1984, Ksiezak-Reding 1994). We have shown that brain-derived tau oligomers from PSP are capable of seeding both 3R and 4R tau, suggesting that differences between diseases in which isoform-specific pathology is seen is likely multifactorial (Gerson, Sengupta et al. 2014). Here, we have evaluated differences in stability between 3R and 4R tau isoforms and association with mutations, another likely cause of conformational differences in disease proteins.

While tau mutations are not associated with AD or PD, they do induce other tauopathies. The identification of around 40 tau mutations that cause familial dementia, such as FTD and Parkinsonism linked to chromosome 17 (FTDP-17) demonstrated that tau alone could cause neurodegeneration (Goedert and Jakes 2005). However, the mechanisms by which tau mutations cause neurological disorders are still unclear, though the effect of mutations on tau aggregation and fibril formation has been studied extensively. While tau protein will not spontaneously aggregate *in vitro* without the addition of polyanionic cofactors, tau fragments expressing point mutations, P301L and ΔK280, rapidly aggregate due to an increase in the shift towards β-sheet structure (von Bergen, Barghorn et al. 2001). Multiple mutations have been identified in the microtubule binding repeat region (P301S, P301L, and P301T), which highlights its importance in aggregation. Additionally, in a study on the effect of mutations on heparin-induced fibrillar assembly, P301L and P301S mutations led to the largest increase in filament formation. G272V also increased filament formation in both 3R and 4R tau, while V337M had a small stimulatory effect only in 3R tau isoforms (Goedert, Jakes et

al. 1999). Studies carried out both *in vitro* and *in vivo* suggest that different mutations may have varied phenotypic and pathogenic effects. The P301S mutation is associated with early onset of clinical symptoms of FTDP-17, reduced microtubule assembly, and increased filament assembly. P301S mice show motor symptoms, including severe partial paralysis of the hindlimbs as well as hyperphosphorylated tau aggregates in the brain and spinal cord, but no signs of apoptosis (Allen, Ingram et al. 2002). On the other hand, P301L mice have increased levels of apoptosis (Götz, Chen et al. 2001, Ho, Xiang et al. 2001). These mice also display motor deficits and NFTs in the brain and spinal cord, as well as deposits in glial cells (Lewis J 2000, Götz, Chen et al. 2001). Meanwhile, the R406W mutation associated with FTDP-17 induces similar clinical symptoms and neuropathology seen in Alzheimer's disease. Mice expressing the mutation develop NFTs of similar morphology to AD inclusions in the forebrain and cognitive deficits, but no sensorimotor symptoms (Tatebayashi, Miyasaka et al. 2002). Another mutation associated with FTD, S320F, produces a Pick's disease-like tauopathy (Rosso, Van Herpen et al. 2002). Moreover, a recent study revealed that mutations give rise to varied tau fibrillar strains (Meyer, Dinkel et al. 2014). Here, we have evaluated the seeding efficiency and stability of two mutations, P301S and S320F, which produce highly distinct pathological and phenotypic traits.

Post-translational modifications are also likely candidates for creating diverse tau oligomeric conformations. Phosphorylated tau is well-known to be altered in disease and to affect aggregation. However, the relationship between phosphorylation state and toxicity is complex, with different sites leading to widely varying effects, as was illustrated recently in a *Drosophila* model of tauopathy (Papanikolopoulou and Skoulakis

2015). In fact, elevated levels of tau phosphatase has been shown to increase toxicity of tau in the extracellular space (Díaz-Hernández, Gómez-Ramos et al. 2010). Moreover in a *Drosophila* model of tauopathy, reduced tau phosphorylation was associated with rescue of phenotype but increased oligomerization of tau (Cowan, Quraishe et al. 2015). Tau has also been shown to be altered by a number of other post-translational modifications including acetylation, glycation, and others (Gerson, Castillo-Carranza et al. 2014), suggesting that this wide variability could have an effect on tau oligomer conformation. Tau has also been shown to form disease-specific fragments that enhance its ability to seed the formation of oligomers and spread through the extracellular space (Wang, Martinez-Vicente et al. 2009, Kanmert, Cantlon et al. 2015, Matsumoto, Motoi et al. 2015). These results suggest that tau modifications could be involved in the formation of unique oligomeric tau conformations that result in different disease phenotypes.

Cell and organelle specificity

Regional and cellular differences in accumulation of tau aggregates between neurodegenerative diseases have been long known and used to differentiate disorders, even prior to the proposal of the prion-like attributes of tau and other aggregant proteins. Alzheimer's disease is characterized by the presence of flame-shaped NFTs in neurons and rarely glial inclusions. In Pick's disease, tau aggregates are primarily found in spherical Pick bodies in neurons, but can also be detected in lower levels in astrocytes and oligodendrocytes, while in both PSP and CBD there is widespread deposition of astrocytic and oligodendroglial tau aggregates (Yoshida 2006). Moreover, we found that while multiple tauopathies contain tau annular protofibrils, cellular specificity of their formation differed by disease, suggesting the presence of disease and cell-specific

conformations (Lasagna-Reeves, Sengupta et al. 2014). Here, we have investigated cell type specificity in tau oligomeric strains detected in human tauopathy tissue for the first time.

In addition to intercellular differences, alterations to the intracellular localization of tau may correlate with disease. While tau in its native state is commonly located in the axonal cytoskeleton, recent studies suggest that it also has a nuclear role, binding and protecting DNA and RNA under oxidative stress conditions that also lead to the formation of toxic tau oligomers (Sultan, Nesslany et al. 2011, Violet, Delattre et al. 2014). In this environment, functional tau translocates from the cytosol to the nucleus, however tau oligomer formation appears to both inhibit the movement of protective tau to the nucleus and lead to the buildup of nuclear tau aggregates (Violet, Chauderlier et al. 2015). Tau may also differentially interact with RNA in disease states in the endoplasmic reticulum (ER) as tau has been shown to be associated with ribosomal and other ER proteins (Meier 2015). The redistribution of tau protein may also underlie synaptic dysfunction seen in tauopathies as tau aggregates have been found in higher proportions in the somatodendritic compartment and can be specifically detected at the pre- and post-synaptic densities in disease states (Meyer, Dinkel et al. 2014).

Spread of tau oligomeric strains

The ability of amyloid and tau fibrils to seed soluble monomers and convert them to aggregates capable of propagating has been known for more than 20 years and is well standardized and documented (Jarrett, Berger et al. 1993, Kelly 2000, Margittai and Langen 2004, O'Nuallain, Williams et al. 2004, Siddiqua and Margittai 2010, Dinkel, Siddiqua et al. 2011). Recently, our lab and others have shown that amyloid oligomers

also can seed and propagate *in vitro* (Kayed, Canto et al. , Lee, Culyba et al. 2011). Moreover, we discovered that tau oligomers prepared from recombinant tau can seed the aggregation of tau *in vitro* (Lasagna-Reeves, Castillo-Carranza et al. 2010). It is hypothesized that brain-derived tau oligomers seed and propagate by a mechanism known as *oligomer-nucleated conformational induction*. Unlike the seeding mechanism proposed for tau fibrils prepared *in vitro*—template-assisted growth (Margittai and Langen 2004, Congdon, Kim et al. 2008)—this mechanism excludes the addition of tau monomer to the nucleus/template, stating instead that all monomers will assemble into oligomers before fibril formation. In this mechanism, different conformational strains of tau oligomers would propagate rapidly, seeding endogenous monomer to oligomerize prior to forming stable fibrillar structures.

There is increasing interest in understanding the spread of disease from the early stages of tauopathies. In the specific case of AD, NFTs progressively spread throughout the brain in an anatomically stereotypical manner (Braak and Braak 1991, Delacourte, Sergeant et al. 2002). Based on these and several other studies, it has been postulated that tau proteins spread via a prion-like mechanism (Clavaguera, Bolmont et al. 2009, Brundin, Melki et al. 2010, Frost and Diamond 2010). Clavaguera and coworkers support this concept by demonstrating that intracerebral injections of brain extracts from mice expressing human P301S tau induce the formation and spread of tau aggregates. The pathology spreads from the injection site to neighboring brain regions 15 months post-injection in mice transgenic for human wild type tau (ALZ17 mouse model) (Clavaguera, Bolmont et al. 2009) and 12 months post-injection in wild-type mice. The observation that some areas of the human brain show evidence of neurodegeneration prior to the

formation of NFTs and clinical diagnosis (Kril, Patel et al. 2002) suggests that tau oligomers could be responsible for neuronal death and the spread of tau pathology. Up to this point, all studies investigating the propagation of tau in cells and *in vivo* have used heterogeneous populations of aggregated tau; therefore, whether tau oligomers—as opposed to other species—play a leading role in tau aggregation and propagation has been difficult to ascertain. A recent publication by Mathias Jucker and Larry Walker demonstrated that soluble Aβ assemblies (oligomers) are the most potent amyloid-inducing factors, 50-fold stronger than large fibrillar aggregates (Langer, Eisele et al. 2011).

These results combined with evidence for the existence of different tau oligomeric strains suggest that varying regional distribution and time to acquire disease may be due to differences in the ability of tau conformers to seed and spread through the brain. Within the same disease, some strains may correlate with certain disease stages. There may be diversity by brain region as well as by cell type as different environmental conditions including pH (Sneideris, Darguzis et al. 2015, Verasdonck, Bousset et al. 2015) and temperature (Tanaka, Collins et al. 2006) could lead to conformational changes. The impact of interactions with chaperones, as well as post-translational modifications are also important areas of exploration as the formation of amyloid strains has been shown to depend on variability of chaperone interaction (Sporn and Hines 2015) and tau is known to interact with a number of different chaperones that affect its aggregation (Petrucelli, Dickson et al. 2004, Dickey, Yue et al. 2006). Additionally, some strains may correlate with different tauopathies, which could yield critical information behind pathological differences in tau diseases.

Tau strains outside the brain

A simple, non-invasive, and inexpensive test for the early detection of neurodegenerative tauopathies is urgently needed. It is well-established that tau levels are increased in the cerebrospinal fluid (CSF) of patients with AD and recent studies have shown tau changes in AD can be distinguished from other tauopathies as well (Magdalinou, Paterson et al. 2015, Wagshal, Sankaranarayanan et al. 2015). As the central nervous system (CNS) is in direct contact with the CSF, an increase in tau oligomers in the CNS would theoretically be evident in the CSF, which can be obtained from living patients. Evidence that amyloidogenic proteins may exhibit different properties in the CSF when compared to the brain was recently provided by a study that investigated seeding potential of Aβ oligomers from brain tissue and CSF. Despite a greater than 10-fold increase in levels of Aβ oligomers in the CSF compared to the brain, when injected in the brains of transgenic mice, these CSF Aβ oligomers were not capable of seeding, while those collected from the brain did seed amyloid aggregation *in vivo* (Fritschi, Langer et al. 2014). Therefore, differences in the conformation and seeding potential of oligomers in the brain versus the CSF may be of great importance for the development of biomarkers and diagnostic tools, though a simple blood test might be preferable. Studies show that Aβ can be detected in the platelets, however high molecular weight tau that may be comprised of oligomers and other aggregates correlates better with disease symptoms (Neumann 2011, Farías 2012).

It is well-known that in addition to the accumulation of toxic amyloidogenic proteins in the parenchyma of the AD brain, there is also a vascular component to the disease. High levels of comorbidity between AD and vascular conditions suggest a possible common mechanism underlying vascular dysfunction and amyloidogenic protein

dysregulation in AD (Yarchoan, Xie et al. 2012, Sudduth, Weekman et al. 2014). Notably, animal models of AD also manifest Aβ-dependent vascular pathology (Kimura, Whitcomb et al. 2014, Lasagna-Reeves, Sengupta et al. 2014, Park, Koizumi et al. 2014). Vascular changes can induce hypoxia, which has been shown to facilitate the formation and deposition of Aβ and tau aggregates (Villarreal, Barron et al. 2014). The strongest correlation between vascular pathology and neurodegenerative disease is for AD, but dementia in PD and LBD has also been associated with vascular alterations (Toledo, Arnold et al. 2013). Deformation of capillaries in the hippocampus and cortex is a common change in AD (Baloyannis 2012), which could result from the over-secretion of vascular endothelial growth factor stimulated by Aβ (Dal Prà, Armato et al. 2014, Kimura, Whitcomb et al. 2014). Importantly, alterations in blood vessel walls may include perforations that could impair blood brain barrier (BBB) function (Scheibel 1989). Notably, BBB impairments have been described in both neurodegenerative disease patients and mouse models (Tanifum, Starosolski et al. 2014).

Additionally, oligomers from amyloidogenic proteins, including Aβ, α-synuclein, and TDP43, are found in the cerebrovasculature of AD patients (Guerrero-Muñoz, Castillo-Carranza et al. 2014). The relationship between tau and the cerebrovasculature in tauopathies has not been thoroughly investigated, although tau has been shown to accumulate in perivascular areas affected by Aβ deposits in the AD brain (Williams, Chalmers et al. 2005). Additionally, the tuft-shaped astrocytes commonly found in PSP brain frequently have vascular contact, and tau deposits are located in close proximity to blood vessels (Shibuya, Yagishita et al. 2011). Decreases in blood pressure in elderly patients with hypertension, which are associated with negative effects on the vasculature,

and chronic cerebral hypoperfusion, which is the leading cause of vascular dementia, lead to increases in phosphorylated tau and memory deficits (Glodzik, Rusinek et al. 2014, Zhao, Gu et al. 2014). Moreover, tau pathology in a mouse model of tauopathy (P301L mice) is associated with brain capillary constriction prior to cell death (Jaworski, Lechat et al. 2011). Collectively, the evidence suggests that vascular dysfunction is a critical component in neurodegenerative disease and that tau aggregation may play a role in this process. However, the extent to which vascular pathology varies among diseases is unknown. More research will be needed to determine whether tau aggregates may present in the cerebrovasculature and if they may differ between disorders or individuals based on conformation.

Further multi-disciplinary study and structural characterization is needed to fully understand the most toxic tau species in disease and the impact of aggregation state and conformation on disease phenotype. The evaluation and unique detection of these structures could lead to the determination of dominant toxic strains as well as potentially neuroprotective conformers. Improved detection of tau oligomeric strains in living patients through imaging technologies such as positron emission tomography (PET) could open doors to new and more accurate diagnostic tools for neurodegenerative disease (Okamura, Harada et al.). These results could have massive impact on diagnostics as well as therapeutic approaches.

We have previously generated a tau oligomer-specific monoclonal antibody (TOMA) clone, H12 that recognizes tau oligomers specifically and does not recognize monomeric functional tau or mature meta-stable NFTs for use in studying tau oligomeric strains. Moreover, we have established the efficacy of H12 TOMA at protecting against

tau oligomer toxicity in four different animal models (Castillo-Carranza, Gerson et al. 2014, Castillo-Carranza, Sengupta et al. 2014, Castillo-Carranza, Guerrero-Muñoz et al. 2015, Gerson J 2015). We have evaluated H12 and three additional TOMA clones for ability to identify unique tau oligomeric strains in human disease tissue. For the first time, we have isolated and characterized tau oligomeric strains from human disease tissue and evaluated their effects *in vivo*.

Methods

Preparation of Recombinant Tau Oligomers

Oligomers of microtubule binding repeat domain fragments of 4R human tau (K18) expressing P301S and S320F mutations (Martin Margittai lab) were used to seed the aggregation of both 3R and 4R recombinant tau monomer. Seven microliters of mutant K18 oligomers (0.3 mg/mL) were added to 3R and 4R tau stock (0.3 mg/ml) and mixed by pipetting for 1 min. Samples were then incubated at room temperature for 1 hr on an orbital shaker. Unseeded tau oligomers were also prepared from 4R tau stock using two different buffers, HFIP and PBS. For HFIP, 0.5 mg of tau was dissolved in 200 µl of hexafluoroisopropanol and incubated for 10–20 min at room temperature. The resulting solution was diluted in 700 µl H2O in a siliconized eppendorf tube with holes placed on top of the cap to allow the evaporation of HFIP. The samples were stirred at 500 r.p.m. using a Teflon-coated micro stir bar for 36 h in a fume hood at room temperature. For PBS, 0.5 mg of tau was dissolved in 700 µl of 0.02 % sodium azide in 1X PBS. The resulting solution was stirred at 500 r.p.m. using a Teflon-coated micro stir bar for 48 h at room temperature.

Brain Tissue Preparation

Frozen brain tissue from the frontal cortex, hippocampus and pons was attained from subjects with AD, DLB, PSP, Pick's disease and age-matched control subjects from the Institute for Brain Aging and Dementia (University of California at Irvine, Irvine, California, USA), the Oregon Brain Bank (Oregon Health and Science University, Portland, Oregon, USA) and the Brain Resource Center at Johns Hopkins (Baltimore, Maryland, USA). Brain tissue was collected with patient consent and protocols were approved by the Institutional Review Board of University of California at Irvine, Oregon Health and Science University and Johns Hopkins. All samples were examined by neuropathologists for diagnosis. Brains were homogenized as described in Chapter 2. FTLD and Alzheimer's disease tissue from additional brain regions including the parietal lobe and temporal lobe were also obtained for sections.

Preparation of Brain-derived Tau Oligomers

In order to collect biologically relevant tau oligomeric strains, tau oligomers were immunoprecipitated from cortex, hippocampus, and pons of AD, DLB, PSP, and Pick's disease brains as described in Chapter 2.

Generation of Tau Oligomer-Specific Monoclonal Antibody (TOMA) Clones

The production of three new TOMA clones was completed as described in Chapter 4 for the production of the H12 TOMA clone (Castillo-Carranza, Gerson et al. 2014, Castillo-Carranza, Sengupta et al. 2014).

Proteinase K (PK) Digestion

In order to evaluate stability of tau oligomeric strains, Proteinase K (PK) enzyme was used to treat tau oligomer samples at a ratio of 1-2.5 ug/mL of enzyme in water. Tris-HCl and NaCl were added to each sample and Proteinase K enzyme mixture so that the final concentrations of Tris-HCl and NaCl were 100 mM and 5 mM respectively. Samples were treated with PK for 1 hour at 37°C. The reaction of the enzyme was stopped by adding 4X SDS-PAGE loading buffer (Npage LDS sample buffer, Invitrogen). Samples were then incubated at 95°C for 5 minutes. The total reaction volume for each sample was immediately used for analysis by Western blot.

Western Blot

Western blot with T22 (1:200), Tau-5 (1:1000) or β-actin (1:3000) was completed as described in Chapter 2.

ELISA

ELISA with TOMA clones (H5, H12, E7 and D9) was completed as described in Chapter 4.

Immunohistochemistry

Immunofluorescence was completed as described in Chapter 3. Prior to permeabilization, sections evaluated for Proteinase K (PK) sensitivity were treated with 10 μg/ml PK for 10 min. All human sections were immersed in 70% ethanol for 5 min, followed by 5 minute incubation with autofluorescence eliminator reagent (Millipore) and 3 washes in 70% ethanol to block lipofuscin fluorescence in aging brain tissue prior to blocking. Mouse tissue sections labeled with antibodies raised in mouse went through a second blocking step with unconjugated Fab anti-mouse IgG (40μg/ml; Jackson Immunoresearch) for 1 hr

to block endogenous IgG immediately following blocking with serum. Sections were labeled with TOMA clones (H5, H12, E7 and D9), GFAP (1:300), T22 (1:250) or incubated for 20 min with Neurotrace 640/660 (1:100) and washed overnight. Those sections unlabeled by Neurotrace were incubated with DAPI (Invitrogen) to label nuclei for 5 min at room temperature. The sections were examined using a Nikon A1R MP laser scanning microscope. An n of 4 was examined for each disease state and control brains and 4 images were randomly taken from each sample. The corrected total cell fluorescence was quantified using Image-J as described in Chapter 2.

In Vitro Seeding

Both 3R and 4R tau monomer were prepared by dissolving lyophilized pellets of recombinant 3R and 4R tau at 0.3 mg/mL in phosphate-buffered saline (PBS) [15,31]. Tau monomer was seeded with tau oligomers prepared from tau microtubule binding repeat domain (K18) fragments expressing mutations: S320F and P301S. Seeds were added at a 1:100 (w/w) dilution to 2.5 μM solutions of monomeric 3R and 4R tau in PBS (in duplicates) with gentle agitation at room temperature as described in Chapter 2.

Animals

Six-month-old homozygous Htau mice (The Jackson laboratory stock #005491)(Andorfer, Kress et al. 2003) were housed at UTMB as described in Chapter 3.

In Vivo Experimental Design

Experimental design for mouse experiments is outlined in Figure 6.4. Briefly, brain-derived tau oligomeric strains from AD, DLB and PSP were injected intravitreally in 6-month-old Htau mice and after two weeks behavioral assessment was completed. Tissue

was collected from each mouse for analysis and tau oligomers were immunoprecipitated from injected mouse tissue. Second generation injections were conducted in another set of 6-month-old Htau mice with the immunoprecipitated tau oligomers from AD and PSP-injected mice. Three weeks after injection, brain tissue was collected for analysis.

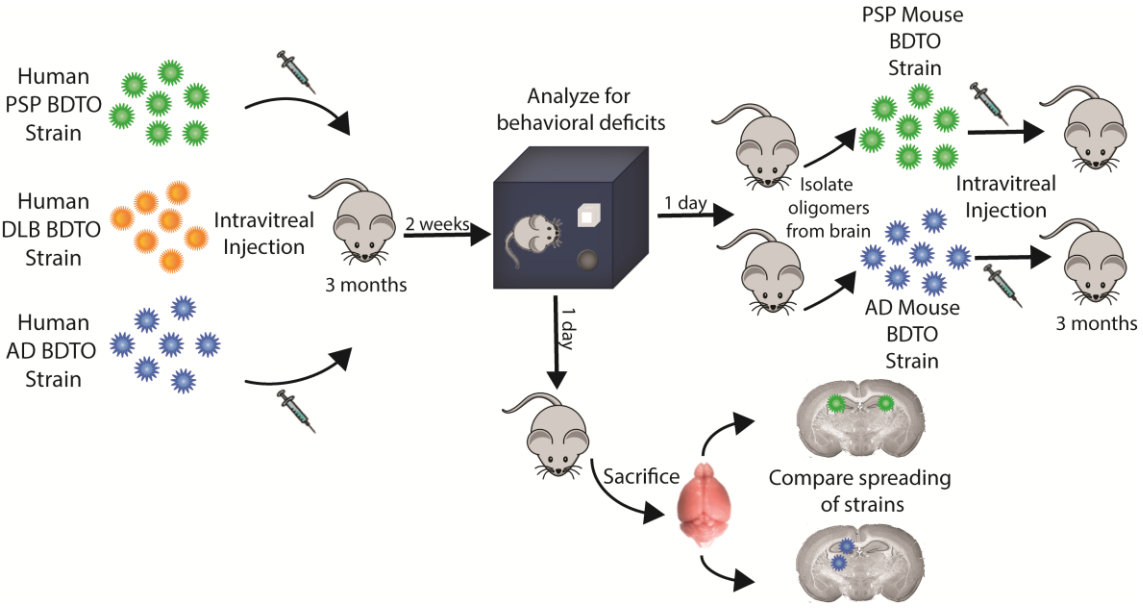

Figure 6.4 Experimental design for *in vivo* experiments. 3-month-old Htau mice were injected intravitreally with PSP, DLB or AD BDTO strains with PBS as a control. Two weeks post-injection mice were evaluated on a battery of behavioral tasks. The following day, tissue was collected from mice for analysis of tau oligomer levels in different brain regions. Tau oligomers were immunoprecipitated from PSP and AD BDTO-injected mouse brains and injected in a new set of 3-month-old Htau mice intravitreally for future analysis of cross-generational stability of strains.

Intravitreal Injection

Mice were anesthetized with a ketamine (80-150 mg/kg)/xylazine (8-16 mg/kg) cocktail. Proparacaine hydrochloride ophthalmic solution (0.5%) was applied as a local anesthetic. Pupils were dilated with tropicamide ophthalmic solution (1%) to facilitate surgery. Intravitreal injections of 2 µL of tau oligomeric strains (0.3 mg/ml) from AD, DLB and PSP were then completed with 33 gauge needles mounted to 10-µl Hamilton syringes. The needle was inserted through the dorsal limbus of the eye using a surgical microscope. Injections were performed slowly throughout a period of 2 minutes. Antibiotic ointment was then applied to the eyes to prevent infection and mice were monitored after surgery until recovery and once a day afterwards for signs of infection.

Behavioral Analysis

Open Field

Two weeks after intravitreal injection of tau oligomeric strains, locomotor activity of mice was evaluated using the open field task. Each mouse was placed in the center of a white open-field arena (55 cm in diameter; 60 cm in height) and allowed to freely explore for 2 minutes prior to testing. Afterwards, exploratory behavior of each mouse was recorded for 15 minutes and measured using ANY-maze software (Stoelting).

Novel Object Recognition

On the next day immediately following the open field task, mice were placed in the same open-field arena for the training phase of novel object recognition, completed as described in Chapter 3.

Novel Arm Y-MazeTask

The animals were placed in a symmetrical Y-shaped maze. Arms were 40 cm long, 8 cm wide, and 12 cm high (San Diego Instruments), beige in color, nonreflective, and randomly designated A, B, or C. Each arm was identical and provided no intramaze cues. Extra-maze cues were placed in the room for mice to navigate. On the first day of testing, one of the arms was closed off for the training trial. Each mouse was placed into one of the arms facing the center (starting arm) and allowed to explore the maze for 12 min. Total arm entries were recorded. On the second day of testing, all three arms were open and each mouse was placed back into the starting arm and allowed to explore the maze for 5 min. The number of entries into each arm was recorded by a blinded researcher. The total arm entries on the training trial as well as number of novel arm entries divided by total entries on the testing trial was calculated for each mouse.

Rotarod

The rotarod task was completed as described in Chapter 5.

Tissue Collection and Analysis

Immediately following behavioral analysis in first generation mice and three weeks after injection in second generation mice, mice were euthanized with CO_2 and brains were removed and divided by hemisphere. The right hemisphere was embedded in OCT for tissue sectioning and the left hemisphere was dissected into olfactory bulb, forebrain and hindbrain. Brain homogenate and sections were prepared as previously described for analysis by Western blot and immunohistochemistry.

Data Analysis

Data were analyzed by One-way Analysis of Variance (ANOVA) and Tukey's post-hoc test using Statview software.

Results

Tau Oligomeric Strains Display Unique PK Digestion Patterns

Recombinant tau oligomers prepared using different buffers show different patterns of PK fragmentation by Western blot with Tau-5 (Figure 6.5A). Seeding of recombinant 3R and 4R tau with P301S and S320F K18 tau mutants revealed both isoform and mutation-specific PK digestion patterns by Western blot with Tau-18 (Figure 6.5B). Evaluation of P301S and S320F seeded samples by AFM also revealed different aggregate composition, with increased fibrillization in P301S-seeded tau samples compared to S320F (Figure 6.5C).

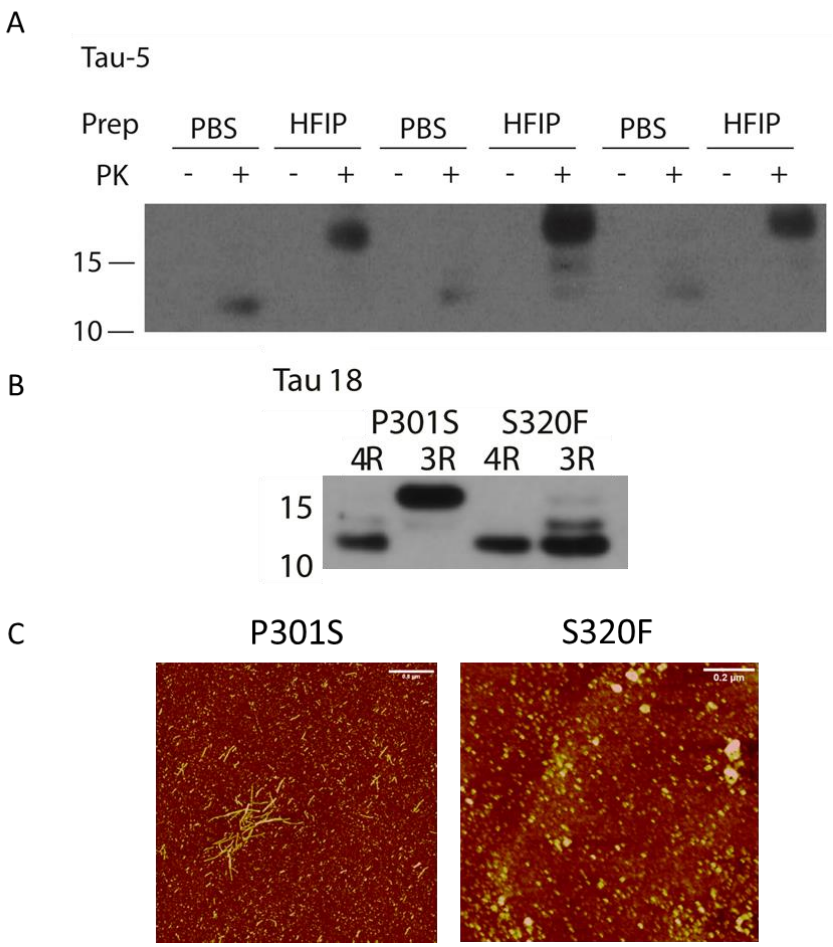

Figure 6.5 Proteinase K digestion detects recombinant tau oligomeric strain stability differences. Preparation of recombinant tau oligomers using PBS and HFIP techniques was repeated in triplicate. Following treatment with PK, samples show consistent fragmentation patterns on a Western blot with Tau-5 that differ between the two preparations (A). 3R and 4R tau oligomers seeded by K18 P301S and S320F mutants can be differentiated by both isoform and mutation in PK stability following analysis by Western blot with Tau 18 (B). Additionally, P301S seeded tau shows increased speed to formation of fibrillar structures by AFM when compared to seeding with S320F (C).

In order to determine whether biologically relevant samples could be differentiated by PK digestion, tau oligomers immunoprecipitated from AD, PD, LBD, PSP and Pick's disease, purified and confirmed to be in oligomeric conformation by AFM (Figure 6.6A) were treated with PK and evaluated by Western blot with Tau-13. Truncation pattern differences were found between diseases (Figure 6.6B).

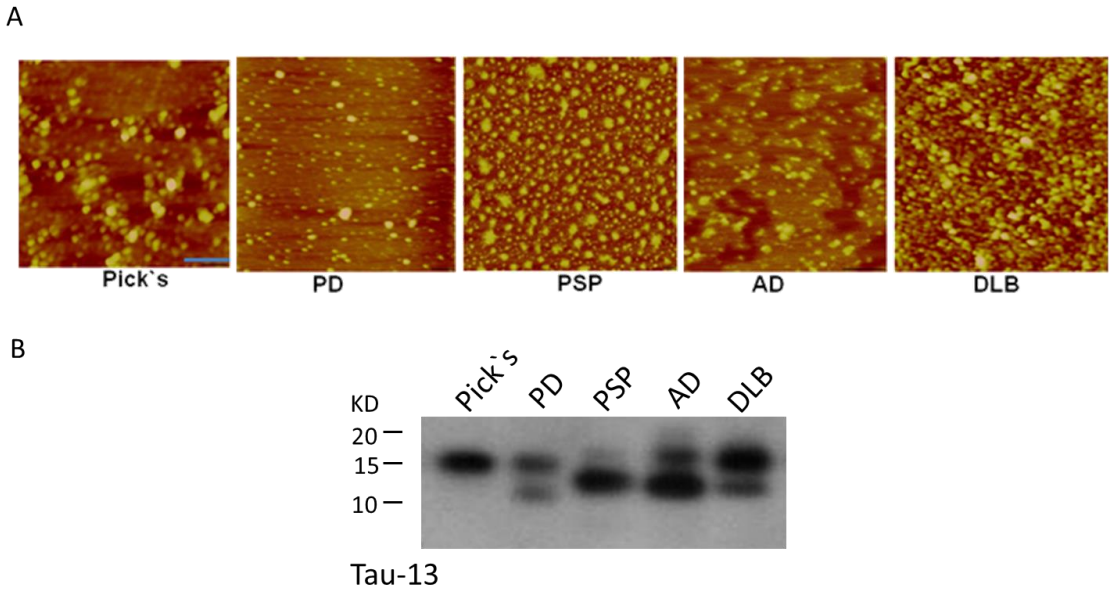

Figure 6.6 Brain-derived tau oligomeric strains treated with PK exhibit different truncation patterns. Tau oligomers immunoprecipitated from Pick's disease, PD, PSP, AD and DLB were characterized by AFM to confirm the presence of spherical, oligomeric structures (A). After treatment with PK, brain-derived tau oligomers were evaluated by Western blot with Tau-13, showing fragmentation differences between each disease (B).

TOMA Clones Differentially Detect Tau Oligomeric Strains

TOMA clones, H12, H5, E7 and D9, were sequenced and specificity for tau oligomers was confirmed by ELISA with recombinant tau monomer and oligomers, as well as Aβ and α-synuclein oligomers (Figure 6.7).

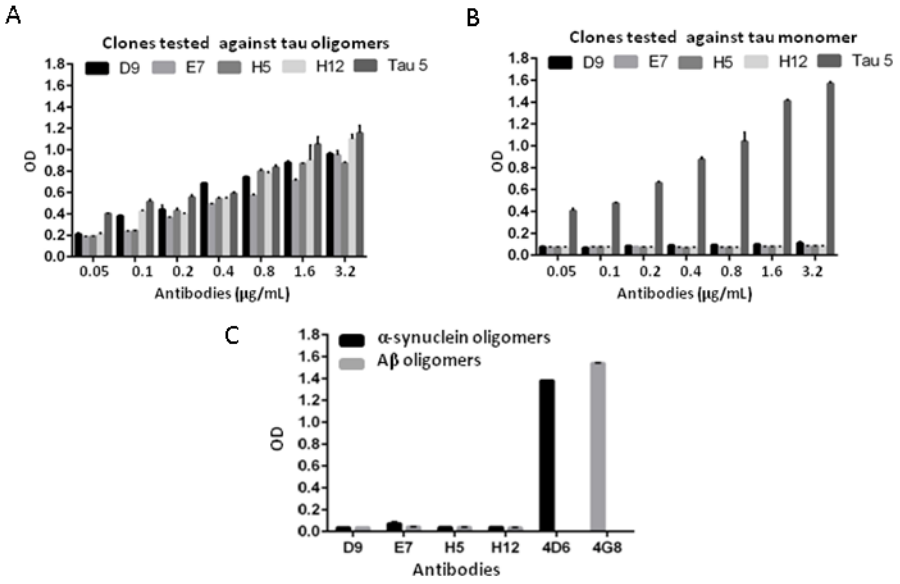

Figure 6.7 TOMA Clone specificity was confirmed by ELISA. All four TOMA clones recognized recombinant tau oligomers with similar efficacy (A). When compared to total tau antibody, Tau-5, none of the four TOMA clones were able to detect recombinant tau monomer (B). TOMA clones do not show cross-reactivity with Aβ or α-synuclein oligomers, compared with Aβ-specific antibody, 4G8 and α-synuclein-specific antibody, 4D6 (C).

TOMA clone recognition of tau oligomeric strains was evaluated first by characterization of brain-derived tau oligomers. ELISA and dot blot with oligomers derived from AD, PD and PSP with all four TOMA clones showed that specificity differed between disease. While all clones were unable to detect either Aβ or α-synuclein oligomers, all were able to detect recombinant tau oligomers. However, in tau oligomers immunoprecipitated from Alzheimer's disease, H5 was the most effective TOMA clone while PD-derived oligomers were recognized at highest levels by H12 and D9. E7 was most effective clone for detection in PSP samples (Figure 6.8A-B). Affinity of TOMA clones for different disease-associated tau oligomeric strains in brain tissue was also evaluated by immunofluorescence in AD hippocampus, frontal cortex, temporal lobe and parietal lobe sections and in frontal cortex from PD and FTLD (Figure 6.8C-E). Similar results were seen with H5 detecting the highest levels of tau oligomers in AD, significantly increased over D9 ($p=0.02$) and D9 recognizing high levels of oligomers in PD sections, significantly higher than E7 ($p=0.04$) and H12 ($p=0.02$). FTLD sections were primarily detected with H5 antibody, levels of which were significantly higher than H12 ($p=0.0003$), E7 ($p<0.0001$) and D9 ($p<0.0001$).

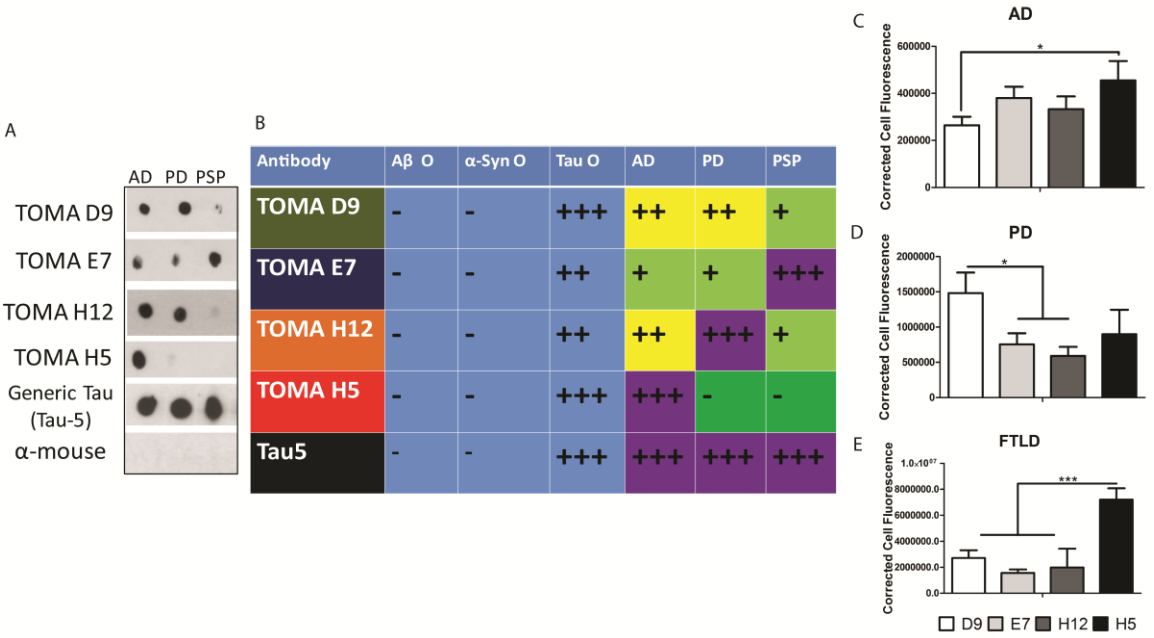

Figure 6.8 Brain-derived tau oligomeric strains are differentially recognized by TOMA clones. Dot blot of AD, PD and PSP-derived tau oligomers show that each TOMA clone detects different levels of oligomeric strains based on disease (A). ELISA with each TOMA clone similarly shows variable levels of recognition of each clone associated with different diseases, with AD being the most highly detected by H5, PD with H12 and D9 and PSP with E7 (B). Immunofluorescence with each TOMA clone in AD hippocampus, frontal cortex, temporal lobe and parietal lobe sections and in frontal cortex from PD and FTLD also showed differing recognition by disease. H5 detected the highest levels of tau oligomers in AD, significantly increased over D9 ($p=0.02$; C) and D9 recognized high levels of oligomers in PD sections, significantly higher than E7 ($p=0.04$) and H12 ($p=0.02$; D). FTLD sections were primarily detected with H5 antibody, levels of which were significantly higher than H12 ($p=0.0003$), E7 ($p<0.0001$) and D9 ($p<0.0001$; E).

TOMA Detection of Tau Oligomeric Strains is Dependent on Brain Region

In order to determine what effect brain region has on tau oligomer conformation and detection with TOMA clones, brain sections from Alzheimer's disease frontal, temporal and parietal cortex, as well as hippocampus were labeled with TOMA clones. To confirm that TOMA clones were detecting biologically important tau oligomeric strains, levels of corrected total cell fluorescence for all TOMA clones was pooled and analyzed by brain region (Figure 6.9A). As expected, levels of tau oligomers were highest in the hippocampus, significantly elevated when compared to the parietal and frontal lobes ($p=0.03$). However, broken down by individual TOMA clones, the patterns of tau oligomer detection in different brain regions varied (Figure 6.9B). D9 detected significantly higher levels of tau oligomers in the hippocampus than in the frontal cortex ($p=0.03$) while E7 detected high levels of tau oligomers in both the hippocampus and the frontal lobe at significantly higher levels than the parietal lobe ($p=0.02$ and $p=0.0495$ respectively). On the other hand, while H5 detected higher levels of tau oligomers than H12, both had similar levels of recognition across all four brain regions.

Tau Oligomeric Strain Detection by TOMA Clones Varies By Cell Type

In order to determine whether tau oligomeric strains recognized by TOMA clones varied by cell type, sections were labeled with TOMA, GFAP and NeuroTrace. Low levels of tau oligomers in a cell type were defined as 1-3 cells positive for tau oligomers, medium levels were determined when 3-5 tau oligomer-positive cells were present and high recognition was defined when levels exceeded 5 TOMA-positive cells. Both E7 and H12 exhibited high levels of neuronal tau oligomeric strains, while H5 had high levels of

astrocytic tau oligomers. D9 recognized medium levels of astrocytic tau oligomers and low levels of neuronal tau strains (Figure 6.9C).

PK Resistance Differs by Tau Oligomeric Strain

To evaluate the effect of PK treatment on tau oligomeric strains in tissue, sections were incubated with PK prior to immunohistochemistry and evaluated for TOMA clone recognition. While all TOMA clones detected PK-resistant tau oligomers, D9 only detected low levels and both H12 and H5 had medium levels of recognition of tau oligomers. On the other hand, E7 detected high levels of PK-resistant tau oligomers (Figure 6.9C).

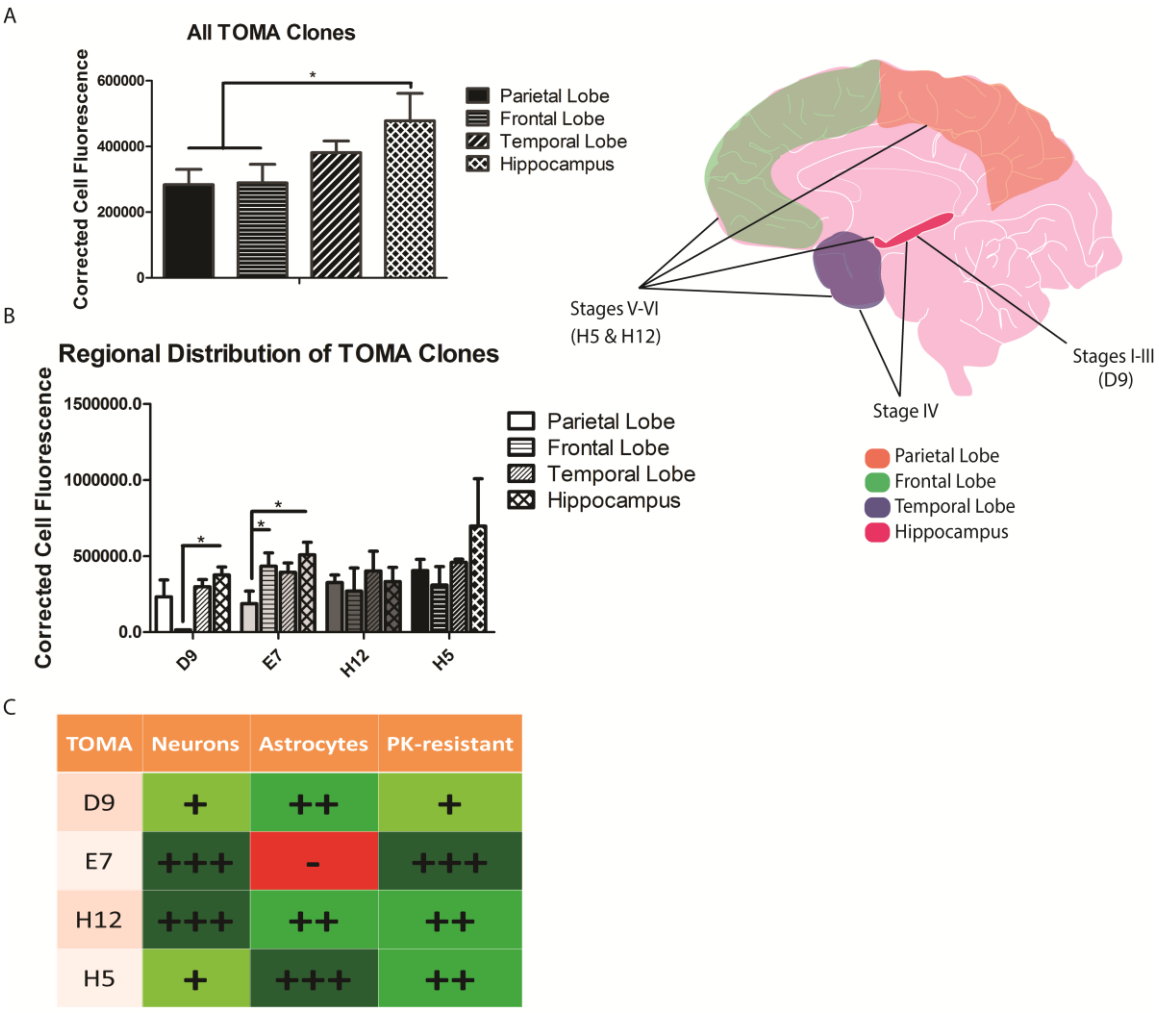

Figure 6.9 Tau oligomers detected by TOMA clones vary by brain region in AD, and cell type and PK resistance in AD and other tauopathies. Corrected total cell fluorescence pooled for all TOMA clones showed that levels of tau oligomers were significantly higher in the hippocampus than the parietal and frontal lobes (p=0.03; A). Patterns of tau oligomer detection in different brain regions varied when broken down for each individual TOMA clone (B). D9 fluorescence was significantly higher in the hippocampus than in the frontal cortex (p=0.03), corresponding to Braak stages I-III. E7 levels in the hippocampus and the frontal lobe were significantly higher than the parietal lobe (p=0.02 and p=0.0495 respectively). However, neither H5 nor H12 showed

differences between brain regions, corresponding to patterns of tau pathology in Braak stages V-VI. High levels of neuronal tau oligomeric strains were detected by H12 and E7. H5 showed high levels tau oligomers associated with astrocytes. D9 recognized medium levels of astrocytic tau oligomers and low levels of neuronal tau strains. After PK treatment, low levels of tau oligomers could be detected by D9. H12 and H5 had medium levels of recognition of PK-resistant tau oligomers and E7 detected high levels of PK-resistant tau. *$p<0.05$; + 1-3 TOMA-positive cells, ++ 3-5 TOMA-positive cells, +++ >5 TOMA-positive cells.

Brain-derived Tau Oligomeric Strains Induce Different Behavioral Deficits

In the open field task, time spent immobile and mobile were measured as determinants of motor activity. PSP tau oligomer-injected Htau mice spent significantly more time immobile than mice injected with AD-derived tau oligomers ($p=0.03$; Figure 6.10A). Likewise, AD mice spent significantly more time mobile than PSP mice ($p=0.03$; Figure 6.10B). Analysis of time spent exploring the edges of the enclosure when compared to the center revealed no differences between treatment group (data not shown). However, analysis of memory deficits with the novel object recognition task showed that AD oligomer-treated mice had significantly lower discrimination index than PSP oligomer-injected mice ($p=0.01$; Figure 6.10C). On the other hand, the spatial memory task, the Y maze, did not reveal any differences between groups in time spent exploring the novel arm (data not shown). However, total arm entries during training were significantly elevated in DLB oligomer-injected mice when compared to PSP ($p=0.04$; Figure 6.10D). In order to specifically evaluate motor coordination, mice were then tested on the rotarod

task and PSP tau oligomer-injected mice remained on the rotating rod for significantly less time than AD oligomer mice (p=0.01; Figure 6.10E).

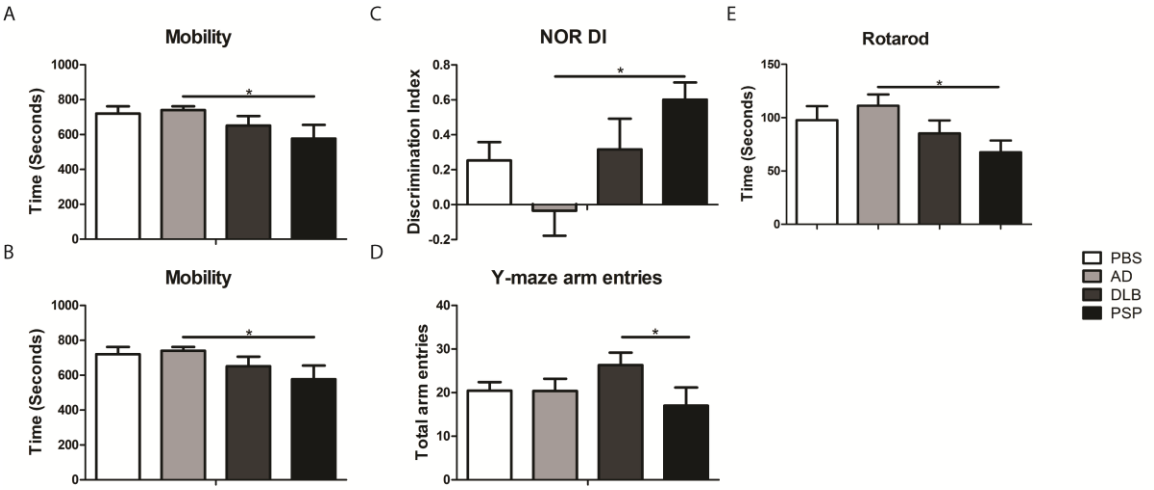

Figure 6.10 Brain-derived tau oligomeric strains injected intravitreally induce diverse behavioral phenotypes in Htau mice. PSP tau oligomer-injected mice spend significantly more time immobile (A) and significantly less time mobile (B) when compared to AD oligomer-injected mice in the Open Field task (p=0.03). On the novel object recognition task, AD oligomer-treated mice had significantly lower discrimination index scores than PSP oligomer-injected mice (p=0.01; C). Total exploration measured by arm entries during training in the Y maze task was significantly elevated in DLB oligomer-injected mice when compared to PSP mice (p=0.04; D). PSP tau oligomer-injected mice remained on a rotating rod for significantly less time than AD oligomer mice in the Rotarod task (p=0.01; E).

In Vivo Seeding of Tau Oligomers Varies by Disease

Brains from mice injected with brain-derived tau oligomeric strains were divided into olfactory bulb, forebrain and hindbrain regions for analysis of tau oligomer levels (Figure 6.11A). Levels of tau oligomers in total brain (pooled from the three regions) were significantly increased in AD and DLB brains when compared to PBS and PSP oligomer-injected mice (Figure 6.11B). Ratios of each brain region to the total brain levels of tau oligomers were then evaluated. DLB tau oligomer-injected mice had significantly higher levels of tau oligomers in the olfactory bulb than PBS and PSP-injected mice and AD oligomer-injected mice had significantly higher levels when compared to PSP oligomer mice (Figure 6.11C). In the forebrain, AD oligomer mice had significantly higher tau oligomer levels than both PBS and PSP-injected mice (Figure 6.11D). However, in the hindbrain, PSP oligomer-injected mice had significantly higher tau oligomer levels than all other groups (Figure 6.11D).

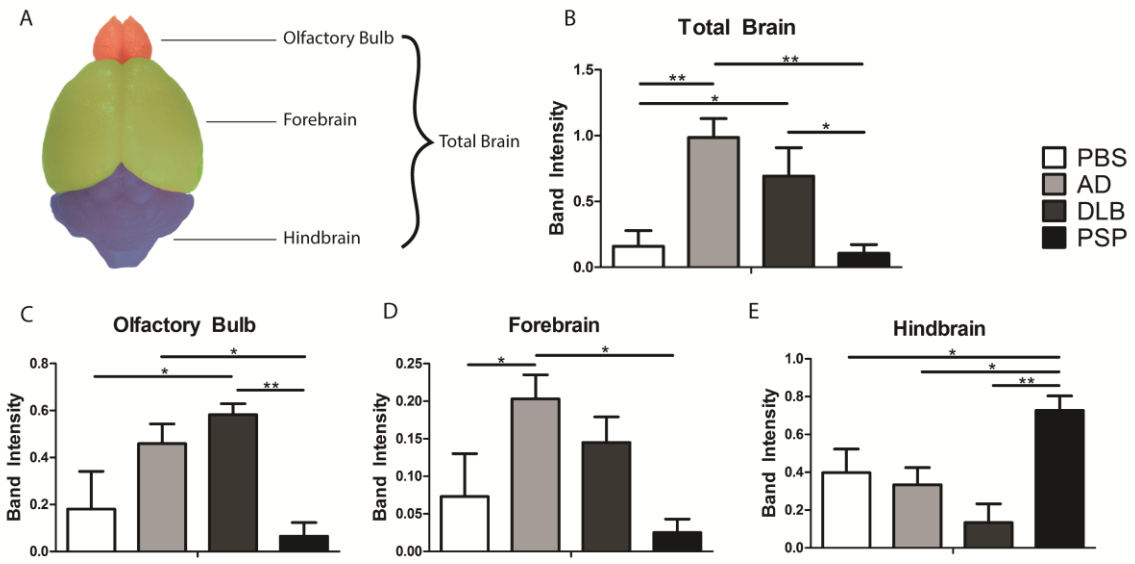

Figure 6.11 Tau oligomer levels in the brain increase following intravitreal injection of brain-derived tau oligomeric strains and ratios by brain region differ by disease. Total brain homogenate as well as olfactory bulb, forebrain and hindbrain regions were evaluated by Western blot with T22 for each group (A). Total brain tau oligomer levels were significantly increased in AD and DLB brains when compared to PBS (p=0.004 and p=0.03 respectively) and PSP (p=0.003 and p=0.02 respectively) oligomer-injected mice (B). DLB tau oligomer-injected mice had a significantly higher ratio of tau oligomers in the olfactory bulb to total brain than PBS (p=0.02) and PSP-injected mice (p=0.006) and AD oligomer-injected mice had significantly higher levels when compared to PSP oligomer mice (p=0.02; C). AD oligomer mice had significantly higher levels of tau oligomers in the forebrain: total brain than both PBS (p=0.04) and PSP-injected mice (p=0.01; D). PSP oligomer-injected mice had significantly higher tau oligomer levels than all other groups in the hindbrain fraction: total brain (PBS: p=0.048, AD: p=0.02, DLB: p=0.003; D).

Discussion

We have reported for the first time the existence of biologically-relevant tau oligomeric strains that can be differentiated by stability, seeding and recognition with tau oligomer-specific antibodies (TOMA clones). The characterization of diverse tau oligomeric strains will be critical for the accurate study of tau pathology in disease.

In order to evaluate tau oligomer conformational diversity, we first evaluated tau oligomeric strains prepared *in vitro*. We found that oligomers from recombinant tau aggregated under different environmental conditions led to differences in stability detected by proteinase K (PK) digestion. Moreover, recombinant tau oligomers seeded by tau fragments expressing biologically relevant tau mutations associated with FTDP-17 exhibited differential PK truncation as well. These results suggested that tau oligomers *in vivo* could also potentially form diverse conformers based on environmental differences between brain regions, organelles or under stress. In order to evaluate differences in tau oligomeric strains that may derive from different tauopathies, we isolated, purified and characterized tau oligomers from mixed pathology tauopathies, AD, PD and DLB as well as pure tauopathy, PSP. Digestion with PK revealed differences in stability between diseases, as well as individual differences. We then determined whether tau oligomer-specific antibody (TOMA) clones—found to be capable of recognizing only oligomers from tau, but not monomer or fibrils, nor oligomers of other proteins—could be used to differentiate between tau oligomers isolated from different disorders. We found that detection with biochemical methods did reveal differences between the TOMA clones, suggesting that they could be used to more effectively characterize toxic tau pathology in different diseases. Thus we collected brains, again from both mixed tauopathies—AD

and PD—and pure tauopathy, FTLD. We found that immunofluorescence detection of tau oligomers in the brains of tauopathy patients also differed by TOMA clone. The diversity of oligomeric strains in mixed pathology versus pure tauopathy disorders may depend on the interaction between tau and other amyloid proteins. However, the question remained of whether tau oligomeric strains may differ based on environmental conditions or disease staging as well.

Therefore, we investigated the specificity of each TOMA clone for detecting tau oligomers in different brain regions in AD. Differences may reflect conformational specificity that relates to the Braak stage of disease, whereby in Stages I-III tau pathology in primarily found surrounding the hippocampus, but by Stage IV tau aggregates have extended to the temporal lobe and late in disease, Stages V-VI, show tau pathology throughout the cortex, in the parietal and frontal lobes (Braak H 1991). Secondly, TOMA clones detected different levels of tau oligomers based on the cell type associated, with some clones recognizing higher levels of glial oligomers and others recognizing neuronal tau oligomers. Thus, TOMA clones may be able to distinguish between strains based on both disease staging and environmental changes. Lastly, we evaluated differences in TOMA clone labeling based on PK resistance, as studies of other amyloid proteins show that conformations that are partially PK resistant may be the most toxic species in disease (Roberts, Wade-Martins et al. 2015). These results collectively illustrate the potential for the use of tau oligomer-specific monoclonal antibodies in the differentiation of different tau disorders.

The association of different tau oligomeric strains with different disorders suggests that they may be partly responsible for the diverse outcomes of tauopathies,

however this hypothesis had never been directly tested. Therefore, we injected oligomers isolated from AD, DLB and PSP and tested their effects *in vivo* in the Htau mouse model that overexpresses non-mutated human tau. Studies investigating the spread of tau in the brain have all previously targeted tau oligomers to one specific brain region, eliminating the ability to study potential differences in tau oligomeric strain seeding when the proteins originate from different areas. Therefore, we used intravitreal injections to evaluate whether tau oligomers can spread from the eye to the brain and to more effectively evaluate the effects of tau oligomeric strains without targeting specific brain regions. Following injection, mice were evaluated for both cognitive and motor dysfunction. Surprisingly, we found that tau oligomeric strains were associated with different behavioral outcomes. PSP-injected mice exhibited an impaired motor phenotype, but no cognitive impairment, while AD-injected mice exhibited problems on a memory task, but no motoric changes. DLB-injected mice on the other hand exhibited hyperactive and anxiolytic behavior that is similar to what is seen in early synucleinopathy models, prior to any gross motor impairment (Graham and Sidhu 2010, Paumier, Sukoff Rizzo et al. 2013). Consistent with the results of the memory test, only around 30% of PSP patients develop any memory dysfunction, while AD is associated with cognitive impairment early, dependent on the origin of pathology in the hippocampus (Braak H 1991). Synucleinopathies including PD and DLB are associated with both motor and cognitive changes, however both occur later in disease, with olfactory dysfunction being an early sign of a disorder, while changes to gait are seen very early in PSP (Rodriguez-Oroz, Jahanshahi et al. 2009, Burciu RG 2015). Therefore,

further study in the future will be needed to determine the impact of tau oligomeric strains on disease phenotype as a function of time following application of toxic tau.

In order to evaluate whether behavioral changes between different tau oligomeric strains were also accompanied by changes in the spread of the toxic species, brains were collected from injected mice and levels of tau oligomers were measured in the olfactory bulb, forebrain and hindbrain regions. Elevation of tau oligomers in the olfactory bulb was specific to AD and DLB oligomer-injected mice. Previous studies of tauopathy mouse models have found that tau pathology increases in the olfactory bulb, suggesting that olfactory dysfunction in neurodegeneration may depend upon tau toxicity (Hu, Ding et al. 2015). Moreover, both AD and PD patients and models exhibit olfactory dysfunction prior to more noticeable defects (Ubeda-Bañon, Saiz-Sanchez et al. 2010, Wesson, Levy et al. 2010, Marigliano 2014). In fact, olfactory tests may predict future conversion to AD in mild cognitively impaired subjects more effectively than hippocampal volume loss (Marigliano 2014). In the forebrain region, levels of tau oligomers were elevated in AD oligomer-injected mice, which is reflective of the impairment on a hippocampal memory task. However, in the hindbrain region, tau oligomer levels as a ratio to the levels in the whole brain were highest in PSP oligomer-injected mice, which is similar to tau pathology seen in the disease. While PD and DLB motor impairment depends primarily on degradation of the basal ganglia, PSP patients have distinguishable motor symptoms that depend on shrinkage of the cerebellum.

Collectively, these results suggest that conformational diversity of tau oligomeric strains may impact disease outcome and be a viable route for research into the design of

biomarkers for diagnostics and personalized therapeutics for different neurodegenerative tauopathies.

CONCLUSION

The misprocessing of tau is a major contributing factor to a number of neurodegenerative disorders. Original studies linked fibrillar aggregates of tau, known as neurofibrillary tangles, to Alzheimer's disease and other tauopathies. However, further research revealed that tau fibrils do not induce toxicity or correlate with symptoms of disease, while smaller, soluble tau aggregates known as oligomers are highly toxic. We then established that tau oligomers can be detected in the brains of Alzheimer's disease patients early in disease and when applied to wildtype mice can cause cognitive impairment and synaptic and mitochondrial dysfunction. Here I established that tau oligomers are an important component of disease not only in Alzheimer's, but also in pure tauopathy, progressive supranuclear palsy, traumatic brain injury and synucleinopathies. Targeting tau oligomers in models of tauopathy and synucleinopathy inhibits toxicity and protects against behavioral deficits.

However, while tau oligomers are present and damaging in all of these disorders, each has a diverse set of symptoms and pathological outcomes. Moreover, tau oligomers are not a homogenous species of proteins and tau can be misfolded and aggregated into innumerable configurations that may vary in stability, seeding efficiency and optimal environmental conditions. Parallels have been drawn between the characteristics of tau and other amyloid proteins with prions, in that they are capable of seeding the spread of pathology from one brain region to another. Likewise, prions are also a heterogeneous population of protein aggregates and the formation of diverse strains of prions leads to different phenotypes. Therefore, I hypothesized that tau oligomeric strains may induce unique outcomes that underlie some of the differences between tauopathies, as well as individual differences between patients with the same disorder. In the studies reported here, tau oligomeric strains were prepared *in vitro* as well as isolated from tauopathy

brain tissue. Strains were capable of seeding recombinant tau *in vitro*, but showed differential seeding and behavioral effects *in vivo* when injected into human tau mice. Tau oligomeric strains exhibited differences in stability and resistance to proteinase K, as well as regional and cell type differences in the brain. Importantly, tau oligomer-specific antibodies could be used to differentiate tau oligomeric strains found in different tauopathies.

These studies represent the first time that tau oligomeric strains have been identified and shown to induce different effects *in vivo*. These results highlight the importance of evaluating tau oligomers not as one homogenous species, but rather as a diverse set of toxic aggregates that may differentially induce disease. Currently, there are no diagnostics that effectively determine any neurodegenerative disease prior to the appearance of severe symptoms. Furthermore, there are no diagnostics that are capable of distinguishing between diseases. The identification of tau oligomeric strains and combinations of strains that are specific to diseases could change the trajectory of the tau field and allow clinicians to identify disease-specific biomarkers for the first time. These studies lay the groundwork for future studies that could make the identification of tau strains possible in patients. Moreover, these results could be used to make effective, personalized therapeutics for individuals with tauopathies and varying pathophysiology, as there are currently no effective treatments for any neurodegenerative tauopathy.

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Vita

Julia Gerson was born on March 20, 1990 in Cleveland, Ohio to James and Teresa Gerson.

Education

Arizona State University, Tempe, AZ
Barrett the Honors College
Double Major: Psychology and Biology—Animal Physiology and Behavior
Graduated Summa Cum Laude May 2012

University of Texas Medical Branch (UTMB), Galveston, TX
Presidential Scholar
PhD Program: Neuroscience
Principal Investigator: Rakez Kayed

Teaching/Mentoring Responsibilities

Teaching responsibilities at Arizona State University:
Supplemental Instruction Leader: Introductory Chemistry Courses 2010-2012

Teaching responsibilities at UTMB:
Teaching:
Facilitator: Biochemistry Course 2013
Laboratory Instructor: Neuroscience and Human Behavior (NHB) 2015
Co-director and Lecturer: Introduction to the Study of Biological Systems 2015

Mentees/Trainees:
Elizabeth Jaworski, Biochemistry Graduate Program, BBSC Rotation, 2013
Kelsey English, School of Medicine, Summer Research Program (MSSRP), 2014
Awarded MSSRP Best Neuroscience Poster Award, Sealy Center Forum on Aging Best
Neuroscience Poster Award, and National Student Research Forum Best Oral Presentation in Neuroscience
Jennifer Deger, Summer Undergraduate Research Program, 2014
Awarded Best Overall Poster UTMB SURP Symposium and Best Presentation UT Austin Fall Undergraduate Research Symposium
Natalie Henson, Ball High school Bench Program, 2014-2015
Ashley Nilson, Neuroscience Graduate Program, BBSC Rotation, 2015

Teaching responsibilities at Texas A&M Galveston:
Lecturer: Toxicology 2015

Teaching responsibilities at College of the Mainland:
Co-director and lecturer: Psyc 2314 Developing Through the Lifespan-Service Learning

Publications

Peer-reviewed Manuscripts

1- **Gerson JE**, Castillo-Carranza DL, Sengupta U, Prough D, Dewitt D, Hawkins B, Kayed R (2016). Tau Oligomers derived from Traumatic Brain Injury cause cognitive impairment and accelerate onset of pathology in Htau mice. ***Journal of Neurotrauma***.

2- Guerrero-Munoz MJ, **Gerson JE**, Castillo-Carranza DL (2015). Tau oligomers: the toxic player at synapses in Alzheimer's Disease. ***Frontiers in Cellular Neuroscience***, doi: 10.3389/fncel.2015.00464.

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4- Deger J, **Gerson JE**, & Kayed R (2015). The interrelationship of proteasome impairment and oligomeric intermediates in neurodegeneration. ***Aging Cell,*** doi: 10.1111/acel.12359

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7- Mennenga SE, Baxter LC, Grunfeld IS, Brewer GA, Aiken LS, Engler-Chiurazzi EB, Camp BW, Acosta JI, Braden BB, Schaefer KR, **Gerson JE**, Lavery CN, Tsang CWS, Hewitt LT, Kingston ML, Koebele SV, Patten KJ, Ball H, McBeath MK*, & Bimonte-Nelson HA* (2014). Navigating to new frontiers in behavioral neuroscience: Traditional neuropsychological tests predict human performance on a rodent-inspired radial-arm maze. ***Frontiers in Behavioral Neuroscience*** 8, 294, doi: 10.3389/fnbeh.2014.00294.

8- Mennenga SE, **Gerson JE**, Dunckley T, & Bimonte-Nelson HA (2014). Harmine treatment enhances short-term memory in old rats: dissociation of cognition and the ability to perform the procedural requirements of maze testing. ***Physiology and Behavior,*** 138, 260-265 doi: 10.1016/j.physbeh.2014.09.001.

9- **Gerson JE**, Castillo-Carranza, DL, & Kayed R (2014). Advances in Therapeutics for Neurodegenerative Tauopathies: Moving Towards the Specific Targeting of the Most Toxic Tau Species. ***ACS Chemical Neuroscience***, 5(9), 752-769, doi: 10.1021/cn500143n.

10- **Gerson JE**, Sengupta U, Lasagna-Reeves C, Guerrero-Muñoz M, Troncoso J, Jackson G, & Kayed R (2014). Characterization of tau oligomeric seeds in Progressive Supranuclear Palsy. ***Acta Neuropathologica Communications***, 2, 73, doi: 10.1186/2051-5960-2-73.

11- Lasagna-Reeves CA, Sengupta U, Castillo-Carranza D, **Gerson JE**, Guerrero-Munoz M, Troncoso JC, Jackson GR, & Kayed R (2014). The formation of tau pore-like structures in tauopathies is prevalent and cell specific: Possible implications for the disease phenotypes. ***Acta Neuropathologica Communications***, 2(1), 56, doi: 10.1186/2051-5960-2-56.

12- Castillo-Carranza DL*, **Gerson JE*,** Sengupta U, Guerrero-Muñoz MJ, Lasagna-Reeves CA, & Kayed R (2014). Specific targeting of tau oligomers in Htau mice prevents cognitive impairment and tau pathology following injection with brain-derived tau oligomeric seeds. ***Journal of Alzheimer's Disease***, 40(0), S97-S111, doi: 10.3233/JAD-132477.

13- Castillo-Carranza DL, Sengupta U, Guerrero-Muñoz MJ, Lasagna-Reeves CA, **Gerson JE,** Singh G, Estes DM, Barrett ADT, Dineley KT, Jackson GR, & Kayed R (2014). Passive immunization with tau oligomer monoclonal antibody reverses tauopathy phenotypes without affecting hyperphosphorylated neurofibrillary tangles. ***Journal of Neuroscience***, 34(12), 4260-4272, doi: 10.1523/JNEUROSCI.3192-13.2014.

14- **Gerson JE** & Kayed R (2013). Formation and propagation of tau oligomeric seeds. ***Frontiers in Neurology***, 4, doi: 10.3389/fneur.2013.00093.

15- Camp BW, **Gerson JE**, Tsang CWS, Villa SR, Acosta JI, Braden BB, Hoffman AN, Conrad CD, & Bimonte-Nelson HA (2012). Higher serum androstenedione levels correlate with impaired memory in the surgically menopausal rat: a replication and new findings. ***European Journal of Neuroscience***, 36, 3086-3095, doi: 10.1111/j.1460-9568.2012.08194.

<u>Publications in Preparation</u>

1-Castillo-Carranza DL, Guerrero-Munoz MJ, Shafiei S, **Gerson JE**, Sengupta U, Kayed R. Tau and alpha-synuclein oligomers coexist in exosomes of PD and LBD brain. (In preparation).

2-**Gerson JE**, Castillo-Carranza DL, Sengupta U, Henson N, Farmer, K, Nilson A, Kayed R. Immunotherapy Against Tau Oligomers Protects Against Toxicity in a Synucleinopathy Mouse Model. (In preparation).

3-**Gerson JE**, Sengupta U, Ha Y, Zhang W, Kayed R. Characterization of novel disease-specific tau oligomeric strains (In preparation).

4-**Gerson JE** and Kayed R. Prion-like strains in tau and other amyloid proteins (In preparation).

Book Chapters

1- **Gerson JE,** Guerrero-Muñoz, MJ, Lo Cascio F, Kayed R. "The Potential of Small Molecules in Preventing Tau Oligomer Formation and Toxicity." Neuroprotection in Alzheimer's Disease; Editor, Illana Gozes, Elsevier, **2016**.

2- **Gerson JE** and Kayed, R. "Advances in Tau Immunotherapy." Research Progress in Alzheimer's Disease and Dementia (VI); Editors, Miao-Kun Sun, Nova Science Publisher, **2016**.

3-**Gerson JE**, Sengupta U, Kayed R. "Tau Oligomers as Pathogenic Seeds: Preparation and Propagation in Vitro and in Vivo." Tau Protein: Methods and Protocols; Editors, Caroline Smet-Nocca, Methods in Molecular Biology, **2016**.

4- **Gerson JE** & Kayed R; "Formation and Propagation of Tau Oligomeric Seeds". Tau Oligomers; Editors, Jesus Avila & Naruhiko Sahara, Frontiers in Neurology, Lausanne, **2014**.

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2- Kayed R, Gerson JE, Castillo-Carranza DL, Guerrero-Muñoz MJ, Sengupta U & Barrett A (2015). Tau oligomers as a therapeutic target for Alzheimer's disease. ***Journal of the Neurological Sciences***, 357(S1), e7.

3- **Gerson JE**, Sengupta U, Guerrero-Muñoz MJ, Castillo-Carranza DL & Kayed R (2015). Alpha-synuclein and tau oligomers interact in synucleinopathies: Tau oligomers as a therapeutic target. ***Neurodegenerative Diseases***, 15(S1), 144.

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6- **Gerson JE,** Sengupta U, Lasagna-Reeves C, Guerrero-Muñoz MJ, Troncoso J, Jackson G & Kayed R (2013). Tau oligomers and annular protofibrils in progressive supranuclear palsy. ***Alzheimer's & Dementia,*** 9(S4), P854-P855.

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Permanent address: 7070 N. Holiday Dr., Galveston, TX 77550

This dissertation was typed by Julia Gerson