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Life of Tau: Oligomerization, CNS Extracellular Clearance and Role as Plasma Biomarker

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Life of Tau: Oligomerization, CNS Extracellular Clearance and Role as Plasma Biomarker

by
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I have always thought of graduate school as learning how to fail. I say this not with any malice or bitterness, but more with respect and a deeper appreciation for the much needed maturation it provided. I learned how to cope with failure and disappointment without necessarily losing the intensity and zeal for science. The process of doing science – choosing a topic, formulating hypotheses, designing experiments, eagerly anticipating positive data, troubleshooting failed experiments – is often frustrating, but immensely rewarding. Although the first few years in the lab were challenging, I became a better scientist at the end of the journey. It was a real privilege to work on a condition as serious as Alzheimer disease, and even though I consider my contribution to the field to be minuscule, my experience has left me more determined to keep working toward my goal to become a physician-scientist.

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Tirth K Patel

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May 2020

Dedicated with much love and eternal gratitude to my grandfather, ‘dadu’

Abstract of the dissertation

Life of Tau: Oligomerization, CNS Extracellular Clearance and Role as Plasma Biomarker

for Arts & Sciences Graduate Students

by

Tirth K Patel

Doctor of Philosophy in Biology and Biomedical Sciences

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The microtubule associated protein tau (MAPT, commonly referred to as tau) is an intrinsically disordered, highly soluble protein predominantly expressed in axons where it binds and stabilizes microtubules. Under normal physiological conditions, soluble monomeric tau is released in the extracellular space in the interstitial fluid (ISF) by neurons. Additionally it undergoes reversible phosphorylation and other extensive modifications inside the cell under the action of a host of enzymes. However in the disease process tau loses this solubility, detaches from microtubules and ultimately migrates to the somatodendritic compartment of the neuron, where it ultimately forms insoluble fibrillar aggregates known as tau tangles. The nature, timeframe, and inciting factors of this transformation are active areas of research and as such, remain only partially understood.

In addition to its role in AD, tau is also implicated in several other neurodegenerative conditions collectively referred to as tauopathies, all of which feature insoluble tau tangles. This list, which is still evolving, includes conditions such as frontotemporal dementia (FTD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), chronic traumatic encephalopathy (CTE), and argyrophilic grain disease (AGD). The prevailing hypothesis about the pathogenesis of tauopathies is that following an initiating event, intracellular tau aggregates and eventually spreads to other parts of the brain where it seeds previously normal tau, in a prion-like manner. These seeding and spreading phenomena have been observed in numerous mouse models. The nature of tau species involved in this spread, as well as the exact mechanism of trans-synaptic spread are not well understood.

Tau's involvement in neurodegeneration has made it an attractive therapeutic target. Passive immunotherapy with monoclonal antibodies has shown promise and is being tested in clinical trials in humans. Vaccination trials are also underway. In addition, other approaches such as antisense oligonucleotides (ASOs) and small molecule inhibitors have been tested in animal models.

Here we have mainly focused on three distinct but related aspects in the "life of tau:"

1. Developing methods to measure tau oligomers
2. Determining clearance kinetics of extracellular tau from the CNS to the periphery
3. Evaluating plasma tau as a potential biomarker for CNS tau pathology

Tau oligomers are thought to be an important intermediate step en route to the eventual fibrillization of soluble monomeric tau. We developed a high-sensitivity assay on a single

molecule detection platform to detect tau oligomers, and demonstrated the effective of this assay to detect tau oligomers *in vitro*, and in brain homogenates of transgenic mice.

Our work characterizing extracellular tau clearance is the first comprehensive work of its type and strongly suggests that dural lymphatic system plays a key role in this process. Mice lacking these lymph vessels retain more tau in the brain as well as show slowed clearance.

Lastly we asked whether plasma tau could serve as a reliable biomarker for soluble CNS tau and give us an accurate snapshot of tau pathology. Administration of an anti-tau antibody resulted in dose dependent increase in plasma tau in both humans and mice, most likely because of its role in extending the half-life of plasma tau from 8 minutes to 3 hours. Furthermore plasma tau levels correlated with soluble CNS tau levels (and therefore, inversely with tau pathology), making this a potentially good strategy to monitor tau pathology load in mouse model of tauopathy and potentially in humans.

The work done here represents an important step in characterizing the role of tau in normal physiology and disease and will help guide future therapy as well as diagnostic approaches.

Chapter 1 Introduction

1.1 Tau in Alzheimer disease

Alzheimer disease (AD) is the leading cause of dementia in the elderly and by some counts the sixth leading cause of death in the United States. With increases in longevity, most experts estimate that the number of people with AD will rise to 50 million over the next few decades(Holtzman, Mandelkow, and Selkoe 2012). The two main neuropathological hallmarks of AD are extracellular plaques of amyloid-beta (abeta) and largely intracellular neurofibrillary tangles (NFT) of tau protein. The amyloid cascade hypothesis holds that the triggering event in AD pathogenesis is the initial accumulation and aggregation of abeta into insoluble extracellular plaques. This is thought to initiate a cascade of events that incite misfolding and aggregation of soluble tau into insoluble neurofibrillary tangles, eventually leading to massive neuronal death (Musiek and Holtzman 2015). NFTs in AD are hyperphosphorylated as well as misfolded. Despite the apparent centrality of abeta in governing the pathogenesis of AD, loss of cognitive function in patients is in fact directly correlated to tangle load, and not plaque load. Because of its involvement in AD pathology, tau has been the focus of intense research efforts designed to unravel its role in the pathogenesis of AD and other forms of neurodegeneration. Tau tangles can be found in the brainstem and limbic regions of healthy asymptomatic individuals(Braak and Del Tredici 2011). But in AD there are extensive tangles in the neocortex in addition to the hippocampus(Braak and Braak 1991), suggesting that the mere aggregation of tau may not be toxic and that it needs a trigger to become toxic.

1.2 Tau in other forms of neurodegeneration

In addition to its key role in AD pathology, tau has also been implicated in a host of other neurodegenerative disorders. Collectively termed tauopathies, these disorders all feature aggregated forms of tau in various neuronal cell types(Mandelkow and Mandelkow 2012a). Primary tauopathies, where tau is thought to be driver of pathogenesis, include conditions like corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), argyrophilic grain disease (AGD), Pick's disease (PiD), primary age-related tauopathy (PART), and more recently chronic traumatic encephalopathy (CTE). A subset of frontotemporal dementias (FTD) – a multifactorial degenerative disease – has also been identified as being caused by mutations in tau and is included in this category(Li and Götz 2017 Tau-based therapies in neurodegeneration: Opportunities and challenges). Several other rare causative mutations have been identified as well. This is clearly an evolving category and will keep getting updated as research continues. Secondary tauopathies are disorders where tau pathology is co-existent, but not necessarily predominant. This category obviously includes AD but also other parkisonisms such as lewy body dementia (DLB), and Parkinson disease dementia (PDD), where the primary culprit is misfolded and aggregated forms of alpha-synuclein protein. Table 1.1summarizes a list of tauopathies as well as the diverse forms of tau aggregates seen in each (adapted from (Li and Götz 2017)).

1.3 Structure and physiological function of tau

The microtubule associated protein tau (MAPT, commonly referred to as tau) is an intrinsically disordered, highly soluble protein predominantly expressed in axons where it binds microtubules. Some expression is seen in testes, muscle and periphery as well. Originally discovered in the late 1970s(Weingarten et al. 1975) its significance in disease was largely overlooked until a series of studies in the 1980s showed that it was the core component of neurofibrillary tangles(Kosik, Joachim, and Selkoe 1987, Luo et al. 2014). Further genetic and protein characterization localized the MAPT locus to chromosome 17(Neve et al. 1986). It was soon discovered that alternative splicing leads to six different isoforms, depending on the presence or absence of an extra repeat domain (which includes the aggregation prone region of the protein) and an extra amino domain. Thus the isoforms range from 0N3R to 2N4R(Goedert et al. 1989).

Tau has a myriad of binding partners apart from microtubules and it interacts with an army of enzymes that modify it in practically every way possible. Tau can get phosphorylated, glycosylated, sumoylated, acetylated, ubiquitination and nitration among many other modifications(for detailed overview see Mandelkow & Mandelkow, 2012). In common with other IDPs such as alpha-synuclein, tau is prone to oligomerization and fibrillization.

Under normal physiological conditions neurons have been shown to release soluble monomeric tau in the interstitial fluid (ISF) (Yamada et al. 2011a)The precise mechanism of this release is not yet clear and studies have suggested exosomes, and extracellular vesicles as potential pathways of release (Yamada 2017). It appears that the release of tau in extracellular space can be modulated, as increasing neuronal activity increases tau secretion(Karch, Jeng, and Goate 2012, Pooler et al. 2013, Yamada, Holth, Liao, Stewart, Mahan, Jiang, Cirrito, Patel, Hochgräfe, et al. 2014), and inhibiting lysosomal function decreases it ((Mohamed et al. 2014). Additionally protein folding chaperones can also regulate secretion of tau (Fontaine et al. 2016).

Surprisingly despite decades of work on tau, its precise function in the neuron is unknown. One of the earliest known function attributed to tau was assembly and stabilization of microtubules (Weingarten et al. 1975). It is thought to play a role in axonal trafficking by perhaps regulating dynein and kinesin motors and by maintaining a concentration gradient (Dixit et al. 2008, Drewes, Ebneth, and Mandelkow 1998 MARKs and microtubule dynamics, Ebneth et al. 1998 mitochondria, and endoplasmic reticulum: Implications for Alzheimer's disease, Mandell and Banker 1996, Trinczek, Ebneth, and Mandelkow 1999), although this is undermined by the observation that ablation of tau does not seem to affect axonal transport (Yuan et al. 2008). So it is likely that tau's role might not be essential and other microtubule binding proteins in the neuron could compensate in the absence of tau. This is supported by the fact that deletion of both tau and

MAP1B, another microtubule binding protein, leads to impaired axonal elongation and development and is lethal *in vivo* (Takei et al. 2000).

(Partial) list of tauopathies		
	Name	**Inclusions**
Primary	Progressive supranuclear palsy (PSP)	Neuronal + Glial
	Corticobasal degeneration (CBD)	Neuronal + Glial
	Pick disease	Largely neuronal
	Argyrophillic grain disease (AGD)	Neuronal + Glial
	Chronic traumatic encephalopathy (CTE)	Neuronal + Glial
	Primary age related tauopathy (PART)	Neuronal
	Globular glial tauopathy (GGT)	Glial
Secondary	Alzheimer disease (AD)	Neuronal

Table 1.1. A list of tauopathies and the diverse type of tau aggregates seen in each (adapted from (Li and Götz 2017))

It has also been proposed that tau plays a role in modulating various cell signaling pathways by binding kinases such as Fyn and Src (Ittner et al. 2010, Sharma et al. 2007).

Any claim to the essential physiological nature of tau in the cell is undermined by the fact that knockout of tau does not seem to have catastrophic consequences. Tau knockout mice do not seem to suffer from any major behavioral or cognitive deficits, nor do they suffer from early mortality (Dawson et al. 2001, Harada et al. 1994, Roberson et al. 2007 Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model.). In fact reduction of tau seems to have a neuroprotective effect. Tau knockout mice are less susceptible to seizures (Holth et al. 2013), and a similar effect is seen when tau mRNA levels are reduced with antisense oligonucleotide (ASO) treatment (DeVos et al. 2013b). The consequences of tau reduction are even more evident when animal models of abeta pathology are used. Tau reduction in APP overexpression mouse models improves behavioral deficits without affecting abeta levels (Ittner et al. 2010, Roberson et al. 2007). A possible mechanism underlying this interplay between tau and abeta might be the kinase Fyn (Ittner et al. 2010, Ittner and Götz 2011, Roberson et al. 2011).

1.4 Prion-like spread of tau pathology in neurodegeneration

In recent years a lot of attention has been devoted to the mechanisms underlying tau's "villainous" transformation from a highly soluble monomer to an insoluble fibril and how this might relate to its disease-mediating properties. This transformation process itself still remains poorly understood but moderate breakthroughs have been made in elucidating how tau might be wreaking

neuropathological havoc in the diseased brain. In the late 1980s and early 1990s, Braak and colleagues noted that in the brains of patients with AD, NFTs exhibited a stereotypical pattern of distribution, that was characteristic enough that they developed a six stage system of classifying the stage of disease based on the location and extent of tangle load (Braak and Braak 1991, 1997, Braak and Braak 1995). Stage I-II are when tangle pathology is limited to transentorhinal cortex, stages III-IV see tangle pathology spread to hippocampus and stages V-VI see extensive tangle pathology in the neocortex. Thus tau pathology appears to be "spreading" to different brain regions.

Recently this theory has gained a lot more traction to the point where it has been developed into a full-fledged model aimed at explaining pathogenesis of not just tauopathies but several other protein aggregation disorders such as Parkinson disease (PD), amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (Jucker and Walker 2013) (Figure 1.1).

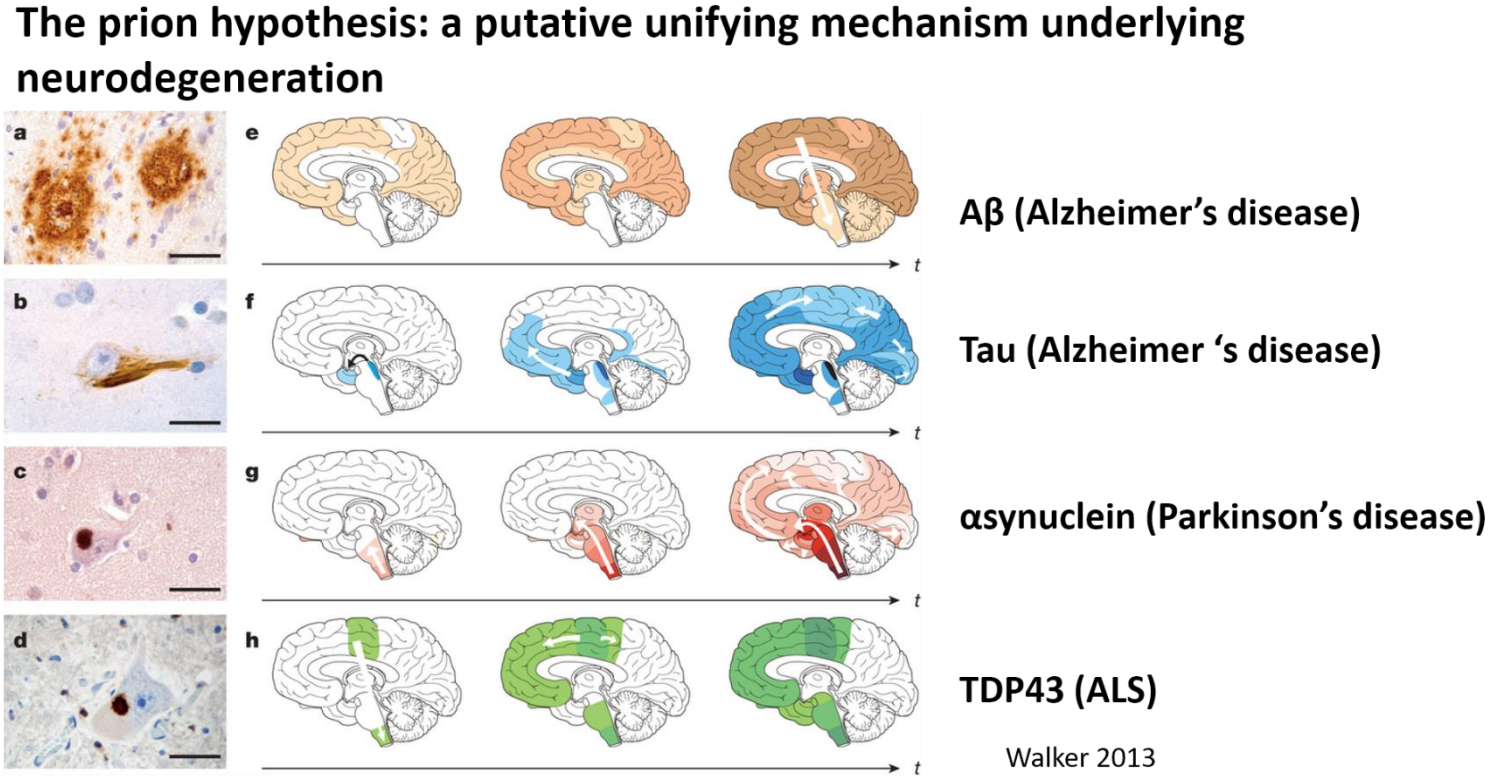


Figure 1.1. The prion hypothesis: a putative unifying mechanism underlying neurodegeneration.

Previously normal version of the implicated protein misfolds and aggregates following an inciting event. This abnormal version then spreads to other parts of the brain where it can seed and cause other normal forms of itself to misfold and aggregate. For each protein (and its corresponding disease) the initial pathology and its subsequent spread to other brain regions follows a stereotypical pattern that can help stage and characterize the disease better. Figure adapted from (Jucker and Walker 2013).

In the context of tau the first study demonstrating the possible cellular internalization and propagation of pathogenic seeds was published in 2009 (Frost, Jacks, and Diamond 2009). A

number of *in vitro* studies have subsequently demonstrated this phenomenon (for instance (Kfoury et al. 2012, Takeda et al. 2015, Wegmann et al. 2016, Wu et al. 2013).

Injection of brain lysate from a transgenic mouse carrying the human P301S mutation into a WT mouse carrying a WT human tau transgene resulted in local seeding and uptake at the site of injection and subsequent spreading to distant brain regions (Clavaguera et al. 2009). In the years since many studies using a variety of inoculates have confirmed this finding. In addition to brain lysates from transgenic mice (Ahmed et al. 2014, Clavaguera et al. 2014), cell lysates (Sanders et al. 2014), synthetic recombinant tau fibrils (Iba et al. 2013, Peeraer et al. 2015) and tau extracts purified from human brains (Boluda et al. 2014, Clavaguera et al. 2013, Guo et al. 2016, He et al. 2018, Lasagna-Reeves, Castillo-Carranza, Sengupta, Guerrero-Munoz, et al. 2012) have also been shown to robustly induce uptake, seeding and spreading in the brain.

Tau seeds can be taken up by the cell via heparan sulfate proteoglycans (HSPGs) (Holmes et al. 2013). The stability of the initial seeds through generations of passaging and the variable seeding strengths of different types of inoculates seems to suggest that there are various proteopathic tau "strains" (Sanders et al. 2014, Sanders et al. 2016). Neuronal activity is known to regulate release of tau in the ISF (Yamada, Holth, Liao, Stewart, Mahan, Jiang, Cirrito, Patel, Hochgräfe, et al. 2014) and also the prion-like spread of tau pathology (Wu et al. 2016). The fact that intraperitoneal injections are enough to trigger seeding and spreading (Clavaguera et al. 2014) indicates that the pathogenic seeds might only need to be introduced in very small amounts in the brain to initiate the prion-like cascade. Although bulk of the research done so far strongly

suggests that the injected seeds are not tau monomers one cell culture study reported that tau monomer was sufficient in inducing seeding (Michel et al. 2014). Similar injection studies have been conducted with alpha-synuclein to show that this mechanism might be shared among several neurogenerative conditions (see (Jucker and Walker 2013) for a discussion of other protein aggregation disorders).

In the midst of all the injection studies, a pair of breakthrough studies demonstrating *in vivo* trans-synaptic spread of tau in an endogenous manner was published in 2012. By restricting expression of human tau to the entorhinal cortex using an inducible tet-off system authors were able to track its movement in the brain. They found that several months following its expression tau was found to spread from entorhinal cortex to the dentate gyrus (de Calignon et al. 2012, Liu et al. 2012).

In humans indirect evidence for this spreading phenomenon outside of bona fide prion diseases is a study showing that a small segment of children inadvertently given growth hormone extracts from diseased pituitary glands developed CJD and abeta aggregates and subsequent amyloid plaque pathology via seeding and spreading as they aged (Jaunmuktane et al. 2015) and another study showing possible transmission of cerebral amyloid angiopathy due to neurosurgery (Jaunmuktane et al. 2018).

The development of a FRET biosenser-based seeding assay (Holmes et al. 2014, Kfoury et al. 2012) has made it possible to assess seeding activity of a variety of samples such as mouse and human brain lysates, cell lysates, synthetic fibrils and microdialysates among others. Tau

extracted from various human brain regions has significant seeding activity, which can be detected in the presence of scant or even prior to the formation of NFT pathology (DeVos et al., 2018; Furman et al., 2017).

1.5 Role of tau oligomers in tau-mediated neurodegeneration

Tau oligomers represent an intermediate step in the fibrillization process of soluble monomeric tau. Much attention has been lavished upon them on their putative role in tau-mediated neurodegeneration. Chapter 2 covers tau oligomers in extensive detail but in brief: emerging evidence suggests that tau oligomers do represent a distinct step in the transformation of tau and are likely involved in some toxicity in neurodegeneration. Since the nature of tau species mediating prion-like spread is not known tau oligomers have emerged as the leading culprits driving this disease process. There is some evidence to back this claim but this area is still largely unexplored.

1.6 Therapeutic strategies against tau

Tau makes an obviously attractive therapeutic target on account of its key role in several neurodegenerative conditions. Several approaches have been tried: active immunization, passive immunization, antisense oligonucleotides and other small molecules (Gallardo and Holtzman 2017, Li and Götz 2017). Active immunization against P301L mutant tau has been shown to

ameliorate cognitive deficits and reduce pathology (Asuni et al. 2007, Bi et al. 2011, Boutajangout, Quartermain, and Sigurdsson 2010). There are two vaccination trials ongoing in humans (Li and Götz 2017)

Several studies have demonstrated efficacy of passive immunization by administering antibodies against various forms of tau. Antibodies against various phosphorylation epitopes can reduce tau pathology (Collin et al. 2014, Gu, Congdon, and Sigurdsson 2013) in mouse models of tauopathy. Additionally p-tau antibodies can also help block TBI (Kondo et al. 2015). Targeting abnormally folded tau with the antibody MC-1 was also successful at reducing pathology (Chai et al. 2011a). Work done in our lab has shown that antibodies that effectively block seeding *in vitro* are also potent at markedly reducing tau pathology *in vivo* and improving brain atrophy (Yanamandra et al. 2013b, Yanamandra et al. 2015). In line with these encouraging results, major clinical trials using monoclonal tau antibodies are in Phase II currently.

Recently antisense oligonucleotides (ASOs) have emerged as attractive therapeutic candidates. By targeting mRNA sequence of a protein, ASOs can reduce protein expression or modify its splicing pattern (Schoch and Miller 2017). Administration of ASO to the P301S mouse model of tauopathy resulted in reduction of hyperphosphorylated tau pathology and improvement in survival and brain atrophy(DeVos et al. 2017). Additionally ASOs can reduce hyperexcitability and protect against seizures by their inhibitory action on tau(DeVos et al. 2013a).

1.7 Scope of this work

The work presented here aims to shed light on three different aspects of tau – a day in the life of tau.

Chapter 2 discusses the structure and formation of tau oligomers, and their role in potentially mediating neurodegeneration and toxicity. We sought to develop a high-sensitivity assay to reliably measure oligomers and explored whether *in vivo* microdialysis could help characterize these species in the interstitial fluid.

Chapter 3 is devoted to the study of tau clearance, a process that is only vaguely understood. Our goal was to characterize this fundamental process and focus on the possible role of dural lymphatics.

Chapter 4 looks at the possibility of using plasma tau as a reliable biomarker for soluble CNS tau. This work has obvious theranostic implications.

Chapter 2 Development of a highly sensitive tau oligomer specific assay for *in vitro* and *in vivo* characterization

This chapter includes work done in collaboration with the laboratory of Marc Diamond (then at Washington University, now at University of Texas, Southwestern) and Janssen pharmaceuticals. Significant contributions were made by Floy Stewart and Hilda Mirbaha.

2.1 Introduction

Very few subjects are as contentious, fuzzy and inscrutable as tau oligomers. That tau undergoes oligomerization en route to its eventual fibrilization and aggregation is widely accepted to be true. The details about this process – kinetics, structure and especially, significance and relevance – are a matter of great discussion. Even the term 'tau oligomer' itself is confusing, since there is no consensus on what its definition. While most studies to call any intermediate soluble multimeric tau species 'oligomers', others have preferred to use a stricter size-based cutoff. To add to the confusion, terms like protofibrils and granular filaments (Maeda et al. 2006, Maeda et al. 2007) are also often used to describe pre-fibrillar tau. Regardless of semantic the chaos surrounding nomenclature there is consensus that understanding the oligomerization (and subsequent fibrillization) of soluble monomeric tau is important for understanding its role in mediating neurodegeneration (Gerson and Kayed 2013 Formation and propagation of tau oligomeric seeds).

2.1.1 Structure and formation of tau oligomers

Despite being soluble even at high concentrations tau is quick to form higher order species such as dimers and trimers (Sahara et al. 2007) especially when a nidus such as heparin or arachidonic

acid is present (Lasagna-Reeves, Castillo-Carranza, Sengupta, Sarmiento, et al. 2012 Identification of oligomers at early stages of tau aggregation in Alzheimer's disease). Cross-seeding by abeta and alpha-synuclein can also promote oligomerization (Lasagna-Reeves et al. 2010). These oligomeric intermediate species are often hyperphosphorylated and may have other modifications (Sahara et al. 2013).

2.1.2 Tau oligomers in neurodegeneration

Tau oligomer researchers are big proponents of the theory that oligomers, and not NFTs, are more potent and the real toxic species in tau-mediated neurodegeneration (Gerson and Kayed 2013). This is because NFT and filament pathology do not correlate well with neuronal cell death in patients and in various mouse models (Andorfer, Kress, Espinoza, De Silva, et al. 2003, Spires et al. 2006, Yoshiyama et al. 2007b). Studies in cell cultures and in inducible transgenic mouse models of tauopathy show that injection of tau oligomers can cause synaptic and mitochondrial dysfunction (Castillo-Carranza, Gerson, et al. 2014) by binding synaptic vesicle proteins and inhibit long-term potentiation (LTP) and memory formation in the hippocampus (Lasagna-Reeves, Castillo-Carranza, Sengupta, Guerrero-Munoz, et al. 2012, Puzzo et al. 2017). Furthermore suppression of soluble tau expression leads to improvement in mitochondrial dysfunction (Kopeikina et al. 2011).

More evidence implicating tau oligomers in disease process comes in the form of neuropathological studies in humans and mouse models. Oligomers have been isolated from post-mortem brains of AD (Lasagna-Reeves, Castillo-Carranza, Sengupta, Sarmiento, et al. 2012, Maeda et al. 2006) patients where they correlate with Braak stage and from CSF (Sengupta et al. 2017) where they are elevated in AD patients compared to non-demented controls. They have also been isolated from PSP brains where they are present in globose inclusions (Gerson et al. 2014). Considering the role of inflammation in mediating neurodegeneration it is notable that oligomers were found to associate with astrocytes and microglia in the retina and brains of transgenic mouse model of tauopathy and post-mortem brains of FTLD and AD patients (Nilson et al. 2017). Cerebrovascular deposition of oligomers was also found in tauopathies, PD, alongside abeta aggregates in a mouse model (Castillo-Carranza et al. 2017) and in a rat model of blast-induced TBI (Gerson et al. 2016)

The lack of a known culprit for mediating prion-like spread of tau pathology has made tau oligomers prime suspects for this role. Cell culture systems have demonstrated uptake and propagation of oligomeric and high-molecular weight tau species (Takeda et al. 2015, Wegmann et al. 2016, Wu et al. 2013). Tau oligomers derived from AD brains with an oligomer specific antibody have been shown to induce local seeding and subsequent spreading to distant regions when injected in the brain of WT mice. These mice also exhibit behavioral deficits (Lasagna-Reeves, Castillo-Carranza, Sengupta, Guerrero-Munoz, et al. 2012). Similarly intracerebral injection of oligomers derived from rat brains of a TBI model causes cognitive deficits in mice overexpressing WT human tau(Gerson et al. 2016).

2.1.3 Therapeutic strategies against tau oligomers

Therapeutic strategies against tau oligomers have mainly consisted of passive immunotherapy. Treatment with an oligomer specific antibody in a mouse model of tauopathy reduced soluble tau oligomer levels, improved behavior and reduced tau pathology (Castillo-Carranza, Sengupta, et al. 2014b) thus suggesting that focusing on oligomers in addition to aggregates can yield good results.

It is clear that proper characterization of tau oligomers *in vivo* can yield valuable insight into the role of tau in pathogenesis of tauopathies.

2.2 Experimental outline

We sought to develop sensitive, robust and reproducible methods to collect and quantify tau oligomers from various physiological compartments such as interstitial fluid (ISF). We addressed two interlinked questions as part of this project:

1) Do tau oligomers exist *in vivo*? And

2) If so can we develop methods to collect and quantify them?

One recent study described collection and analysis of high molecular weight soluble tau species from mouse ISF using HPLC. These tau species were able to propagate in a cell culture propagation system(Takeda et al. 2015). The isolation of these species depends on size-exclusion columns and the measurement was carried out with a tau monomer ELISA, meaning there is still a need for a true quantative oligomer specific assay.

2.3 Results

Here we report the development of tau oligomer specific sandwich ELISAs (using the same capture and detection antibody). Using these ELISAs we measured tau oligomers in lysates from various brain regions from aged P301S tau transgenic mice.

Further we demonstrate that microdialysis is a viable method for assessing tau oligomers. Our results from various *in vitro* experiments show ~1% recovery of full length tau fibrils with a 1 million Da probe and a ~3% recovery when using purified tau oligomers of different sizes. Recovery of oligomers was confirmed by direct fluorescence reading and by the FRET based tau seeding assay.

Since putative tau oligomers are likely present in very small amounts in physiological compartments like ISF or CSF, it is important to develop high-sensitivity assays to aid in their detection. To this end we describe preliminary results regarding the successful translation of our oligomer-specific ELISA to the Simoa HD1 Analyzer, a high-sensitivity single-molecule

detection system. We also describe results from our initial attempts to measure tau oligomers by *in vivo* microdialysis in P301S tau transgenic animals.

2.3.1 Successful collection of repeat domain (RD) tau oligomers by microdialysis

Microdialysis is a versatile technique that allows access to analytes in the ISF of a living, awake, freely moving animal and has proven valuable especially in neurodegeneration by enabling real-time monitoring of abeta (Cirrito et al. 2003b, Cirrito et al. 2005, Kang et al. 2009) and tau (Yamada et al., 2011). Previous work in our lab using *in vivo* microdialysis in P301S tau transgenic animals showed that soluble tau monomers are released physiologically in the ISF in an activity dependent manner and soluble tau decreases as mice age and pathology increases (Yamada et al. 2011a In Vivo Microdialysis Reveals Age-Dependent Decrease of Brain Interstitial Fluid Tau Levels in P301S Human Tau Transgenic Mice, Yamada, Holth, Liao, Stewart, Mahan, Jiang, Cirrito, Patel, Hochgrafe, et al. 2014 Neuronal activity regulates extracellular tau in vivo). Before using microdialysis to attempt to collect putative tau oligomers from mice, we tested the ability of microdialysis to tau oligomers *in vitro*. Figure 2.1 shows the schematic for *in vitro* microdialysis.

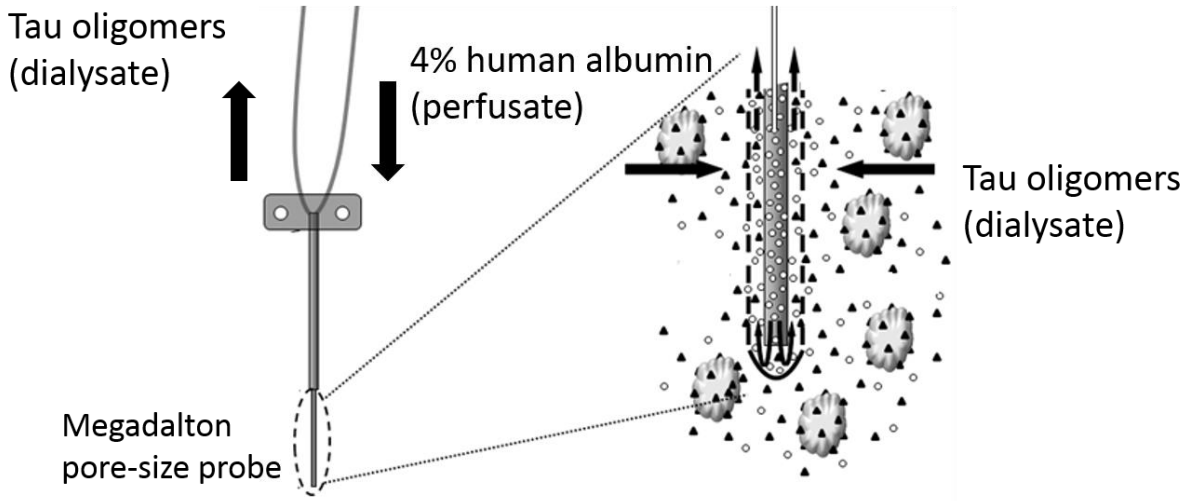


Figure 2.1. In vitro microdialysis for tau oligomers.

(adapted and modified from Wikimedia.org)

Microdialysis probe with a 1 million Dalton cutoff size was inserted in a solution of tau fibrils of known concentration (1-2 ug/ml). As a good first step this was performed by using tau fibrils made of the aggregation-prone repeat domain (RD) of tau. To measure any oligomers that may have been collected by this benchtop *in vitro* microdialysis setup, we developed a RD tau oligomer specific ELISA using the same capture and detection antibody, HJ 9.3. The epitope for this antibody lies in RD of tau, making it an ideal candidate for a sensitive ELISA. Microdialysis was performed with different flow rates to extrapolate and ultimately calculate a hypothetical 'zero flow' recovery concentration. Results are shown in Figure 2.2 Recovery for monomeric tau under similar conditions is ~30% but recovery for RD fibrils was substantially higher than that, at ~65%.

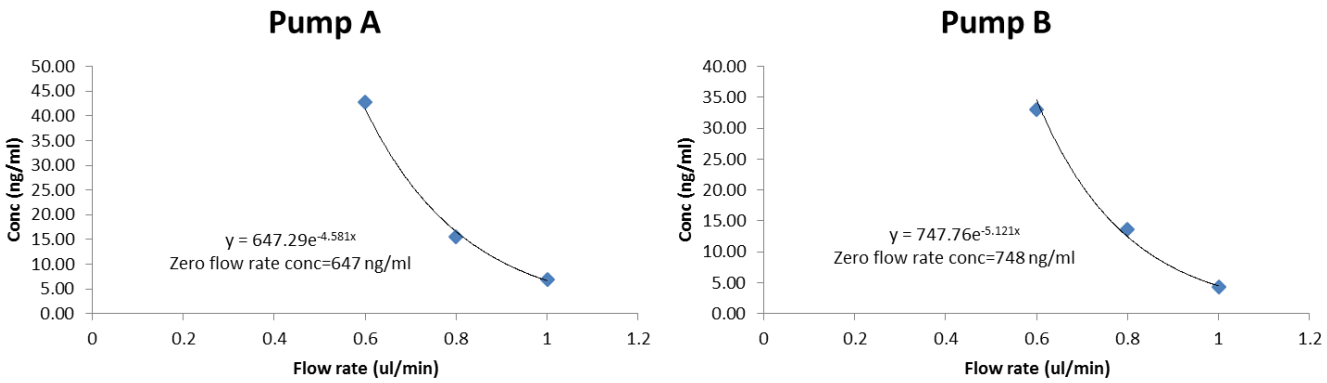


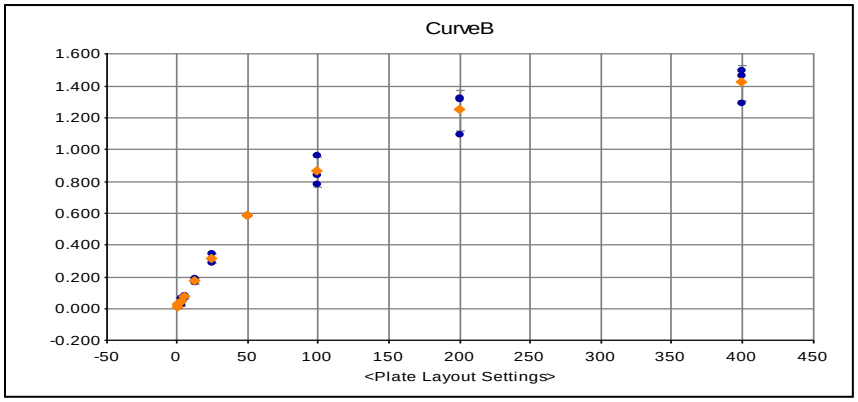
Figure 2.2. In vitro microdialysis for RD tau oligomers.

This proof of concept experiment demonstrates about 65% recovery rate for RD tau oligomers by in vitro tau microdialysis.

2.3.2 Successful collection of full-length (FL) tau oligomers by microdialysis

Next we attempted to measure full-length (FL) tau oligomers using microdialysis. *In vitro* microdialysis for these oligomers was performed similar to RD tau oligomer microdialysis. After a thorough screen of a panel of anti-tau antibodies, two viable candidates (HJ 8.7 and HJ 8.2) were chosen for further optimization and characterization. As shown in Figure 2.3 the sensitivity of these assays is ~1 ng/ml based on a standard curve generated using FL tau fibrils. Since HJ 8.7 is a n-terminal antibody and HJ 8.2 is a c-terminal antibody, having two separate assays targeting two distinct regions of tau could be useful in generating a more complete profile of all the different tau species potentially present in ISF or CSF.

HJ 8.7/8.7b									
200 ng/ml	100 ng/ml	50 ng/ml	25 ng/ml	12.5 ng/ml	6.25 ng/ml	3.125 ng/ml	1.5625 ng/ml	Blank	Monomer (2 ng/ml)
1.592	1.368	0.888	0.525	0.315	0.204	0.146	0.103	0.081	0.086
1.567	1.24	0.816	0.488	0.284	0.186	0.14	0.1	0.07	0.072
1.619	1.405	0.988	0.452	0.287	0.161	0.123	0.098	0.087	0.071



HJ 8.2/8.2b									
200 ng/ml	100 ng/ml	50 ng/ml	25 ng/ml	12.5 ng/ml	6.25 ng/ml	3.125 ng/ml	1.5625 ng/ml	Blank	Monomer (2 ng/ml)
2.313	1.987	1.479	0.97	0.591	0.332	0.2	0.132	0.066	0.07
2.287	1.943	1.442	0.873	0.555	0.306	0.186	0.128	0.068	0.065
2.202	1.917	1.405	0.909	0.543	0.318	0.197	0.138	0.062	0.065

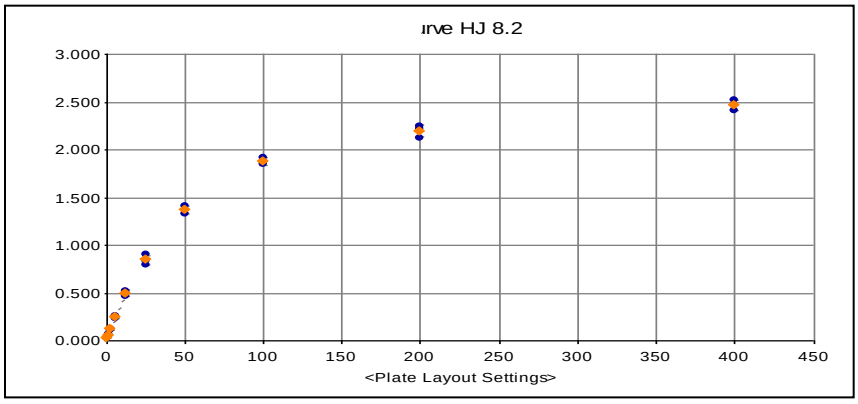


Figure 2.3. Representative standard curves from two tau oligomer-specific ELISAs.

Two separate oligomer-specific ELISAs (one directed at an n-terminal epitope and another at a c-terminal epitope) were used to confirm successful collection of full-length (FL) tau oligomers from in vitro microdialysis.

After successful recovery of FL tau oligomers and measurement by two separate oligomer-specific ELISAs we used HJ 8.7, the n-terminal antibody, for further *in vitro* microdialysis experiments with FL tau oligomers as well as brain lysates from aged P301S tau transgenic mice.

Table 2.1 summarizes concentrations of oligomers as measured by the HJ 8.7/8.7b ELISA from various *in vitro* experiments.

Experiment	Initial (ng/ml)	Recovered (ng/ml)	%recovery
IVMD 1	2000	4.86	0.24
IVMD 2	2000	10.02	0.50
IVMD 3	1000	1.41	0.14
IVMD 4	1000	3.14	0.31

RAB 1	5036.8	60.75	1.21
RAB 2	7887.2	47.41	0.60

Table 2.1. Recovery of FL tau oligomers as measured by HJ 8.7/8.7b ELISA.

Series of in vitro microdialysis experiments with FL tau oligomers and brain lysates from aged P301S tau transgenic mice. (IVMD=In vitro microdialysis; RAB=Brain lysates from 9mo male P301S tau transgenic mice in reassembly buffer (soluble fraction)).

Fluorescently labeled purified FL tau oligomers were obtained from the Diamond lab. In addition to measuring their concentration by ELISA, we also confirmed collection of oligomers by direct fluorescence. These results are summarized in Table 2.2 and Table 2.3.

Purified oligomers	Initial (nM)	Recovered (nM)	%recovery
n=2	20.92	0.47	2.27
n=3	11.22	0.38	3.40
n=10	3.81	0.13	3.35

Table 2.2 Recovery of purified tau oligomers as measured by HJ 8.7/8.7b ELISA.

In vitro microdialysis was performed on fluorescent FL tau oligomers. Recovered amount calculated after mass correction.

Based on results from Tables 2.1 and 2.2, it appears that recovery for purified oligomers is more efficient than for just fibrils. Table 2.3 confirms recovery of purified oligomers by fluorescence reading.

Purified oligomers	Initial fluorescence	Recovered fluorescence	%recovery
n=2	2688	337	12.54
n=3	3514	603	17.16
n=10	2004	291	14.52

Table 2.3. Recovery of purified tau fibrils as measured by direct fluorescence reading.

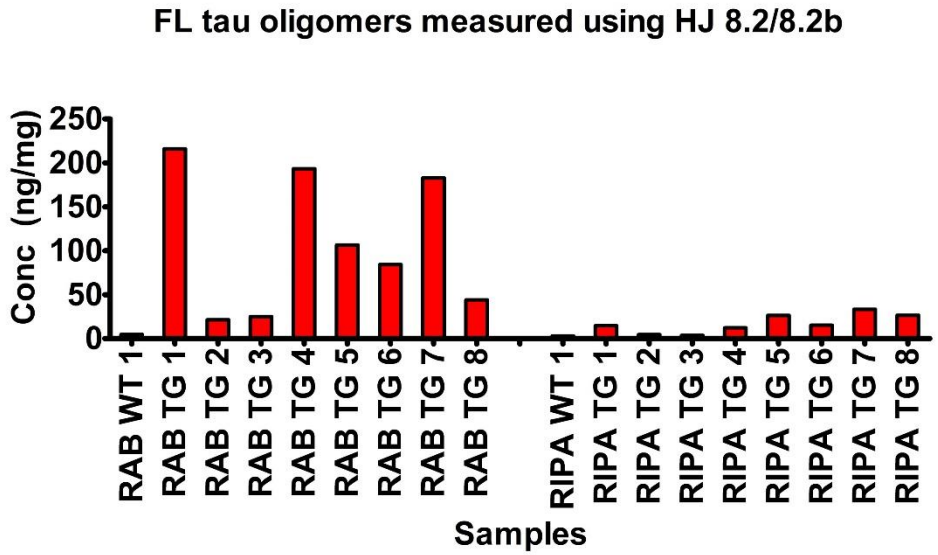
Amount of fluorescent fibrils recovered by in vitro microdialysis were also measured using a spectrophotometer.

Together the results from these *in vitro* experiments are highly encouraging, because they confirm that microdialysis is a viable technique for assessment and measurement of tau oligomers.

2.3.3 Measurement of tau oligomers from cortex, brainstem and hippocampus of P301S mice

As a next step we tested both assays using biological samples. Serial extraction was performed on cortices of wild-type and 9 mo P301S animals. The RAB fraction contains largely soluble monomeric tau and the RIPA fraction contains insoluble tau in addition to some soluble tau as well. We took these RAB and RIPA fractions and tested them using both the HJ 8.7/8.7b and the HJ 8.2/8.2b assays.

As shown in Figure 2.4, concentration of tau oligomers is 3-7x higher in RAB fraction than the RIPA fraction, as measured by both assays.


FL tau oligomers measured using HJ 8.2/8.2b
Conc (ng/mg)
250
200
150
100
50
0
RAB WT 1
RAB TG 1
RAB TG 2
RAB TG 3
RAB TG 4
RAB TG 5
RAB TG 6
RAB TG 7
RAB TG 8
RIPA WT 1
RIPA TG 1
RIPA TG 2
RIPA TG 3
RIPA TG 4
RIPA TG 5
RIPA TG 6
RIPA TG 7
RIPA TG 8
Samples

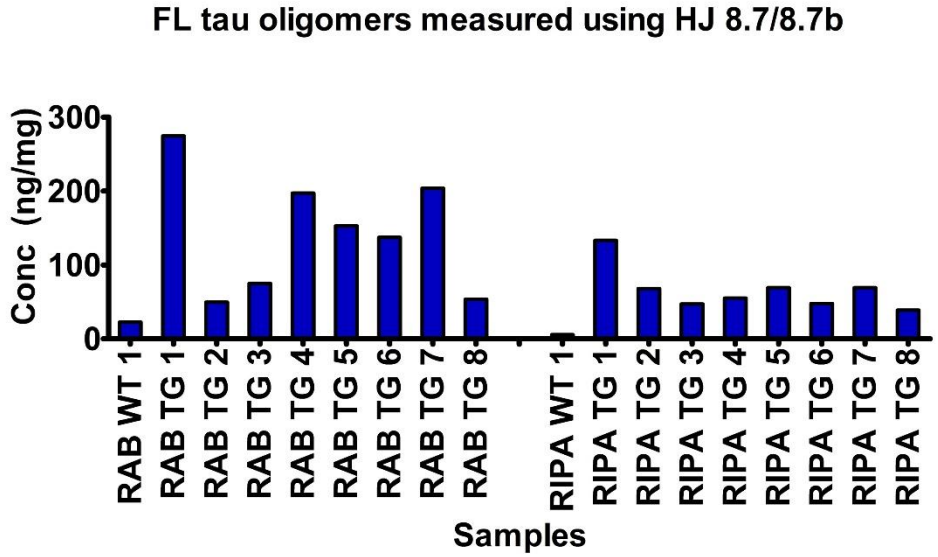
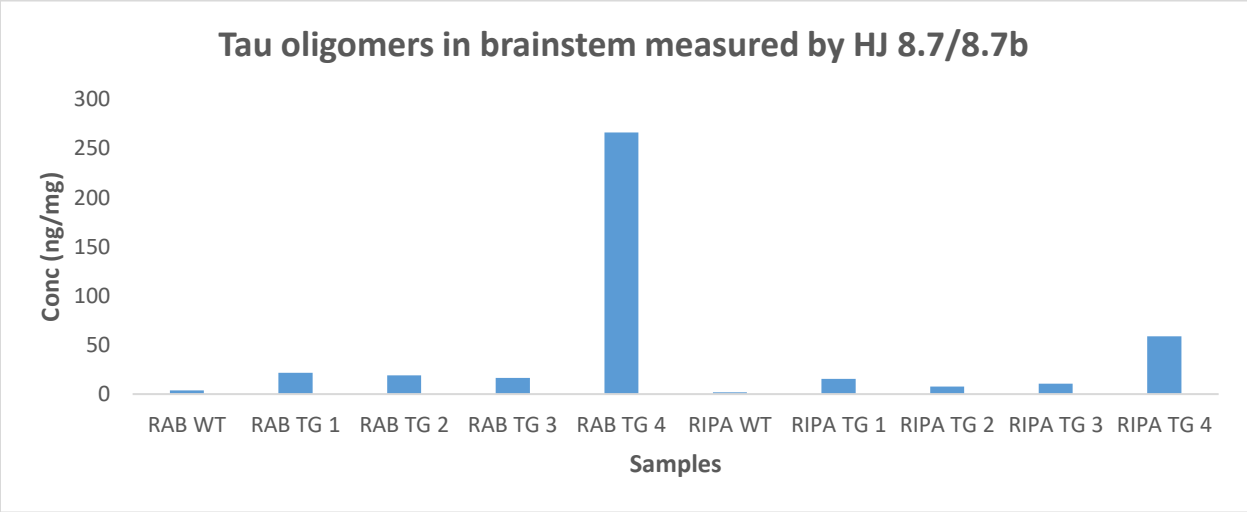

FL tau oligomers measured using HJ 8.7/8.7b
Conc (ng/mg)
300
200
100
0
RAB WT 1
RAB TG 1
RAB TG 2
RAB TG 3
RAB TG 4
RAB TG 5
RAB TG 6
RAB TG 7
RAB TG 8
RIPA WT 1
RIPA TG 1
RIPA TG 2
RIPA TG 3
RIPA TG 4
RIPA TG 5
RIPA TG 6
RIPA TG 7
RIPA TG 8
Samples

Figure 2.4. Measurement of tau oligomers in brain lysate fractions of the cortex in aged P301S mice.

Both the n-terminal and the c-terminal based oligomer assays are effective at quantifying tau oligomers from brain lysates. (Values are in ng/mg of protein present in the wet weight of tissue extracted. RAB represents salt-soluble fraction; RIPA is detergent soluble fraction)

Further we measured oligomers from hippocampus and brainstem of 8.5 old male P301S mice using HJ 8.7/8.7b assay. As seen with cortex, majority of tau oligomers in the hippocampus and brainstem are collected in the soluble RAB fraction.



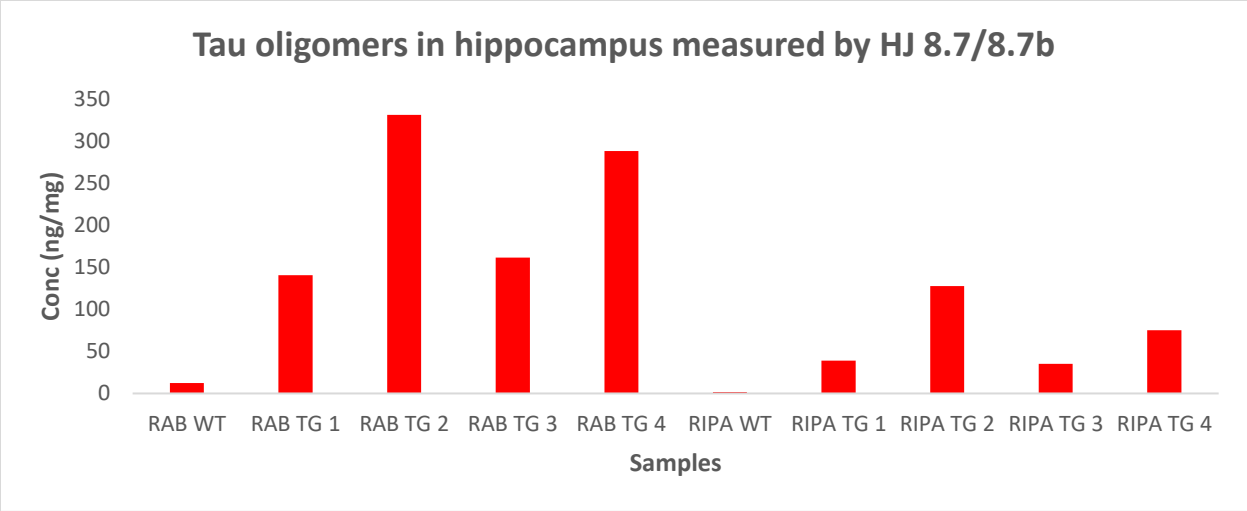


Figure 2.5. Tau oligomers in brainstem and hippocampus of aged P301S mice.

2.3.4 Towards a high-sensitivity assay: optimization on the Simoa analyzer

If tau oligomers are present in ISF, their concentration is likely very low. Thus we need to have reliable assays with high sensitivity if we aim to measure their concentration. Our currently oligomer-specific ELISAs (HJ 8.7/8.7b and HJ 8.2/8.2b) have a lower limit of detection of ~1 ng/ml. A 20-80x increase in sensitivity of these assays would enable us to detect putative oligomers with much better sensitivity. One such high sensitivity platform is the Simoa analyzer, developed by Quanterix Corp. First described in 2010 (Rissin et al. 2010b), the Simoa uses femtoliter microwells just large enough to trap a single bead coated capture antibody. Antigen concentration is titrated enough to get into a range where Poisson statistics dictate that each positive well is a single-molecule detection event (Figure 2.6). Recently this platform was used to calculate tau concentration in the plasma of Swedish hockey players before and after

concussion. The limit of detection was ~20 fg/ml, an astonishing 1000x increase in sensitivity for monomeric tau.

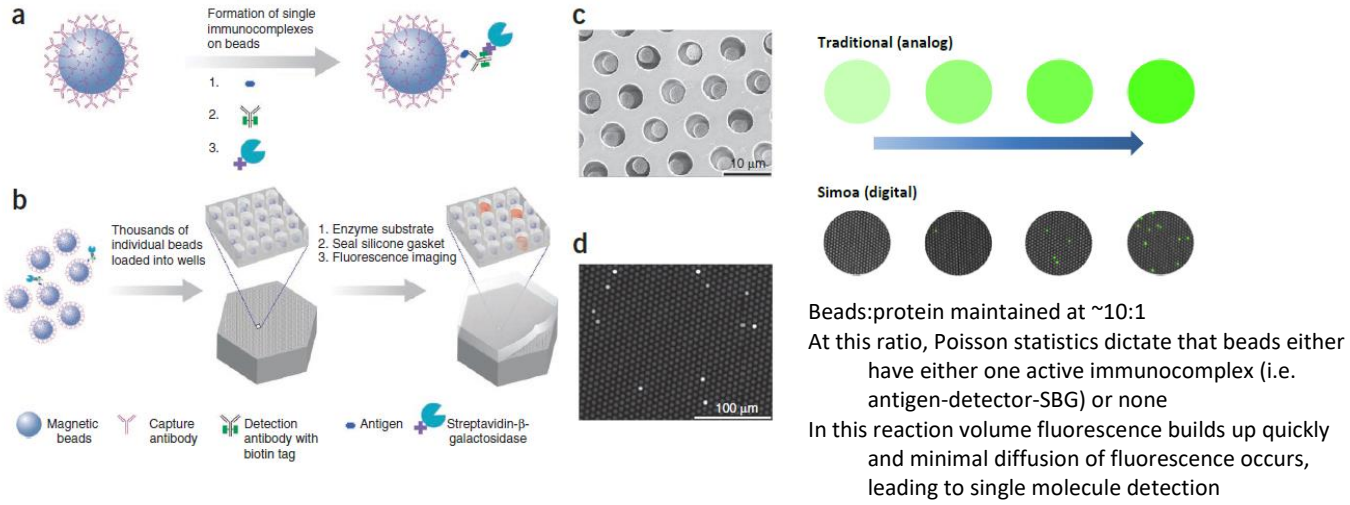


Figure 2.6. Working principle of the Simoa

(adapted from ((Rissin et al. 2010b);Simoa white paper)

To see if we can translate our oligomer-specific ELISA to this platform, we worked with Quanterix scientists for a week to optimize our assay on the Simoa. We were able to achieve ~100x increase in sensitivity in our HJ 8.7/8.7b ELISA after preliminary work. Based on a standard curve generated using FL WT tau fibrils (just as in our ELISA), our LOD was ~30 pg/ml. This is shown in Figure 2.7.

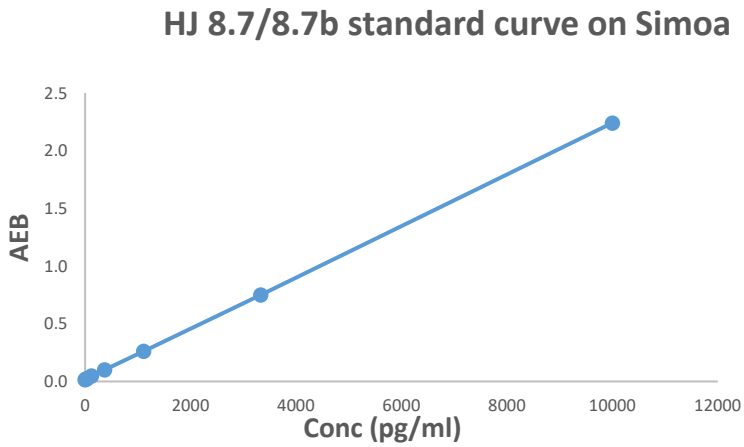


Figure 2.7. Standard curve demonstrating optimization of the HJ 8.7/8.7b ELISA on the Simoa.

Using the same antibody as the plate-based ELISA we created a new oligomer assay for the Simoa platform with a LOD ~30 pg/ml, about 100x more sensitive than our conventional assay. (AEB=avg enzyme per bead, analogous to OD value in an ELISA).

Conc (pg/ml)	Mean AEB	SD AEB	%CV AEB
0	0.0153	0.0004	2.69
13.7	0.0198	0.0008	3.84
41.2	0.0259	0.0008	2.97
123.5	0.0478	0.0019	3.87
370.4	0.1015	0.0008	0.76
1111.1	0.2608	0.0133	5.1
3333.3	0.7492	0.0783	10.4
10000	2.2409	0.2324	10.3

After the successful generation of a standard curve, we measured tau oligomers in cortex from 9 month old male P301S mice using the Simoa HD analyzer (Figure 2.8). Although the trend of higher concentration in RAB fraction still holds, the values are ~10x higher than what was measured by the conventional ELISA. Further optimization is needed in this case.

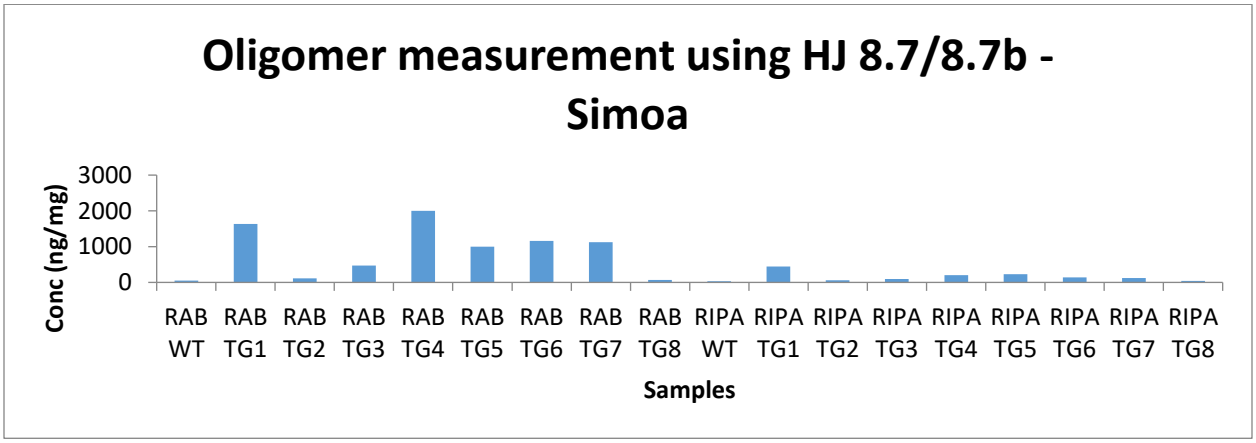


Figure 2.8. Tau oligomers from aged P301S brain lysates as measured by the Simoa

2.3.5 Attempts to measure tau oligomers *in vivo* by microdialysis

After the initial success of translating the HJ 8.7/8.7b assay to the Simoa, we attempted to measure oligomers in ISF by *in vivo* microdialysis. For this purpose we performed *in vivo* microdialysis with a 1 megadalton probe on both young (3 mo) and old (9 mo) mice. ISF was collected over 48 hours and divided into two pools for analysis. Each fraction represents 90

minutes of collection (~45 ul of ISF at the slower flow rate we used). Samples were loaded at three different dilutions. Table 2.4 shows the results from this experiment, which strongly suggests a slight increase in oligomer specific signal in older mice compared to the younger mice.

Standards	**Avg AEB**
0	0.070455
10	0.082665
30	0.100395
100	0.159579
300	0.310008
1000	0.704024
3000	2.770925
10000	7.415564

3 mo P301S males			9 mo P301S males		
Mouse	Dilution	Avg AEB	Mouse	Dilution	Avg AEB
41-1	5x	0.081	152-1	5x	0.123
	10x	0.087		10x	0.104
	20x	0.090		20x	0.099
41-2	5x	0.084	152-2	5x	0.087
	10x	0.086		10x	0.094
	20x	0.092		20x	0.124
46-1	5x	0.085	153-1	5x	0.102
	10x	0.089		10x	0.096
	20x	0.095		20x	0.106
46-2	5x	0.083	153-2	5x	0.102
	10x	0.091		10x	0.097
	20x	0.092		20x	0.104

Table 2.4. Slight increase in oligomer specific signal in aged P301s animals (particular animal #152)

In order to establish that we were indeed detecting faint signal from tau oligomers from ISF we decided to treat P301S animals with the seizure inducing cholinergic pilocarpine. Previous work

from the lab showed that causing widespread seizures and cell death with pilocarpine leads to a huge increase in tau in ISF (see Figure 2.9). Based on this we expected that injecting older P301S mice with pilocarpine would cause a corresponding increase in signal as measured by the HJ 8.7 oligomer assay.

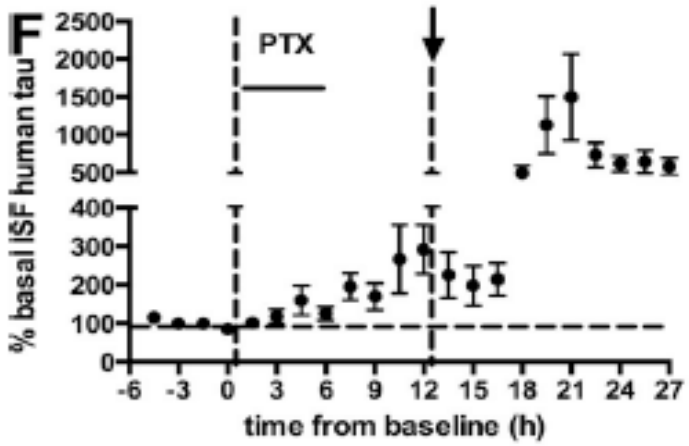


Figure 2.9. 10-15 fold increase in tau concentration in ISF following treatment with the cholinergic pilocarpine (arrow)

(adapted from (Yamada, Holth, Liao, Stewart, Mahan, Jiang, Cirrito, Patel, Hochgrafe, et al. 2014))

Baseline ISF was collected from 7 mo P301S females. Pilocarpine was given at 22 hours to induce seizures and cell death. Seizures were terminated 10 minutes post-injection and ISF was collected subsequently for 24 hours. To obtain some temporal resolution ISF fractions were

divided into three separate pools (baseline, post-pilocarpine 1, post-pilocarpine 2). Table 2.5 shows results from this experiment. Although the signal was above background there was no noticeable increase in oligomeric tau post-seizure induction in any of the mice.

Standards	Avg AEB
0	0.053317
25	0.06307
50	0.066886
100	0.073667
200	0.092079
400	0.128666
800	0.191301
1600	0.338736

7mo P301S females

Mouse	Avg AEB
72-1	0.059113
72-2	0.060292
72-3	0.061105
74-1	0.06694
74-2	0.068982
74-3	0.072391
83-1	0.075192
83-2	0.073025
83-3	0.065133
87-1	0.074156
87-2	0.071086
87-3	0.071035

-Pool 1: pre-pilocarpine (1-15)
-Pool 2: post-pilocarpine 1 (16-24)
-Pool 3: post-piocarpine 2 (25-33)

Table 2.5. No increase in oligomeric tau post pilocarpine treatment in older P301S animals

The levels of tau in ISF rise significantly in the first few fractions after probe placement due to injury. Thinking that this would result in an increase in tau oligomers as well, we measured the first few fractions using the Simoa. Results are depicted in Table 2.6 and they show a modest increase in two animals.

Mouse	Mean AEB	SD	Concentration (pg/ml)	Calculated conc (pg/ml)
1	0.0461	0.00072	12.6108	63.0541
2	0.0525	0.00283	32.0126	160.0628
3	0.0503	0.00815	25.5013	127.5065
4	0.0464	0.00276	13.3527	66.7634

Table 2.6. Slight increase in oligomers immediately following probe placement.

This increase likely represents release of tau oligomers into the interstitial space following injury caused by placement of microdialysis probe.

In order to show the utility of the assay we measured tau oligomers from media of two cell lines: one expressing tau aggregates and one not. As shown in Table 2.7 below, amount of total tau was similar between the two cell lines. However there is a big increase in the amount of tau oligomers in the cell media of the cell line that expresses tau aggregates.

Culture media	Mean AEB	SD	Oligomer concentration (pg/ml)	Calculated oligomer concentration (pg/ml)	Total tau (ng/ml)
20	0.0448	0.00215	<8*	<40	13.89
20.11	0.0582	0.00756	48.91	244.55	16.19

Table 2.7. Successful detection of tau oligomers in cell media from cell lines.

Media was collected from two HEK293T cell lines, one expression RD tau aggregates, and one without. The Simoa platform is able to detect and quantify tau oligomers.

2.3.6 Investigating the potential of a larger 3 megadalton probe in collecting tau oligomers

All our microdialysis experiments thus far have used a 1 megadalton probe to collect tau. To increase our probability of collecting higher order tau species we decided to test a 3 megadalton microdialysis probe. *In vitro* microdialysis was performed as before. 1 ug/ml of monomer tau was prepared and the probe was inserted into this solution. Fractions were collected in different perfusion buffers (4% or 2% or 0.15% human albumin) at different flow rates (ranging from 0.5 ul/min – 2 ul/min).

We noticed big changes in volumes of the stock solution from experiment to experiment, perhaps indicating that instead of microdialysis these bigger probes are ultrafiltrating. We also noticed at the lower flow rates the probe tends to get clogged. These inconsistencies make the 3 megadalton probe not a very viable option for microdialysis.

	Conc of tau recovered (ng/ml) [initial=1 ug/ml]						
Flow rate (ul/min)	Exp 1(4% albumin)	Exp 2(4% albumin)	Exp 3(2% albumin)	Exp 4(2% albumin)	Exp 5(2% albumin)	Exp 6(0.15% albumin)	Exp 7(0.15% albumin)
0.5	1.02	0.572	21.2	n/a	n/a	No recovery	
0.8	0.855	0.376	30.86	0.202	0.191		
1	0.684	0.327	42.3	0.189	0.132		
1.5	0.645	0.386	48.6	0.219	0.193		
2	0.413	0.373	43.11	0.203	0.233		

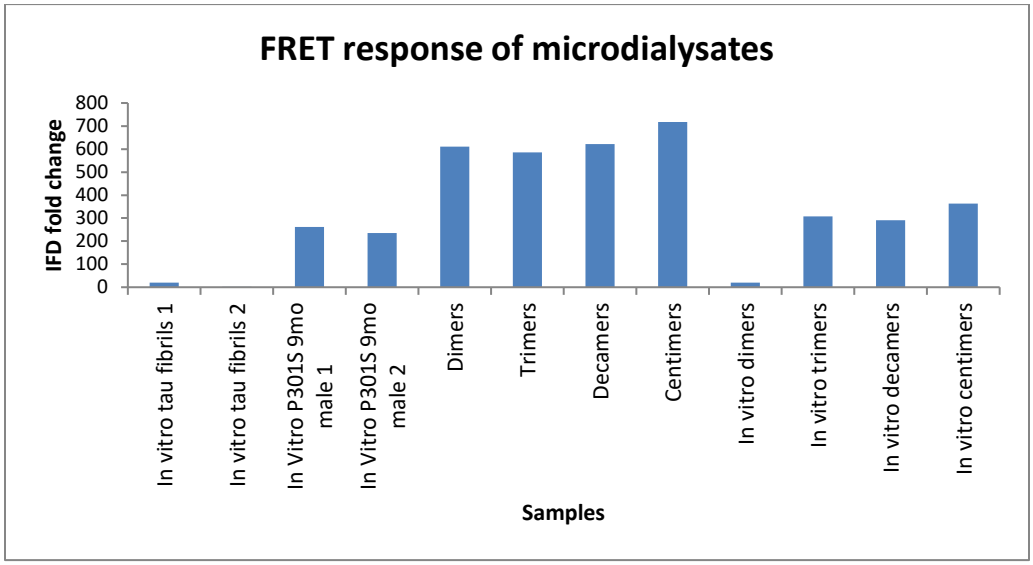
Table 2.8.. Initial in vivo microdialysis experiments using a larger 3 megadalton probe.

Benchtop microdialysis was performed to assess zero-flow rate recovery of known amount of tau, with varying concentrations of albumin. The probe was not successful in recovering tau oligomers.

2.3.7 Assessment of seeding activity of various microdialysates

In addition to the ELISA we were looking for other methods to validate our collection of oligomers by microdialysis. The FRET-based seeding assay using HEK 293T biosensor cells as developed by the lab of Marc Diamond (Holmes et al. 2014) can detect presence of tau ‘seeds’ in a given sample with high sensitivity. We tested seeding response to various microdialysates and compared that to a FRET ‘standard curve’ generated using serially diluted sonicated full-length fibrils. Results are shown in Figure 2.10. We were not able to detect seeding from WT fibrils perhaps because they were not sonicated before adding to the biosensor line. However

microdialysates from brain lysates and purified tau oligomers all show very robust seeding response.



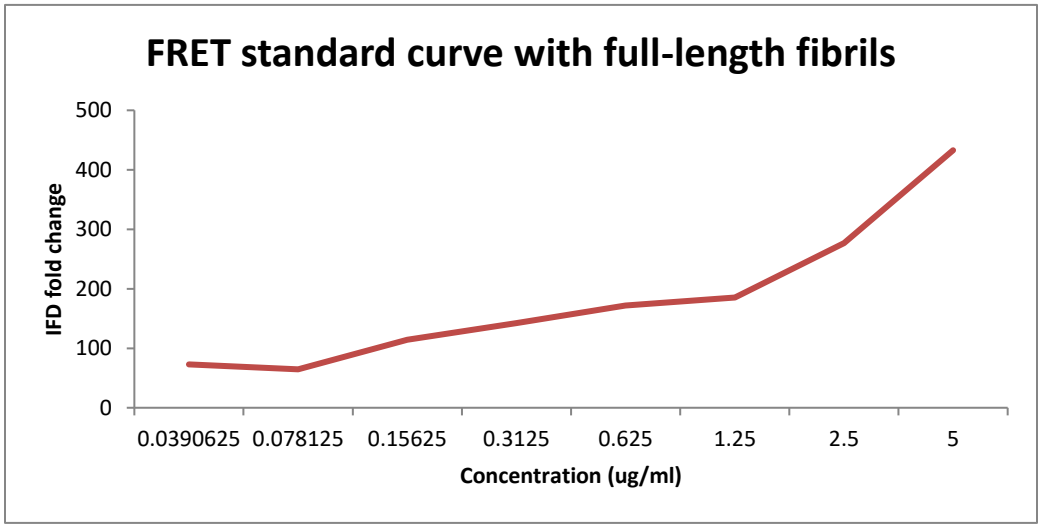


Figure 2.10 Seeding response of various microdialysates as measured by the FRET-based tau seeding assay.

Synthetic purified tau oligomers (i.e. dimers, trimers and higher-order species) gave a much more robust FRET signal on the seeding assay than larger fibrils.

2.4 Discussion

Here we have systematically described the development and optimization of multiple tau oligomer specific ELISAs: one specific for truncated RD tau oligomers and two for full-length tau oligomers. Since microdialysis is a versatile technique allowing us to sample a broad range of analytes from awake, freely moving animals, we naturally asked whether it could be adapted for collection of putative tau oligomers. Before moving on to perform *in vivo* microdialysis in animals we performed a series of benchtop *in vitro* experiments to demonstrate the viability of the microdialysis in collecting tau oligomers. Our results show that we are able to recover a small but measurable amount of oligomers from various input solutions. In addition we are able to recover purified oligomers and oligomers from cell culture media. We confirmed the presence of oligomers in the dialysate by performing the FRET-based tau seeding assay.

Separate from the optimization of microdialysis protocol, we used the oligomer-specific assay to measure tau oligomers from brain lysates from different regions. We observed that a majority of tau oligomers are present in the salt soluble fraction.

Since putative tau oligomers are likely present in very small amounts *in vivo* in the interstitial fluid we invested in the Simoa HD1 Analyzer, a single-molecule detection system. We successfully translated our HJ 8.7/8.7b oligomer-specific assay to this new platform and noticed a dramatic 80x increase in sensitivity.

Our initial attempts at measuring tau oligomers in ISF showed a slight increase in signal in one animal. We are actively working on other methods of detecting tau oligomers. Recent development of cerebral open flow microperfusion (cOFM) has opened the possibility of using it to detect large tau species in ISF. Unlike microdialysis, which is inherently restricted by the cutoff size of the membrane, OFM uses a micromesh that is open to the extracellular milieu where the probe is implanted, and thus allows unrestricted access to molecules of all sizes(Birngruber et al. 2013, Birngruber and Sinner 2016, Ghosh et al. 2014). In theory this should allow for collection of oligomers.

In summary we have developed robust and sensitive tools that should enable us to characterize and quantify tau oligomers from biological samples which should hopefully yield further insight into the role of extracellular tau and its various species in pathogenesis of tauopathies.

2.5 Methods

Preparation of fibrils

FL tau fibrils were prepared according to the protocol outlined in detail in previous work[1,2].

10 µM FL tau was prepared and used in experiments. To induce fibrillization of tau monomer,

10 uM FL tau was incubated with 2mM for 45 min at room temperature. After that a solution of 10mM HEPES, 100mM NaCl, 8uM Heparin was added. This was incubated at 37^0 C for a week.

In vitro microdialysis

In vitro microdialysis was carried out (with some modifications) using the protocols outlined[1,3].

In brief: Solution of FL tau fibrils (1 µg/ml) in 0.15% human albumin/PBS was used. For brain lysates and purified oligomers, initial concentration was measured by HJ 8.7/8.7b ELISA before and after microdialysis. A microdialysis probe with 3 megadalton pore size (MAB) was then inserted in the different preps. Microdialysis samples were collected at various flow rates (0.5 – 2 ul/min). Fractions were collected and kept at 4^0 C but the experiment itself was conducted at room temperature. After fraction collection, samples were loaded onto a half-well ELISA plate (Costar) for the tau oligomer ELISA

HJ 8.7/8.7b ELISA (FL tau oligomers)

Epitope: N-terminal region (117-122 aa)

Capture antibody: HJ 8.7 at 20 ug/ml in coating buffer

Detection antibody: HJ 8.7b at 0.1 ug/ml in 1% BSA/PBS

Blocking: 1 hour in 4% BSA/PBS at 37^0 C

Standard curve (generated using FL fibrils): 200 ng/ml to 1.15625 ng/ml

Simoa procotols

HJ 8.7=0.5 ug/ml

HJ 8.7b=0.8 ug/ml conjugated to 60x biotin

SBG=250pM

Standards=10 pg/ml – 10 ng/ml

Standard ELISA buffer (as described in the ELISA protocol references) was used as sample diluent.

Pilocarpine treatment conditions

20 minutes prior to seizure induction, mice were given scopolamine (1 mg/kg) i.p to block peripheral effects of pilocarpine. Mice were given 250 mg/kg pilocarpine i.p to cause seizures and cell death. Seizures were terminated 10 minutes later with pentobarbital given i.p.

Seeding assay

Seeding was performed following protocols and methods published in (Holmes et al. 2014)

Purified tau oligomers

These were obtained from the laboratory of Marc Diamond.

Sonication and size exclusion chromatography (SEC)- Labeled fibrils prepared in 3 separate batches were sonicated using a Q700 Sonicator (QSonica) at a power of 100-110 watt (Amplitude 65), each for different periods of time (60, 90, and 120 min). Samples were then centrifuged at 10,000 x g for 10 min and 1 mL of supernatant was loaded into a HiPrep 16/60 Sephacryl S-500 HR column (GE Healthcare) and eluted in PBS buffer at 4°C. After measuring the protein content of each fraction with a Micro BCA assay (Thermo Scientific) and fluorescence using a plate reader (Tecan M1000), they were aliquoted and stored at -80°C until use. Each aliquot was thawed immediately before use. The molecular weight of proteins in each fraction was initially determined by running gel filtration standards (Bio-Rad) followed by FCS analysis.

Chapter 3 Dural lymphatics regulate clearance of extracellular tau from the CNS

This chapter is adapted from a published journal article. The full citation is:

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3.1 Abstract

Background

Alzheimer's disease is characterized by two main neuropathological hallmarks: extracellular plaques of amyloid-β (Aβ) protein and intracellular aggregates of tau protein. Although tau is normally a soluble monomer that bind microtubules, in disease it forms insoluble, hyperphosphorylated aggregates in the cell body. Aside from its role in AD, tau is also involved in several other neurodegenerative disorders collectively called tauopathies, such as progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), some forms of frontotemporal dementia, and argyrophilic grain disease (AGD). The prion hypothesis suggests that after an initial trigger event, misfolded forms of tau are released into the extracellular space, where they spread through different brain regions, enter cells, and seeding previously normal forms. Thus understanding mechanisms regulating the clearance of extracellular tau from the CNS is important. The discovery of a true lymphatic system in the dura and its potential role in mediating Aβ pathology prompted us to investigate its role in regulating extracellular tau clearance.

Methods

To study clearance of extracellular tau from the brain, we conjugated monomeric human tau with a near-infrared dye cypate, and injected this labeled tau in the parenchyma of both wild-type and K14-VEGFR3-Ig transgenic mice, which lack a functional CNS lymphatic system. Following

injection we performed longitudinal imaging using fluorescence molecular tomography (FMT) and quantified fluorescence to calculate clearance of tau from the brain. To complement this, we also measured tau clearance to the periphery by measuring plasma tau in both groups of mice.

Results

Our results show that a significantly higher amount of tau is retained in the brains of K14-VEGFR3-Ig vs. wild type mice at 48 and 72 hrs post-injection and its subsequent clearance to the periphery is delayed. We found that clearance of reference tracer human serum albumin (HSA) was also significantly delayed in the K14-VEGFR3-Ig mice.

Conclusions

The dural lymphatic system appears to play an important role in clearance of extracellular tau, since tau clearance is impaired in the absence of functional lymphatics. Based on our baseline characterization of extracellular tau clearance, future studies are warranted to look at the interaction between tau pathology and efficiency of lymphatic function.

3.2 Background

Alzheimer's disease (AD) is the leading cause of dementia in the elderly and currently affects more than five million people in the United States. The two main neuropathological hallmarks of AD are extracellular plaques of amyloid-β (Aβ) and intracellular accumulations of aggregated, hyperphosphorylated forms of tau in structures such as neurofibrillary tangles (NFT) (Holtzman, Mandelkow, and Selkoe 2012). The amyloid cascade hypothesis holds that the triggering event in AD pathogenesis is the initial accumulation and aggregation of Aβ into oligomers and insoluble extracellular plaques (Musiek and Holtzman 2015). This initiates a cascade that incites the misfolding and aggregation of soluble tau into insoluble forms, eventually leading to neurodegeneration. Loss of cognitive function in AD and other tauopathies is correlated with the amount of aggregated tau accumulation. In addition to its key role in AD pathology, tau has also been implicated in a host of other neurodegenerative disorders such as progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), certain forms of frontotemporal dementia (FTD) and argyrophilic grain disease (AGD). Collectively termed tauopathies, these disorders all feature aggregated forms of tau in the CNS (Mandelkow and Mandelkow 2012a, Li and Götz 2017).

One model explaining part of the pathogenesis of tauopathies is the prion hypothesis, which states that misfolded forms of tau can exit the cell, spread to distant regions of the brain where they can re-enter cells and “seed” previously normal forms of the protein, much like prion

protein (Jucker and Walker 2013, Davis, Leyns, and Holtzman 2018) Expression of mutant human tau in neurons in the entorhinal cortex shows spread of tau pathology to synaptically connected regions in the dentate gyrus of the hippocampus in mice (de Calignon et al. 2012, Liu et al. 2012). In addition to seeding and uptake in cell culture (Holmes et al. 2014, Kfoury et al. 2012, Frost, Jacks, and Diamond 2009, Wu et al. 2013), injection of brain lysates from transgenic mice (Ahmed et al. 2014, Clavaguera et al. 2014), cell lysates (Sanders et al. 2014), synthetic recombinant tau fibrils (Iba et al. 2013, Peeraer et al. 2015) and tau extracts purified from human brains (Clavaguera et al. 2013, Boluda et al. 2014, He et al. 2018, Lasagna-Reeves, Castillo-Carranza, Sengupta, Guerrero-Munoz, et al. 2012, Guo et al. 2016)into mouse models have also been shown to robustly induce uptake, seeding and spreading of tau pathology. If this model of tau propagation is correct, it is likely that extracellular tau plays a key role in mediating pathogenesis and progression of tauopathy. As a result understanding the mechanisms that regulate tau fate in the extracellular space of the CNS and its eventual clearance to the periphery is important.

Regulated clearance of substances out of the CNS to the periphery is vital to healthy functioning of the CNS, and as such is an important and active area of research. For all the attention focused on the brain in health and disease, little was known about fundamental clearance mechanisms until relatively recently. For decades, it was thought the brain enjoyed a ‘true’ immune privileged status because of an apparent lack of lymphatic drainage from the CNS. Early tracer injection studies showed that peptides and solutes injected in the CSF and parenchyma eventually make

their way to deep cervical lymph nodes (dCLNs) by traveling along olfactory sinuses and the cribriform plates (Cserr, Harling-Berg, and Knopf 1992, Rennels et al. 1985).

More recent work, also largely done with tracers, refined this model further by proposing that solutes are cleared across paravascular routes and are aided in this process by astrocytes. The role of astrocytes in mediating this clearance led to this model being called the 'glymphatic' (a portmanteau of glia and lymphatic) system (Xie et al. 2013). The water channel aquaporin 4 (AQP4), predominantly localized to astrocytic end-feet, was shown to be key to glymphatic clearance, as its deletion impaired clearance of solutes to dCLNs (Iliff, Lee, et al. 2013, Iliff, Wang, et al. 2013, Plog et al. 2015). This model proposes that the interstitial fluid (ISF) compartment of the brain exchanges metabolites and macromolecules with the CSF compartment. This is driven by arterial pulsation (Iliff, Wang, et al. 2013), which causes solutes to exit the brain by following paravascular pathways aided by AQP4.

The characterization of this system has implications for aging and disease. Prolonged exposure to Aβ aggregates has been shown to impair glymphatic function in mouse models (Peng et al. 2016). Deleting AQP4 leads to reduced transport of biomarkers of neuronal injury in a mouse model of TBI (Plog et al. 2015) and exacerbates existing tau pathology, presumably because tau aggregates are not cleared out of the brain properly (Iliff et al. 2014).

More recent work has upended accepted dogma by conclusively showing that the brain does indeed have a 'true' lymphatic system responsible for draining macromolecules and cells from the deep parenchyma. These studies demonstrated and rigorously characterized a network of

lymphatic vessels localized to the dura of the meninges using state of the art microscopy and high resolution fluorescent imaging (Louveau et al. 2015, Aspelund et al. 2015). These dural lymphatics were shown to track along superior sagittal and transverse sinuses, ultimately draining into the deep cervical lymph nodes. Ablating dural lymphatics with either genetic manipulation or surgery resulted in significantly slower clearance of injected macromolecules in the deep parenchyma, perhaps indicating that the glymphatic and lymphatic system exist in parallel and might even be linked (Aspelund et al. 2015, Louveau et al. 2017). Furthermore, it was shown that impaired lymphatic drainage can exacerbate amyloid pathology, particularly in the meninges (Da Mesquita et al. 2018).

Although it is known that extracellular clearance of Aβ is regulated by the transporters at the BBB such as RAGE, LRP1, LDLR and P-GP (Deane et al. 2003, Castellano et al. 2012a, Shibata et al. 2000, Elali and Rivest 2013, Tarasoff-Conway et al. 2015), by cellular enzymes such as neprilysin (Iwata et al. 2001) and insulin-degrading enzyme (Qiu et al. 1998), and by glymphatic flow (Iliff et al. 2012, Kress et al. 2014), data surrounding what regulates extracellular clearance of tau is limited. Extracellular tau in various CNS compartments can be detected in the periphery (Iliff et al. 2014, Yanamandra et al. 2017, Shahim et al. 2014), but it is unclear what regulates this process. A recent study showed that various isoforms of tau are readily detected in plasma following injection in the ventricles (Banks et al. 2016) but the mechanisms mediating this process are unknown. There is also some evidence that the glymphatic system is involved (Iliff et al. 2014); however, the role of the dural lymphatic system has not yet been studied. This lack of

information and proper understanding of tau clearance prompted us to investigate the role of dural lymphatic system in clearance of extracellular tau.

3.3 Results

3.3.1 Extracellular tau clearance is impaired and significantly more tau is retained in the brain of K14-VEGFR3-Ig mice

We utilized the K14-VEGFR3-Ig transgenic mice for our studies. These mice lack a functional dural lymphatic system (Aspelund et al. 2015)and thus provide a model to study the contribution of this system to clearance of substances from the brain. We first confirmed that these mice lack dural lymphatics by immunohistochemistry for LYVE1, a lymph endothelial cell marker (Figure 3.1). WT mice show clear distribution of lymphatic vessels along superior sagittal and transverse sinuses, while K14-VEGFR3-Ig mice have no LYVE1 staining, indicating a complete lack of lymphatic vessels.

To assess clearance of extracellular tau from the CNS over time, we performed serial FMT – an optical imaging modality that allows for in vivo imaging of fluorescent probes through layers of skin, bone and tissue – of mice injected with recombinant monomeric human tau labeled with cypate, a near-infrared dye that can be easily conjugated to a number of different tracers, probes and molecules for optical imaging of tumors and other physiological processes (Zhang et al. 2012, Achilefu et al. 2000, Ye et al. 2003, Ye et al. 2005). In this experiment, 4-6mo old WT and K14-VEGFR3-Ig mice were imaged at 1, 2, 24, 48, 72 and 168 h post-injection of tau (Figure

3.2a). Fluorescence was quantified in the brain to calculate amount of tau retained at each time point. Signal for each mouse at each time point was normalized to the 1 h time point. As we imaged mice over several days, we observed a clear result: tau clearance is delayed from the brain in K14-VEGFR3-Ig mice. Significantly more tau is retained at 48 h (85% vs 52%) and 72 h (52% vs 22%) post-injection in the K14-VEGFR3-Ig mice, strongly suggesting that in the absence of dural lymphatics, extracellular tau is not as effectively cleared from the CNS to the periphery (Figure 3.2b). This is reflected in the half-life of tau in both groups (Figure 3.2d): tau has a shorter half-life of 39.67 h in WT mice compared to 154 h in the K14-VEGFR3-Ig mice.

3.3.2 Clearance of HSA from CNS is also impaired in K14-VEGFR3-Ig mice

To get a more clear understanding of tau clearance by lymphatics we repeated our experiment by injecting HSA-cypate to serve as a reference tracer. Since it is known that albumin is cleared by lymphatic vessels out of the CNS (Louveau et al. 2015, Aspelund et al. 2015), we hypothesized delayed clearance of HSA in K14-VEGFR3-Ig mice. Longitudinal FMT of 4–6 mo WT and K14-VEGFR3-Ig mice injected with HSA-cypate reveals that significantly more HSA is retained in the brain of the transgenic mice at 24 h (57% vs 29%) and 48 h (36% vs 15%) compared to WT mice (Figure 3.2c), suggesting that similar to tau, clearance of HSA is delayed in mice lacking a functional dural lymphatic system. Additionally we found that half-life of HSA was longer in K14-VEGFR3-Ig mice (26 h) compared to WT mice (~ 13 h). This is in agreement with published reports of rat albumin's half-life in rat brain (Cserr et al. 1981, Yamada et al. 1991)being in the range of 11–18 h, thus further validating this method to measuring CNS

clearance of proteins. The fact that HSA is cleared faster than tau could perhaps indicate uptake and/or additional extracellular retention of tau within CNS.

3.3.3 Measurement of plasma tau reveals a trend toward delayed clearance in K14-VEGFR3-Ig mice

As we showed there is delayed clearance of extracellular tau from the brain, one would predict that the amount of tau appearing in the blood would also be delayed. In order to study this directly, we measured plasma tau in both groups of mice to complement FMT experiments looking at brain retention of tau to get a more complete picture of tau clearance. We used an antibody-mediated approach (Yanamandra et al. 2017) to increase half-life of soluble tau in the plasma to allow us to measure it reliably. This was done because the half-life of tau once it reaches the plasma is only 8.49 min; however, in the presence of the anti-tau antibody HJ8.5, the half-life of plasma tau increases to 3.4 h allowing one to measure it there in a more facile fashion (Yanamandra et al. 2017). Following intraperitoneal antibody administration of HJ 8.5 (an anti-human tau antibody), WT and K14-VEGFR3-Ig mice were injected in the hippocampus with 1.1 μg tau. Blood was collected at 2, 24, 48, 72 and 168 h post-injection and plasma human tau was measured by a high-sensitivity ELISA on a Simoa analyzer. The plasma tau assay uses capture antibody HJ8.7 (which binds to N-terminal epitopes 117–122 of tau) and biotinylated detection antibody BT2 (which binds to epitopes 194–198 of tau). We found that K14-VEGFR3-Ig mice, which retained a significantly higher amount of tau in the brain, correspondingly also show delayed appearance of tau in the periphery (Figure 3.3) compared to WT mice. The amount

of human tau in the plasma peaked earlier in the WT mice than in the K14-VEGFR3-Ig mice (24 h vs. 48 h). Significantly higher plasma tau was present in K14-VEGFR3-Ig mice at 48 h, when the WT mice had already cleared most of the tau from the periphery. Together with the fluorescence data from the brain, these plasma tau measurements strongly suggest that impaired dural lymphatic function leads to increased retention in the brain as well as delayed clearance of tau to the periphery.

3.4 Discussion

While intracellular forms of tau are critical for its normal function as well as its role in neurodegenerative diseases, extracellular forms of tau appear to have an important role in the process of tau aggregate spreading transynaptically in different tauopathies as well as possibly in a component of tau toxicity. Given this, it is important to understand what regulates the clearance of extracellular tau from the brain. One important recently identified pathway that is involved in extracellular protein clearance from the brain is the dural lymphatics. Because of the recent discovery of bona fide lymph vessels in the meninges, we particularly focused on their role in this process. By using the K14-VEGFR3-Ig mouse model, which lacks dural lymphatics and shows delayed clearance of CNS macromolecules and tracers (Aspelund et al. 2015), we demonstrated that extracellular tau clearance from the CNS is significantly impaired in the absence of a functioning dural lymphatic system. A significantly higher amount of tau is retained in the brain of the K14-VEGFR3-Ig mice and its eventual clearance and appearance in the periphery is also delayed.

As our work was done to establish ‘baseline’ clearance mechanism of monomeric tau, it opens several exciting future directions: does disruption of lymphatic vessels exacerbate tau pathology? What effect does age have on lymphatic clearance of tau? Is clearance of aggregated tau markedly different than monomeric tau? The mice we used in our work are otherwise healthy and do not show any neuropathological alterations (such as tau aggregation). As a result it would be interesting to determine whether the clearance of tau is altered in the presence of significant AD or other pathology as this will help us to understand if there is a link between CNS lymphatics and neurodegeneration.

Since the glymphatic system appears to play a key role in the ISF-CSF exchange of solutes, we can propose a model of extracellular tau clearance that traces the fate of ISF tau: following its release into the ISF compartment, tau is cleared into the CSF space by the glymphatic system. Once in the CSF it is drained to the cervical lymph nodes by the dural lymphatic system. Thus the glymphatic and lymphatic systems most likely work in tandem to accomplish the eventual clearance of tau to the periphery.

However one key observation from our FMT experiments in K14-VEGFR3-Ig mice is that despite complete lack of lymphatic vessels, extracellular tau is still able to be cleared out of the brain to the periphery, albeit at a much slower rate than in healthy WT mice. This implies the existence of alternate paths for tau clearance and/or uptake within the CNS. Coupled with the fact that passive clearance of HSA appears to be faster than passive clearance of tau (despite

their similar molecular weights) this leads to the hypothesis that the dural lymphatic system might show some specificity in transporting macromolecules out of the CNS. The potential existence of alternate clearance mechanisms needs to be investigated further. K14-VEGFR3-Ig mice lack a functional lymphatic system owing to lack of signaling through the VEGFR3 receptor. Mice show complete lack of lymph vasculature and profound lymphedema. They survive the neonatal period despite these defects and subsequently even show regeneration of lymph vasculature in the periphery. In adulthood they only lack the dural lymphatic system (Aspelund et al. 2015, Mäkinen et al. 2001). Though these mice do not have any defects in vascular permeability that are detectable, it is possible that abnormal development of peripheral lymphatics might also lead to disturbance in degradation and eventual clearance of plasma tau.

Finally, in the process of investigating the contribution of dural lymphatics to extracellular clearance we made extensive use of FMT, a technique that has previously been used mostly to study tumor evolution and other related cellular processes in vivo (Zhang et al. 2012). We have thus established a novel use for this versatile technique, which can hopefully be used for more extensive clearance studies in the future.

3.5 Conclusions

We demonstrate a novel use of FMT in studying clearance of extracellular tau from the CNS. Using a mouse model lacking functional lymphatics we performed longitudinal FMT to show that tau clearance is notably impaired in the absence of functional lymphatics and its eventual clearance to the periphery is significantly delayed, thus establishing a key role for the dural

lymphatic system in mediating extracellular tau clearance. Our study highlights a likely link between the glymphatic and lymphatic systems in mediating extracellular clearance and opens new avenues to investigate the interplay between neurodegeneration and the dural lymphatic system.

3.6 Methods

3.6.1 Animal surgeries and husbandry

Initial breeding pairs of K14-VEGFR3-Ig mice (Mäkinen et al. 2001) were obtained from Dr. Kari Alitalo at the University of Helsinki and the colony was further expanded and maintained at Washington University School of Medicine. The transgenic mice are crossed with C57/BL6 mice. WT and TG mice used in all the experiments were littermates.

WT and K14-VEGFR3-Ig mice were anesthetized with ketamine/xylazine cocktail before being placed in the stereotactic surgery frame. Intrahippocampal injection of recombinant monomeric human tau (labeled and un-labeled) and human serum albumin (HSA) was performed at the following site: bregma − 2.5 mm, lateral 2 mm from midline, 2 mm ventral to the dura. All procedures were performed following protocols approved by the Animal Studies Committee and Washington University School of Medicine.

3.6.2 Tau and HSA conjugation with cypate

Recombinant monomeric human tau (1N4R, tau-412, rPeptide) and HSA were conjugated with near-infrared dye cypate in the following manner: Cypate conjugation to proteins was carried out by NHS ester chemistry (Nanda and Lorsch 2014). Cypate-NHS was prepared by dissolving 31.3 mg (0.05 mmol) cypate in 200 µl DMSO. Next a solution of 5.8 mg (0.05 mmol) NHS, and 4.8 mg (0.025 mmol) EDC was prepared in another 200 µl DMSO solution. Finally the two solutions were mixed and incubated overnight at room temperature. For conjugation, 100 µg tau was reconstituted with 90 µl pH 7.4 1 X PBS buffer. Then 0.75 µl Cypate-NHS was diluted with DMSO into 10 µl, and then added to the 90 µl tau solution and allowed to incubate for 2 h at room temperature.

For HSA conjugation 1.0 mg HSA was dissolved in 200 µl pH 7.4 1 X PBS buffer. Next 1.5 µl, Cypate-NHS DMSO solution was diluted with DMSO into 20 µl, added to the 200 µl HSA PBS solution, and then incubated at room temperature for overnight. Reaction mixtures were dialyzed with 1 X PBS, pH 7.4 at 4 °C for overnight, and then lyophilized. Dye and protein concentrations were determined by protein assay (Bio-Rad) and UV-Vis absorption spectrum, to give dye to protein molar ratio.

3.6.3 In vivo FMT of tau-cypate and HSA-cypate drainage

In vivo FMT was performed on a Perkin Elmer FMT4000 imaging system. Following intra-hippocampal injection of cypate labeled tau, mice were anesthetized with 2% isoflurane for imaging. Both WT and K14-VEGFR3-Ig mice were imaged at 1, 2, 24, 48, 72 and 168 h post-

injection of tau. A 2 µl of solution (1.1 µg) of monomeric human tau or HSA was injected at a flow-rate of 0.2 µl/min. Fluorescence was quantified based on instrument calibration with cypate phantoms. Total fluorescence of tau and HSA fluorescent conjugates was measured in manually drawn ROIs approximating the brain and lymph nodes. To control for variability caused by injection, data for each mouse at each time point were normalized to the fluorescent signal at the 1 h time point.

3.6.4 Plasma tau measurement

For plasma measurement, 50 mg/kg of anti-human antibody HJ 8.5 was injected intraperitoneally as described in (Yanamandra et al. 2017). Mice were injected with 2 µl of solution (1.1 µg) of unlabeled, monomeric tau 1 h post-antibody administration. Blood was collected at 2, 24, 48, 72, 168 h post-tau injection. Samples were spun at 8000 g for 10 min to obtain plasma. The Simoa HD-1 analyzer (Quanterix Corp) was used to measure human tau in plasma as previously described (Yanamandra et al. 2017).

3.6.5 Meningeal extraction and immunohistochemistry

Mice were anesthetized by intraperitoneal injection of pentobarbital (200 mg/kg) for perfusion with ice-cold Dulbecco's PBS with Heparin. Following perfusion, the skull cap was carefully removed and fixed overnight in 4% paraformaldehyde. Intact meninges were then peeled off and stored as floating sections in PBS until immunohistochemistry. For LYVE1 staining, meninges were washed in PBS-Triton X-100 (0.5%) and blocked in PBS-T with 0.5% BSA at room temperature. Sections were incubated with LYVE1-e660 (ThermoFisher) at 1:200 overnight at

40 C. Sections were washed in PBS-T prior to mounting on slides in Prolong Gold antifade reagent with DAPI (ThermoFisher) mounting medium. Slides were imaged on Zeiss Axio Imager Z2 fluorescence microscope and Cytation 5 imaging reader (Biotek Imaging, Inc.). Images were processed on ZEN software suite (Carl Zeiss, Inc.) and Cytation 5 imaging reader.

3.6.6 Statistics

Bulk of data plotting and statistical analysis were done on GraphPad Prism 5. For tau-cypate (n = 6–9) and HSA-cypate (n = 4–6) injection and brain retention studies, a two-way ANOVA with a Bonferroni post-test by was used to analyze the two groups of mice at each time point (i.e. 2, 24, 48, 72 and 168 h). For plasma tau measurement a mixed effects linear model was used to compare plasma tau (pg/ml) for the two groups (WT n = 5, K14-VEGFR3-Ig n = 6) across five times (2, 24, 48, 72, and 168 h). Akaike's AIC was used to evaluate 15 ovariance structures to determine the best fit model for the two analyses. Least square estimates of the differences between groups (i.e., mouse types or drug treatment) at each time period were used to compare the trajectory of response over time. All tests were conducted at the alpha = 0.05 level of significance. All tests were run in PROC MIXED of SAS 9.4.

3.7 Figures

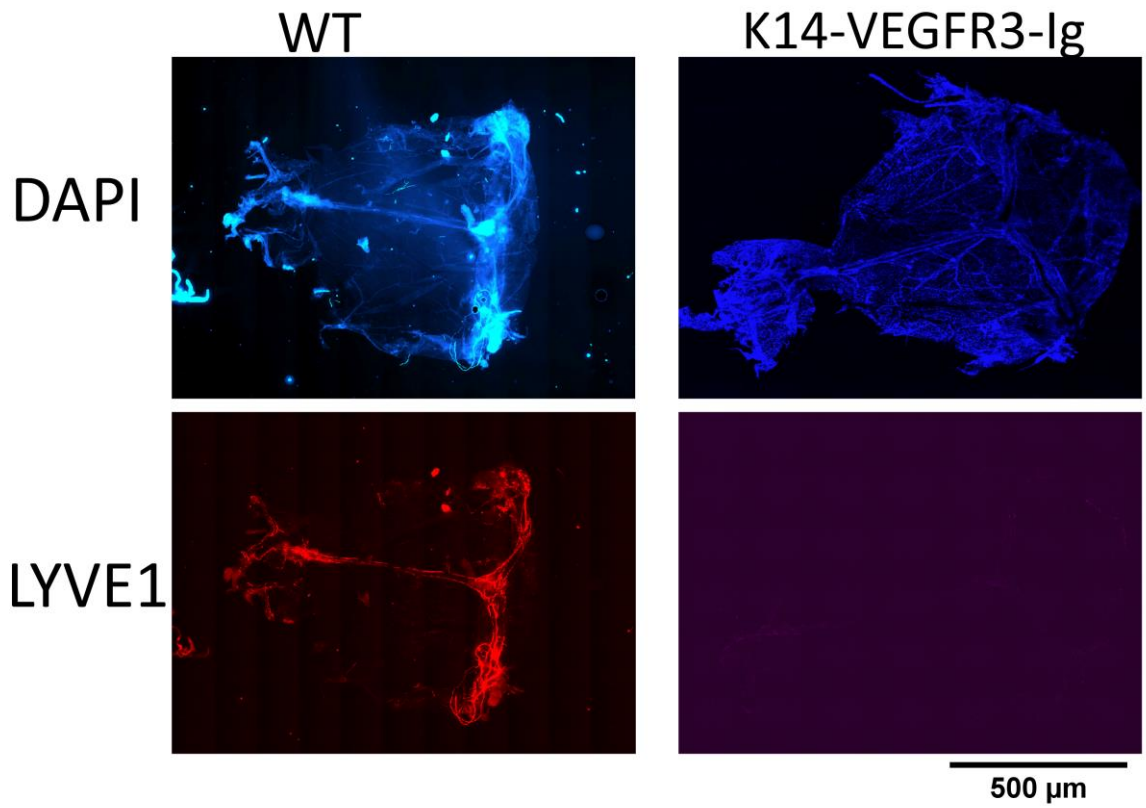
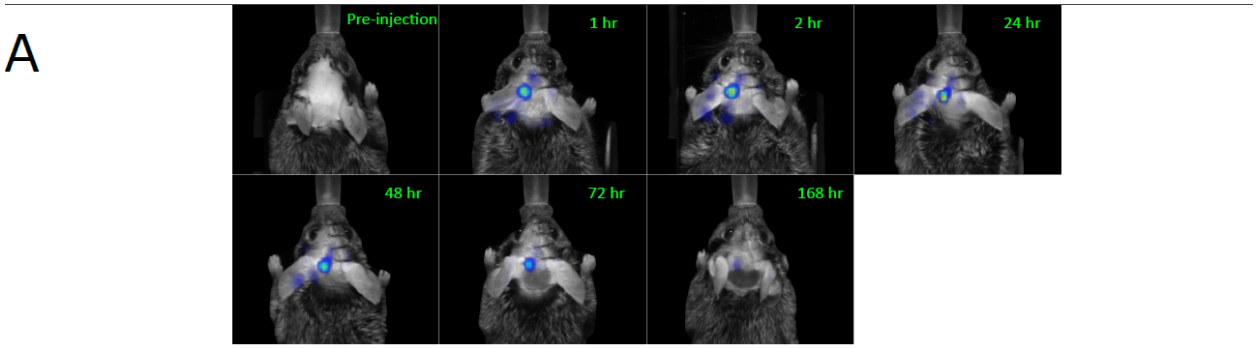
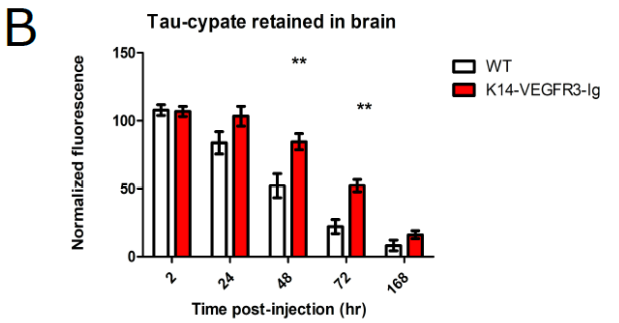
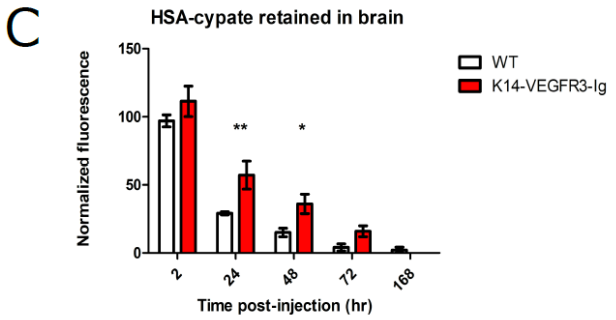


Figure 3.1. Histological characterization of the dural lymphatic system.

Intact meninges from both WT and K14-VEGFR3-Ig mice were stained for cellular marker DAPI (top) and the lymphatic endothelial marker LYVE1 (bottom). Representative sections are shown here. WT mice show the characteristic distribution of dural lymph vessels along the superior sagittal and transverse sinuses (as delineated by LYVE1 staining). K14-VEGFR3-Ig mice have no LYVE1 staining.







D

Half-life (hrs)		
	Genotype	
Protein	WT	K14-VEGFR3-Ig
Tau	39.67	154
HSA	12.63	26.01

Figure 3.2. K14-VEGFR3-Ig mice retain significantly more tau in the brain following intra-CNS injection.

A. Panel showing distribution and gradual clearance of tau-cypate (as measured by longitudinal FMT) in a representative WT mouse from pre-injection through 168 hrs post-injection. Monomeric recombinant human tau was conjugated with the near-infrared fluorescent dye cypate and stereotactically injected in the hippocampus of WT and K14-VEGFR3-Ig mice. Mice were longitudinally imaged with FMT at the time-points indicated. The same injection and imaging timeline was followed for experiments involving clearance of HSA-cypate in WT and K14-VEGFR3-Ig mice B. Significantly more tau is retained in the brain of K14-VEGFR3-Ig

mice, indicating delayed clearance. 4-6 mo old WT (n=9; 5 males, 4 females) and K14-VEGFR3-Ig mice (n=6; 3 males, 3 females) were injected with tau-cypate in the hippocampus. They were imaged with FMT at 1, 2, 24, 48, 72 and 168 hrs post injection. The amount of fluorescence in the brain was quantified and normalized to values for the 1 hr time point. A significantly higher amount of tau was retained in the K14-VEGFR3-Ig mice compared to WT mice at 48hrs (85% vs 52% retention) and 72hrs (52% vs 22% retention), following injection. Data was analyzed by a two-way ANOVA with a Bonferroni post-test. (** p-value <0.01). C. Significantly more HSA is retained in the brain of K14-VEGFR3-Ig mice, indicating delayed clearance. 4-6 mo old WT (n=4; 1 male, 3 females) and K14-VEGFR3-Ig mice (n=6; 4 males, 2 females) were injected with HSA-cypate in the hippocampus. They were imaged with FMT at 1, 2, 24, 48, 72 and 168 hrs post injection. The amount of fluorescence in the brain was quantified and normalized to values for the 1 hr time point. A significantly higher amount of HSA was retained in the K14-VEGFR3-Ig mice compared to WT mice at 24hrs (57% vs 29% retention) and 48hrs (36% vs 15% retention) following injection. Data was analyzed by a two-way ANOVA with a Bonferroni post-test. (** p-value <0.01, * p-value <0.05). D. Table summarizing half-life of tau and HSA in the CNS following their injection in both WT and K14-VEGFR3-Ig mice. The half-life of both proteins is longer in the K14-VEGFR3-Ig mice.

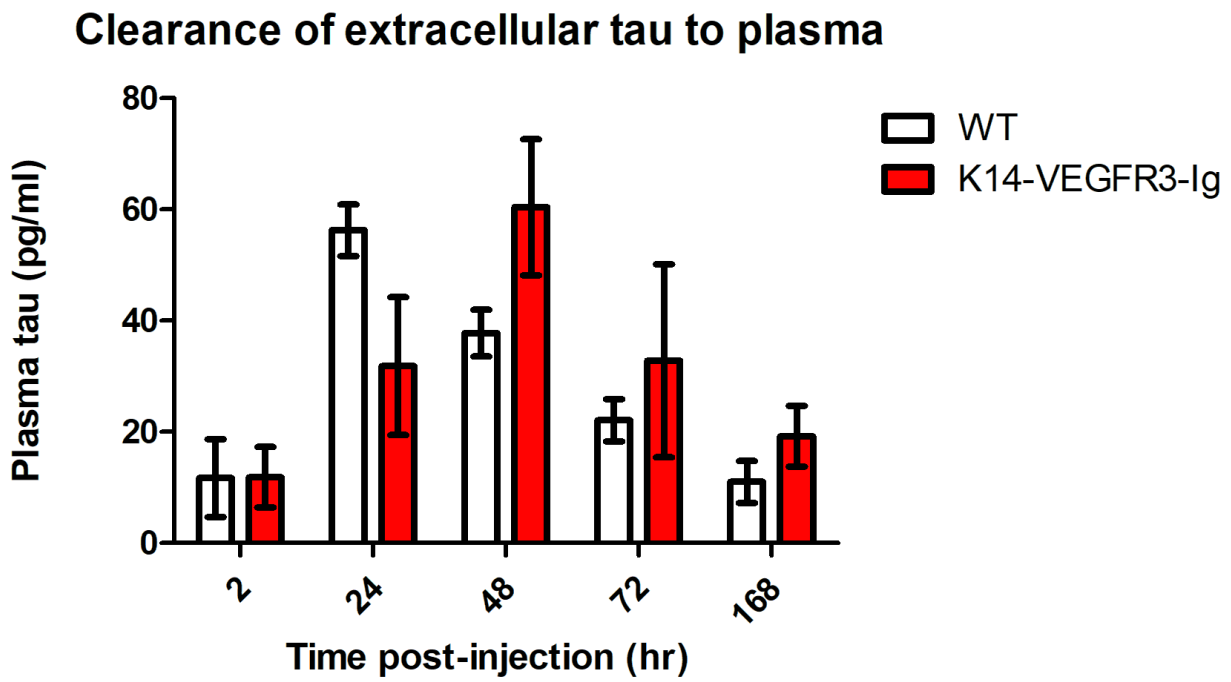


Figure 3.3. K14-VEGFR3-Ig mice show delayed clearance of extracellular tau to the plasma after intra CNS injection.

4-6mo old WT (n=6; 3 males, 3 females) and K14-VEGFR3-Ig mice (n=5; 3 males, 2 females) were injected with anti-tau antibody HJ 8.5 to stabilize tau entering the plasma from the CNS to allow for its measurement. An hour later recombinant monomeric human tau was injected in the hippocampus and blood was collected at time points indicated. Plasma tau was measured using the ultrasensitive Simoa HD1-Analyzer platform. Though the overall difference between tau for the two mouse groups was not significant ($p=0.6766$), the interaction between the two mouse groups over time was significant ($p=0.0191$). Plasma tau peaks earlier in WT mice compared to K14-VEGFR3-Ig mice (24 vs 48 hrs). Amount of plasma tau is significantly higher in K14-VEGFR3-Ig mice at 48 hrs compared to WT mice ($p=0.0260$), indicative of delayed clearance of tau due to impaired lymphatics. Data was analyzed by mixed effects linear model. Akaike's AIC was used to evaluate 15 covariance structures to determine the best fit model for this analysis. Least square estimates of the differences between the two mouse groups at each time period were used to compare the trajectory of response over time.

3.8 Acknowledgements

We would like to thank Jason D. Ulrich and Mary Beth Finn at Washington University School of Medicine for discussions and valuable technical input at various times in the project.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

3.9 Authors' contributions

TKP and DMH conceived and designed the study with critical input from LHG. TKP and DMH wrote, and ESM and SA assisted with editing the manuscript with input from all co-authors. TKP performed stereotactic surgeries, plasma collection, Simoa assays, immunohistochemistry, image analysis. LHG, XG, BX performed protein conjugation. TKP and LHG performed FMT experiments. LHG performed FMT image analysis. KA graciously provided K14-VEGFR3-Ig mice for the study and provided supporting data. CAM and TKP performed meningeal extraction. PWS and TKP performed imaging of meningeal sections. CX and DC performed additional statistical analysis. All authors read and approved the final manuscript.

Chapter 4 Anti-tau antibody administration increases plasma tau in transgenic mice and patients with tauopathy

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4.1 Abstract

Tauopathies are a group of disorders in which the cytosolic protein tau aggregates and accumulates in cells within the brain, resulting in neurodegeneration. A promising treatment being explored for tauopathies is passive immunization with anti-tau antibodies. We previously found that administration of an anti-tau antibody to human tau transgenic mice increased the concentration of plasma tau. We further explored the effects of administering an anti-tau antibody on plasma tau. After peripheral administration of an anti-tau antibody to human patients with tauopathy and to mice expressing human tau in the central nervous system, there was a dose-dependent increase in plasma tau. In mouse plasma, we found that tau had a short half-life of 8 min that increased to more than 3 hours after administration of anti-tau antibody. As tau transgenic mice accumulated insoluble tau in the brain, brain soluble and interstitial fluid tau decreased. Administration of anti-tau antibody to tau transgenic mice that had decreased brain soluble tau and interstitial fluid tau resulted in an increase in plasma tau, but this increase was less than that observed in tau transgenic mice without these brain changes. Tau transgenic mice subjected to acute neuronal injury using 3-nitropropionic acid showed increased interstitial fluid tau and plasma tau. These data suggest that peripheral administration of an anti-tau antibody results in increased plasma tau, which correlates with the concentration of extracellular and soluble tau in the brain.

4.2 Introduction

Tauopathies including Alzheimer disease (AD), Down syndrome dementia, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick disease, certain forms of frontotemporal lobar degeneration (FTLD), and chronic traumatic encephalopathy (CTE) are a group of neurodegenerative disorders characterized by the hyperphosphorylation and aggregation of the protein tau in the central nervous system (CNS) (Mandelkow and Mandelkow 2012b). Tau is a cytosolic microtubule-associated protein that under normal conditions is localized primarily in neuronal axons (Drechsel et al. 1992, Trinczek et al. 1995, Mandelkow and Mandelkow 2012b) . Its aggregation in specific brain regions in different tauopathies is directly associated with neurodegeneration in those brain regions (Probst et al. 1988, Probst et al. 1996, Falcon et al. 2014, Saito et al. 2004) . Emerging evidence suggests that once tau aggregates in the specific brain regions that are characteristic for each tauopathy, pathological forms of tau then spread through synaptically connected neural networks (de Calignon et al. 2012, Liu et al. 2012).

As tauopathies are currently untreatable, a variety of approaches have been taken attempting to target tau therapeutically. Despite the fact that tau is predominantly a cytosolic protein, it is normally released into the extracellular space from neural cells and its release is regulated by excitatory neuronal activity (Pooler et al. 2013, Yamada, Holth, Liao, Stewart, Mahan, Jiang, Cirrito, Patel, Hochgrafe, et al. 2014). A number of studies in animal models have shown that both active and passive immunization targeting tau can have beneficial effects on reducing tau pathology and improving function (Yanamandra et al. 2013a, Asuni et al. 2007, Boutajangout et

al. 2011, Castillo-Carranza, Sengupta, et al. 2014b, Chai et al. 2011b, Troquier et al. 2012, Schroeder et al. 2016). One mechanism that may account for these effects is via the ability of antibodies to block the intercellular spread of tau pathology. In recent studies, we found that a mouse monoclonal anti-tau antibody, HJ8.5, was able to block the cellular seeding activity of externally applied tau aggregates as well as decrease insoluble tau in the brain and improve function (Yanamandra et al. 2013a). In exploring the potential mechanisms by which this anti-tau antibody might exert its effects, we found that peripheral administration of HJ8.5 to tau transgenic mice that express human tau predominantly in the CNS, markedly increased plasma tau (Yanamandra et al. 2015). This suggested that further exploration of the effects of anti-tau antibodies and plasma tau might provide insight into tau metabolism in the CNS.

To further determine both the origin of plasma tau as well as the potential utility of plasma tau measurements as a marker of CNS tau, we assessed plasma tau concentrations before and after anti-tau antibody administration in a variety of mice expressing human tau as well as in human subjects with PSP. Herein, we report that tau increased in the plasma of mice expressing human tau as well as in the plasma of PSP patients following administration of an anti-tau antibody.

4.3 Results

4.3.1 Anti-tau antibody dose-dependently increases plasma tau in transgenic mice and human patients with tauopathy

We previously assessed the effects of administering the mouse monoclonal anti-tau antibody, HJ8.5, to P301S Tau transgenic (Tg) mice that develop tau pathology in the brain beginning at ~5–6 months of age (Yoshiyama et al. 2007a). HJ8.5 is a high affinity antibody specific to human tau that is directed to an epitope in the N-terminus amino acids 25–30 (Yanamandra et al. 2013a). In agreement with our previous report (Yanamandra et al. 2015), intraperitoneal (i.p.) administration of HJ8.5 at doses ranging from 10–200 mg/kg to 3-month-old P301S Tau Tg mice resulted in a large, dose-dependent increase in plasma tau 48 hours after antibody injection (Figure 4.1A). Plasma tau in Tg mice was assessed using single molecule array (Simoa) technology, a digital enzyme-linked immunosorbent assay (ELISA) detection system developed by Quanterix Inc. (Rissin et al. 2010a, Rissin et al. 2011). Given that HJ8.5 has strong effects on decreasing tau pathology and improving function when given chronically to P301S Tau Tg mice over months, a humanized version of HJ8.5 was developed as a potential treatment for tauopathies. It was administered to an individual with PSP as part of an expanded access treatment protocol. Several doses of the humanized antibody were administered monthly. Tau in human plasma was assessed using a plate-based human plasma tau ELISA (in contrast to the Simoa technology) both prior to dosing administration and following a dose of 7.5 mg/kg given intravenously. Consistent with peripheral administration of HJ8.5 to mice, intravenous

administration of the humanized antibody to one PSP patient resulted in a robust increase in plasma tau (Figure 4.1B). Baseline plasma tau in this individual was not detectable with the human plasma tau assay used (lower limit of detection, 10 pg/ml) prior to antibody administration. Following antibody administration, tau increased rapidly over 24 hours with concentrations of tau reaching ~250 pg/ml over several days (Figure 4.1B). This is about 60-fold higher than the amount of tau previously reported in human plasma by others (~4 pg/ml) using a more sensitive assay (Zetterberg et al. 2013). To confirm this observation, we also measured plasma tau in 3 additional participants receiving the same antibody in a phase 1 single dose study in subjects with PSP. These 3 individuals received a single dose of 15 mg/kg and their plasma tau was assessed (Figure 4.1C). As with the first individual administered the antibody, baseline plasma tau was not detectable (< 10 pg/ml) with the human plasma tau assay utilized. However, following antibody administration, plasma tau increased to ~150–300 pg/ml after 7–14 days. Thus, consistent with our observations in Tg mice expressing human tau, plasma tau markedly increased 50–100 fold over baseline at the doses used after peripheral administration of an anti-tau antibody to humans.

4.3.2 Antibody-mediated increase in plasma tau half-life enables tau detection

One possible mechanism by which tau antibodies could increase plasma tau is by altering the half-life of tau once it enters the plasma. To test this hypothesis, we injected recombinant human tau intravenously into the jugular vein of wild-type mice in the presence and absence of the anti-tau antibody HJ8.5 and assessed the clearance of tau from the plasma. Plasma tau was measured

with the Simoa ELISA. We found that the half-life of intravenously injected human tau in plasma was 8.49 minutes (Figure 4.2A, D). Administration of HJ8.5 (50 mg/kg, i.p.) one hour prior to jugular vein injection of recombinant human tau extended the half-life of tau to ~3.4 hours (Figure 4.2B, D). We next co-incubated recombinant human tau with a 10-fold molar excess of HJ8.5 and administered the tau-antibody complex intravenously to wild-type mice. Co-incubation of HJ8.5 with tau increased the half-life of tau within the plasma to an even greater extent ~13.2 hours (Figure 4.2C, D). These data suggested that the peripheral administration of an anti-tau antibody increased plasma tau by binding to tau and extending the half-life of tau in the plasma (Figure 4.2B, C, D).

To determine the extent to which tau in the extracellular space of the CNS enters the plasma and is measurable in the plasma, we injected recombinant human tau into the cisterna magna of wild-type mice in the presence or absence of peripherally administered HJ8.5. In control mice, plasma tau was detectable in the plasma within minutes after cisterna magna injection and exhibited a half-life of 1.6 hours (Figure 4.2E, G). The increased half-life of CNS-injected tau versus venous-injected tau likely reflected continued entry of tau from the CSF into plasma over several hours following a single bolus injection. In HJ8.5-injected mice, human tau was detected in the plasma within minutes after CNS injection, but the half-life was increased to almost 24 hours (Figure 4.2F, G). This again suggested that the HJ8.5-tau antibody complex had a prolonged half-life in plasma relative to tau alone, which likely accounted, at least in part, for the HJ8.5-dependent increase in plasma tau.

4.3.3 HJ8.5-induced increase in plasma tau occurs in different mouse models expressing human tau

To further characterize the effects of HJ8.5 on plasma tau as well as to determine its origin, we administered HJ8.5 at a dose of 50 mg/kg i.p. to a variety of human tau Tg mice. P301S Tau Tg mice express human tau under the control of the prion promoter, which drives transgene expression predominantly in the CNS, but also in peripheral tissues such as muscle. Therefore, plasma tau is likely to be detectable under basal conditions in P301S Tau Tg mice due to some expression of tau in peripheral tissues outside of the CNS (Figure 4.3A). We wanted to verify that the increase in plasma tau in Tg mice following HJ8.5 administration was attributable to CNS-derived tau. We administered HJ8.5 to two different mouse models, the hTau mouse, which expresses human tau under control of the endogenous tau promoter, and in another mouse model in which adeno-associated virus (AAV) 2/8-mediates expression of human tau with the neuronal-specific synapsin promoter in the brain (Andorfer, Kress, Espinoza, de Silva, et al. 2003). We injected AAV type 2/8 expressing human P301S tau (AAV-syn-P301S) intracerebroventricularly (ICV) into the brain of postnatal day 0 (P0) wild-type mice. This resulted in strong CNS expression of human tau by 1-month of age, at levels comparable to those observed in 3 month-old P301S Tau Tg mice (Figure 4.3B). Unlike P301S Tau Tg mice, human tau was not detectable in the plasma of either 2-month old hTau mice or 1-month old AAV-tau mice under basal

conditions (Figure 4.3A). Plasma tau in different Tg mice was assessed using Simoa technology. The lower limit of detection in the plasma tau Simoa assay we utilized in mice was ~1 pg/ml. Injection with HJ8.5 increased plasma tau in hTau mice to 93 pg/ml and to ~20ng/mL for AAV-tau mice (Figure 4.3A). Given the large difference between hTau and AAV-tau mice regarding plasma tau detected following HJ8.5 administration, we decided to measure soluble human tau in the mouse brain using a plate-based human tau-specific ELISA. We found that soluble tau was much lower in hTau mice compared to P301S and AAV-tau mice (Figure 4.3C), suggesting that the different plasma tau concentrations observed following HJ8.5 administration reflected different concentrations of soluble tau within the CNS compartment. However, because of major differences in the promoters used in these mice and both the amount and location of tau expression between the human tau expressing mouse models, we decided to further test this idea in just one model.

4.3.4 HJ8.5-induced increase in plasma tau is correlated with soluble brain tau

Given that we saw differences in plasma tau elevation upon antibody injection that appeared to be potentially related to differences in soluble tau in the brain, we hypothesized that we might be able to detect pathology-dependent alterations in soluble tau using antibody-dependent measurements in plasma tau. In previous studies, we characterized the amount of tau within the interstitial fluid (ISF) as well as brain soluble and insoluble tau in P301S Tau Tg mice (Yamada et al. 2011b). ISF tau represents tau present in the extracellular space of the brain that is released

from neurons (Yamada, Holth, Liao, Stewart, Mahan, Jiang, Cirrito, Patel, Hochgräfe, et al. 2014). It is only a small fraction of the soluble brain tau assessed biochemically, which is predominantly composed of cytosolic tau. In accordance with previous results, we observed an age-dependent decline in soluble tau, an age-dependent increase in insoluble tau, and no age-dependent change in the detergent-containing radioimmunoprecipitation assay (RIPA) buffer solubilized tau (Figure 4.4A–C). Furthermore, we found higher ISF tau in young, pre-symptomatic P301S mice compared to aged 9-month-old P301S mice (Figure 4.4D). The amount of ISF tau in 9-month-old mice was ~60% lower than in 3-month-old mice. No changes were observed in total human tau expression by qPCR (Figure 4.4E).

Having established that aged mice bearing tau pathology exhibited decreased total soluble and ISF tau, we next tested whether we would detect differences in antibody-dependent increases in plasma tau between P301S mice of different ages before and after the onset of tau pathology. After i.p. injection of HJ8.5 (50 mg/kg), plasma tau assessed with the Simoa assay increased over 1–6 hours and remained elevated for more than a week compared to basal conditions in 3-month, 6-month, and 9-month-old P301S mice (Figure 4.5A–C). As a control group, anti-human Aβ antibody HJ3.4 at 50 mg/kg was injected into P301S Tau Tg mice. No change in plasma tau was observed in these groups (Figure 4.5A–C). Interestingly, the amount of plasma tau in the HJ8.5-injected mice was decreased by 60% in the 9-month-old mice during the first 24 hours following HJ8.5 injection compared to the amount in the 3-month and 6-month-old groups (Figure 4.5D, E). This result was in accordance with the observed decrease in total brain soluble tau and ISF tau in 9-month-old compared to 3-month-old P301S mice (Figure 4.4A,D). To

further investigate this relationship we plotted the amount of plasma tau in HJ8.5-treated mice as a function of soluble brain tau and found a positive correlation between the amount of soluble brain tau and plasma tau (Figure 4.5F). Together, these results suggest that brain extracellular tau in ISF is in equilibrium with biochemically extractable salt-soluble pool of tau within the brain and that the ISF pool of tau is able enter the plasma where it was captured and detected by an anti-tau antibody.

4.3.5 Changes in mouse brain ISF and plasma tau detected by HJ8.5 after neuronal injury

Whereas the data suggest that decreases in soluble brain tau and ISF tau that occur in 9-month-old versus 3-month-old P301S mice maybe reflected in plasma following HJ8.5 injection, we wanted to determine whether injury-induced increases in extracellular tau could also be detected in the blood following peripheral injection of the anti-tau antibody. We decided to assess the effects of unilateral striatal infusion of a neuronal toxin, 3-nitropropionic acid (3-NP), on ISF tau. Baseline striatal ISF tau was assessed in a group of P301S Tau Tg mice followed by infusion of 3-NP through reverse microdialysis. This treatment resulted in a 10–20 fold increase in striatal ISF tau over several hours (Figure 4.6A). We then stereotactically injected a group of P301S Tau Tg mice bilaterally with either 3-NP or PBS into the striatum as described previously (Musiek et al. 2016). Four hours later, mice received an i.p. injection of HJ8.5 (50 mg/kg). Plasma tau was assessed prior to and after 3-NP or PBS injection using the Simoa tau assay. Plasma tau increased in all mice administered HJ8.5 (Figure 4.6B). However, there was a significanty larger

increase in plasma tau in mice given 3-NP compared to those given PBS ($p<0.01$) (Figure 4.6B). This suggested that not only decreases but also increases in ISF tau could be detected in plasma after treatment with an anti-tau antibody.

4.4 Discussion

Disease-modifying therapies including immunotherapies are emerging as potential treatments for neurodegenerative diseases. Antibodies targeting Aβ are furthest along in this process (Spencer and Masliah 2014); however, antibodies targeting tau and α-synuclein have also shown promising results in animal models and have entered clinical trials (Valera, Spencer, and Masliah 2016, Pedersen and Sigurdsson 2015). A challenge moving forward in clinical trials for neurodegenerative diseases characterized by protein aggregation is screening for patients that have the aggregated form of the protein being targeted in the CNS, as well as determining whether the therapy being utilized is hitting its target. In this study, we found that peripheral administration of anti-tau antibody to mice expressing human tau as well as to humans with PSP resulted in a marked increase in plasma tau. Our data indicated that the antibody-dependent increase of plasma tau resulted primarily from CNS-derived tau and that plasma tau concentrations appeared to reflect soluble, extracellular tau in the brain. The results of this study in both humans and mouse models suggest a direct relationship between extracellular tau in the CNS and plasma tau.

Expression of tau is almost exclusively in neurons where it is present in the cytosol. It is also released by neurons in the brain physiologically and can be detected in soluble forms in brain ISF and CSF (Pooler et al. 2013, Yamada, Holth, Liao, Stewart, Mahan, Jiang, Cirrito, Patel, Hochgrafe, et al. 2014). In brain ISF, tau has a long half-life, ~11 days in mice (Yamada et al. 2015). In human plasma, it is detectable in only low amounts ~4 pg/ml in humans (Zetterberg et al. 2013). Our data suggest that when tau enters the plasma from the CNS, it is rapidly cleared (Figure 4.2A). In the presence of a tau antibody, tau that enters the plasma binds to the antibody and this extends the plasma half-life of detectable tau (Figure 4.2). It is also possible that some antibody-tau complexes form initially in the CNS and then are cleared via the plasma with normal clearance of antibody from the brain to the blood. Interestingly, our data suggest that the amount of tau bound to anti-tau antibody in plasma is related to brain soluble tau and perhaps more directly to extracellular tau such as that in the ISF. For example, after neuronal injury, ISF tau and plasma tau both increased (Figure 4.6), possibly due to extracellular release of cytosolic tau in the brain after injury. Also, we detected lower tau in the plasma of older P301S Tau Tg mice with tau pathology following anti-tau antibody administration reflecting lower ISF tau and brain soluble tau (Figure 4.5). While there was a difference in salt-containing reassembly buffer (RAB) soluble tau in mice before versus after establishment of tau pathology between age groups, there was no correlation between RAB soluble tau and plasma tau after administration of anti-tau antibody within each age group. This may be similar to the observed relationship between CSF Aβ42 and amyloid deposition in the brains of human patients with Alzheimer's

disease pathology and mouse models of amyloidosis in which once Aβ has aggregated then CSF and ISF Aβ42 decreases (Fagan et al. 2006, Roh et al. 2012, Potter et al. 2013). However, low CSF Aβ42 in individuals with amyloid deposition does not correlate with the absolute amount of Aβ deposition within this group. Even though CSF Aβ42 does not correlate with the absolute amount of amyloid deposition, it may be useful for clinical trial patient selection based on its ability to discriminate between the presence or absence of amyloid deposition. Whether increases in plasma tau following anti-tau antibody administration would be different in humans with a primary tauopathy compared to healthy controls is currently not known but should be tested in future studies.

We, and others, have found that administration of certain anti-Aβ antibodies results in a large increase in plasma Aβ in both mice and humans that is predominantly derived from the brain (DeMattos et al. 2001, DeMattos, Bales, Cummins, et al. 2002, Farlow et al. 2012, Asami-Odaka et al. 2005, Winkler et al. 2010). The large increase in plasma Aβ seen with certain antibodies may be due to a prolongation of plasma Aβ half-life when bound to antibody, perhaps similar to what we report here with tau. Animal studies suggest that such changes in Aβ may also reflect the amount of extracellular soluble Aβ in the CNS that is entering the blood (DeMattos, Bales, Cummins, et al. 2002, Winkler et al. 2010). In fact, manipulations that alter brain to blood clearance of Aβ, such as the brain expression of the low-density lipoprotein receptor, affect the concentration of Aβ detectable in the blood following anti-Aβ antibody administration

(Castellano et al. 2012b). There are, however, differences between extracellular CNS-derived Aβ and tau. The half-life of ISF Aβ in mice is ~1.5–3 hours; the half-life of ISF tau in mice is ~ 11 days (Cirrito et al. 2003a, Yamada et al. 2015).

There are several limitations to this study. First, whether all anti-tau antibodies will increase plasma tau as we report here is not yet known although others have also observed increases in serum tau after administration of different anti-tau antibodies to human Tau Tg mice (Castillo-Carranza, Sengupta, et al. 2014a, d'Abramo et al. 2016). Second, the differences in plasma tau following anti-tau antibody administration that were observed in the presence or absence of tau pathology or after neuronal injury in mice have only been studied in one mouse model, P301S tau transgenic mice. Whether similar differences will be observed in other animal models of tauopathy is not clear. Third, it is not yet known whether increases in plasma tau in PSP patients that were observed following the administration of the anti-human tau antibody used herein will reflect brain extracellular tau in humans. Given these limitations, as anti-tau antibodies have now moved forward into clinical trials, it will be important to assess plasma tau across different patient populations with both primary tauopathies such as PSP, corticobasal degeneration, Pick disease, chronic-traumatic encephalopathy, and frontotemporal lobar degeneration (FTLD) with MAPT mutations versus AD in which there is both Aβ and tau accumulation and changes in CSF tau. Notably, whereas CSF tau is elevated in AD and following acute neuronal injury such as stroke or traumatic brain injury compared to controls, CSF tau is not elevated in the primary

tauopathy PSP. In fact, CSF tau is lower in PSP patients than in age-matched controls (Arai et al. 1997, Wagshal et al. 2015, Perrin, Fagan, and Holtzman 2009).

A well-established observation is that there is an elevation of CSF tau in AD (Holtzman, Morris, and Goate 2011). This is likely a completely different phenomenon than what takes place in primary tauopathies. In AD, CSF tau is likely elevated due to neuronal and synaptic membrane damage secondary to amyloid deposition, not directly due to tau aggregation. This was demonstrated in a recent study where it was shown that murine CSF tau is normal in young amyloid precursor protein (APP) Tg mice prior to amyloid deposition, but then progressively increases once amyloid deposition begins to accumulate in the brain (Maia et al. 2013). Given that these mice do not develop neurofibrillary tangles or tau aggregation, it suggests that amyloid-linked neuronal/synaptic injury leads to an increase in ISF/CSF tau release independent of tau aggregation. We report a similar phenomenon following acute brain injury in P301S Tau Tg mice. In this mouse model of acute CNS neuronal injury following striatal 3-NP administration, we found an increase in brain ISF tau, and also higher plasma tau following anti-tau antibody administration compared to mice given an anti-tau antibody with no neuronal injury. Given these results, we would predict in patients with amyloid deposition and in individuals with acute brain injury from stroke or head trauma that there would be an increase in plasma tau compared to age-matched healthy controls following anti-tau antibody administration.

Given our findings, it will be important to determine whether differences in plasma tau following anti-tau antibody administration can be utilized to screen for individuals with primary tauopathies (where tau is the only major protein aggregating in the brain), for those with AD (where both Aβ and tau are 2 major proteins aggregating in the brain), and for those with different forms of acute versus chronic neuronal/synaptic damage. Finally, as clinical trials move forward with anti-tau antibodies, it will be important to determine whether a reduction in tau accumulation detected by tau imaging as well as any clinical response following anti-tau antibody therapy correlates with measurements of plasma tau. In addition, understanding how plasma and CSF tau correlate before and after anti-tau antibody administration may also provide insights into the mechanism of action of anti-tau antibodies.

4.5 Materials and methods

4.5.1 Study design

The aim of this study was to assess concentrations of plasma tau in patients with PSP and in mouse models expressing human tau before and after administration of an anti-tau antibody. In mouse models, another goal was to determine the half-life of plasma tau in the presence and absence of the anti-tau antibody as well as any differences in plasma tau after antibody administration that were due to tau pathology. All mice and human subjects in the studies were

randomly assigned to experimental groups. All studies were performed by investigators blinded to treatment groups and sample identity. Replication numbers for experiments are listed in the figure legends.

4.5.2 Administration of humanized HJ8.5 antibody to patients with PSP

The mouse monoclonal antibody was cloned from the hybridoma expressing HJ8.5 and the variable regions were cloned to a human IgG4 Fc domain followed by humanization of several amino acids outside of the CDR region. A humanized version of HJ8.5 was administered to an individual with PSP following a treatment protocol under an expanded access program (expanded access IND 119404) by C2N Diagnostics. Informed consent was obtained from the individual and all procedures were approved by an Institutional Review Board. The individual was administered multiple doses of the antibody once per month. Blood samples were collected at various time points before and after start of drug infusion for the purpose of measuring drug concentrations as well as biomarkers. The same antibody was also administered to individuals as part of a phase I single dose study entitled “Safety, Tolerability, and Pharmacokinetics of C2N-8E12 in subjects with Progressive Supranuclear Palsy”, NCT02494024. Informed consent was obtained from all participants and all procedures were approved by an Institutional Review Board.

4.5.3 Tau transgenic mice and treatment with anti-tau antibody

All animal procedure and experiments were performed by the guidelines approved by the animal studies committee at Washington University School of Medicine. We utilized several different

human Tau Tg mice. P301S Tau Tg mice (Jackson Laboratories) overexpress the human T34 isoform of human tau (1N4R) with the P301S mutation under the control of prion promoter (21). These mice are on B6C3 background and develop tau pathology by 5–6 months of age. Human tau (hTau) mice (male and female) were utilized that express all six isoforms of hTau but do not express mouse tau (25). These mice develop hyperphosphorylated tau by 6 months of age (25). For adeno-associated virus (AAV) injections, postnatal day (P) 0 pups of C57BL/6 mice were used. Pregnant C57BL/6 mice were purchased from Charles River Labs. Plasma was collected from two month-old hTau mice, 1-month-old AAV injected mice, and 3-month-old male P301S Tau Tg mice one day before HJ8.5 injection and 48hrs after HJ8.5 (50 mg/kg) i.p. injection. For dose response experiments, 3 month-old male P301S mice were administered HJ8.5 at 10, 50 and 200 mg/kg by i.p injection. Control mice were administered phosphate buffer saline (PBS). All the plasma samples were stored at −80°C until use. For experiments involving P301S mice at different ages (3-months, 6-months and 9-months), only male mice were utilized and plasma samples were collected 1 day before HJ8.5 injection and at various time intervals (1, 6, 24, 72, 168 hours) after HJ8.5 (50 mg/kg) i.p injection. Anti-human Aβ antibody HJ3.4 was used as control antibody. All plasma samples were stored at −80°C until use.

4.5.4 AAV viral particle injection into mouse brain

P301S human tau was cloned from P301S transgenic mouse cDNA and inserted into an AAV-synapsin driven promoter vector. AAV 2/8 serotype was produced at the Hope Center Viral Vectors Core (Washington University in St. Louis). C57BL/6 mice post-natal day 0 (P0) pups

were intraventricularly injected bilaterally with 2 µl (1 × 1013 vg/mL) of AAV2/8 carrying human tau with the P301S mutation (AAV-P301S) under the control of the synapsin promoter using a 10 µl Hamilton syringe (Sigma-Aldrich). One month after viral infection, plasma was collected from tail bleeds one day before injection and 48 hours after an i.p. injection of HJ8.5 (50 mg/kg). Plasma samples were stored at −80°C until use.

4.5.5 Collecting blood samples from mice

Blood samples were collected at various time points from tail bleeds for Tg mice and retro-orbital bleeds for wild-type mice. Heparinized capillary tubes (Kimble & Chase) were used to collect blood and transferred into 1.5 ml Eppendorf tubes along with 1 µl of 0.5M Ethylenediamine tetraacetic acid (EDTA). The last time point of blood collection was from anesthetized mice (50 mg/kg of sodium pentobarbital) from the right ventricle of the heart with a 1 ml insulin syringe filled with 20 µl of 0.5M EDTA. Blood samples were spun at 8000 rpm (6000 × g) for 10 minutes at 4°C to obtain plasma. Plasma samples were stored at −80°C until use.

4.5.6 Measurements of plasma tau half-life in mice

For experiments involving assessment of human tau half-life in the plasma of mice, recombinant human tau (rPeptide), at 1 µg per 100 µl of saline, was injected into the jugular vein under isoflurane anesthesia in 3 month old male B6C3 wild type mice, and retro-orbital bleeds were collected at various time intervals (after 2, 4, 8, 12, 18, and 25 minutes). Recombinant human tau (1 µg, rPeptide) was pre-incubated at room temperature with 10 times molar excess of HJ8.5 (2.3

μM) and injected into the jugular vein of 3 month old B6C3 wild-type mice. Plasma was collected from tail bleeds at different time intervals of 10 minutes, 6, 24, 48, and 72 hours. In some experiments, 1 μg of recombinant human tau was injected into the jugular vein 1 hour following the i.p. administration of HJ8.5 (50 mg/kg) and plasma was collected at 10 minutes, 1 hour, 6 hours, and 24 hours after injection. To determine the half-life of human tau in plasma after administration into the cerebrospinal fluid in the cisterna magna, we anesthetized 3-month old wild type male B6C3 mice by i.p injection of xylazine (10mg/kg)/ ketamine (100mg/kg) in normal saline. Then mice were injected with recombinant human tau (2 μg/2 μl, rPeptide) into the cisterna magna compartment as described previously (DeMattos, Bales, Parsadanian, et al. 2002). Plasma was collected from tail bleeds at different time intervals of 25 minutes, 1, 2, 3, and 6 hours. For experiments in the presence of HJ8.5 antibody, 10 minutes after i.p. injection of HJ8.5 (50 mg/kg), human tau was injected into the cisterna magna. Plasma was collected at various time intervals of 10 minutes, 1, 6, 24, 48, 72 hours. All Plasma samples were analyzed on Simoa HD-1 analyzer. Half-life of tau was calculated by first order kinetics, determining slope of linear regression from semi-log plot of concentration vs. time. Elimination rate constant (Ke) and half-life t1/2 were calculated by using Ke= 2.0303/slope; t1/2=0.693/Ke as previously described (Yamada, Holth, Liao, Stewart, Mahan, Jiang, Cirrito, Patel, Hochgrafe, et al. 2014, Cirrito et al. 2003a, Castellano et al. 2011).

4.5.7 Immunohistochemistry to detect human tau

Immunostaining was performed as described previously (Yanamandra et al. 2013a). To determine the expression of human tau in different transgenic mice, we stained brain sections with biotinylated HJ8.5 antibody. For staining, 3 brain sections of 50 µm thickness and 300 µm apart corresponding approximately to sections at Bregma coordinates −1.4, −1.7, and −2.0 mm in the mouse brain atlas were used (Franklin and Paxinos 1997). Brain sections were blocked with 3% milk in Tris-buffered saline and 0.25% (vol/vol) Triton-X followed by incubation at 4°C overnight with the biotinylated HJ8.5 antibody. C57BL/6 and B6C3 wild type mice brain sections were used as negative controls.

4.5.8 Measurements of plasma tau using the Simoa assay in mice

To measure tau in mouse plasma we utilized a Single molecule array – (Simoa) assay, a sensitive digital ELISA platform (Wilson et al. 2016). Homebrew assays specific for total tau were developed according to the manufacturer's recommendations (Quanterix Corp). All plasma tau levels measurements were analyzed using the Simoa HD-1 Analyzer (Quanterix Corp). Simoa HD-1 consumables were purchased from Quanterix Corp. The paramagnetic beads were pre-coated with a mouse monoclonal anti-tau HJ8.7 capture antibody. In addition to the coated beads, diluted plasma samples, biotinylated detector anti-tau antibody BT2 (Pierce), streptavidin β-galactosidase (sβg), and enzyme substrate (resorufin β-D galactopyranoside) were added to the Simoa HD-1 Analyzer. Recombinant human tau was included in each run to generate a standard

curve. Assays were performed according to the manufacturer's instructions (Quanterix Corp). The presence of HJ8.5 in this assay does not interfere with or influence the assay results.

4.5.9 ELISA assay for plasma tau in PSP patients

To determine tau in human plasma samples, we utilized an ELISA in which plates were first coated with 1 µg/mL of BT2 antibody (Thermo Scientific) in PBS supplemented with 20% glycerol and left to incubate overnight at 4°C. The next day, plates were blocked with 2% BSA in PBS supplemented with Tween-20 0.05% and 20% glycerol for 2 h at RT. Human plasma samples were diluted 1:5 in sample diluent (0.20% BSA/300mM Tris PH 8.0/0.05% Tween 20/20 µg/mL mIgG/0.05% Proclin-300/PBS) while calibrator (recombinant tau 412, rPeptide) was prepared in calibrator diluent (0.20% BSA/300mM Tris PH 8.0/0.05% tween-20/20 µg/mL mIgG/20% pooled normal plasma samples/0.05% Proclin-300/PBS). Calibrators and samples were incubated overnight at 4°C. The next day, plates were washed 4 times with PBS followed by incubation with biotinylated mouse monoclonal anti-tau antibody HJ8.7 (1 µg/mL) in 1% BSA in PBS for 2 h at RT. Plates were then washed 4 times with PBS followed by incubation with streptavidin-poly-horseradish peroxidase-40 (1:6000, Fitzgerald) for 1 h at RT. Plates were washed 4 times with PBS and then developed with Super Slow ELISA TMB (Sigma) and absorbance read at 650 nm.

4.5.10 Biochemical extraction of mouse brain tissue

Biochemical extractions were performed as described previously (DeMattos, Bales, Parsadanian, et al. 2002). Briefly, cortical brain samples were homogenized in 30 µl/mg (v/w) in reassembly

buffer - RAB (100 mM MES, 0.5mM MgSO4, 1 mM EDTA, 2mM DTT, 0.75M NaCl, 1 mM Na3VO4, pH 6.8) supplemented with 1× protease and phosphatase inhibitors (Roche). Samples were spun at 50,000g for 20 minutes, and the supernatant was saved as a RAB-soluble fraction. Pellet was dissolved in 30 µl/mg (v/w) in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, 0.5% deoxycholic acid, 1% TritonX-100, 0.5% SDS, 25 mM EDTA, pH 8.0) supplemented with protease and phosphatase inhibitors (Roche) and spun at 50,000g for 20 minutes. The supernatant was collected as a RIPA-soluble fraction. The pellets were further dissolved in 70% formic acid (FA) at 10 µl/mg, sonicated, centrifuged at 50,000g for 20 minutes. The supernatant was collected as FA-soluble fractions. All fractions were stored at −80°C until analyzed. Samples were analyzed using a human tau specific ELISA.

4.5.11 Human tau specific ELISA to measure tau in mouse brain

Human tau specific enzyme-linked immunosorbent assay (ELISA) that was utilized to measure brain tissue tau levels was performed as described previously (Yanamandra et al. 2015). For assessing human tau levels in Figure 4, biochemical cortical brain extraction samples were analyzed by coating ELISA 96 well plates with mouse monoclonal anti-tau antibody Tau5 (20 µg/ml, gift of L. Binder) overnight. Plates were washed 5 times with PBS and blocked with 4% BSA in PBS for 1 hr at 37°C. Cortical brain extraction samples were diluted in 0.25% BSA in BSA, 300 mM Tris, pH 8.0 supplemented with protease inhibitors and kept at 4°C for overnight on a shaker. The next day, plates were washed 8 times with PBS followed by the addition of

mouse monoclonal anti-human tau-specific biotinylated HT7 antibody (0.2 µg/ml, Thermo Scientific) in 1% BSA in PBS for 1.5 hr at 37°C. Next, plates were washed 8 times with PBS followed by addition of streptavidin-poly-horseradish peroxidase-40 (1:6000, Fitzgerald) for 1.5 h in the dark at room temperature. Then plates were washed 8 times with PBS, developed with Super Slow ELISA TMB (Sigma) and absorbance read at 650 nm. For assessing human tau levels as shown in Figure 3C, cortical brain extracts of different transgenic mice were analyzed by coating ELISA plates with anti-tau antibody BT2 (1 µg/ml) in PBS supplemented with 20% glycerol and incubated overnight at 4°C. Plates were blocked with 2% BSA in PBS supplemented with Tween-20 0.05% and 20% glycerol for 2 h at RT. Next plates were washed 4 times with PBS followed by the addition of mouse (Yamada et al. 2011a, Yamada, Holth, Liao, Stewart, Mahan, Jiang, Cirrito, Patel, Hochgrafe, et al. 2014)monoclonal anti-human tau-specific biotinylated HT7 antibody (0.2 µg/ml, Thermo Scientific) in 1% BSA in PBS for 1.5 h at 37°C. Then plates were washed 4 times with PBS followed by incubation with streptavidin-poly-horseradish peroxidase-40 (1:6000 Fitzgerald) for 1 h at RT. Plates were washed 4 times with PBS and then developed with Super Slow ELISA TMB (Sigma) and absorbance read at 650 nm.

4.5.12 Quantitative PCR to measure human MAPT RNA in mouse brain tissue

For the isolation of RNA, brain tissues were homogenized in TRIzol (Invitrogen). Chloroform was added (1:5 ratio), samples were vigorously shaken for 15 seconds at room temperature, and

then centrifuged at 12,000 × g for 15 minutes at 4°C. The aqueous phase of the sample was removed by angling the tube at 45° and pipetting the solution out. Then samples were diluted (1:1) in 100% isopropanol and incubated for 10 minutes at room temperature. Samples were centrifuged at 12,000 × g for 10 minutes at 4°C. The pellet was washed in 75% ethanol, and then centrifuged at 7500 × g for 10 minutes. Supernatant was discarded and the RNA pellet air-dried for 10 minutes before resuspension in RNase-free water. Reverse transcription was performed by using a high-capacity RNA-cDNA kit (Applied Biosystems (ABI)). Quantitative PCR was performed using an ABI TaqMan primer targeting human MAPT (Product no: Hs00902194_m1, Life Technologies) and reagents on an ABI Prizm 7500 thermocycler according to the manufacturer's instructions. All mRNA measurements were normalized to the total cDNA

4.5.13 Microdialysis and 3-NP treatment of P301S Tau Tg mice

P301S hippocampal microdialysis was performed in P301S male mice with some modifications (Yamada et al. 2011a, Yamada, Holth, Liao, Stewart, Mahan, Jiang, Cirrito, Patel, Hochgrafe, et al. 2014). In brief, a microdialysis probe with 100 kDa cutoff (Amuza Inc.) was implanted in the left hippocampus through a guide cannula (Amuza Inc.). Artificial cerebral spinal fluid (ACSF) containing 4% human albumin solution was used as perfusion buffer for ISF collection. ISF was collected in 90-minute fractions for 24 hours following probe implantation. Striatal microdialysis with 3-nitropropionic acid (3-NP) treatment was performed in 8–9-month-old P301S mice using a microdialysis probe with 1000 kDa cutoff (Amuza Inc.) implanted in the left striatum. ACSF

containing 4% human albumin solution was used as perfusion buffer for ISF collection. After stable baseline ISF collection 200 µM 3-NP (Sigma-Aldrich) was dissolved in perfusion buffer and administered directly in the striatum for 3 hours via reverse microdialysis. For bilateral striatal 3-NP injections combined with plasma tau measurements, a 100 nmol/µl solution of 3-NP (Sigma-Aldrich) was prepared in PBS and 0.5 µl of this solution was injected bilaterally in the striatum of 5 month old P301S mice via stereotaxic surgery at Bregma coordinates − +0.98 mm, +/− 1.5 mm mediolateral, and a depth of 2.6 mm as described (Musiek et al. 2016).

4.5.14 Statistical analysis

The amount of plasma tau in P301S mice of different ages was compared by 2-way ANOVA with post hoc Dunnett test using GraphPad Prism 6.0 (GraphPad Software Inc.). Non-parametric Spearman correlation analyses were performed with Graph Pad Prism 6.0 software. The Area Under the Curve (AUC) for tau in plasma during the 24 hours following injection of anti-tau antibody was calculated using the basic non-comparmental pharmacokinetics (PK) package for R. R is a free software environment for statistical computing and graphics. The mean AUCs and error estimates for each group were calculated by PK and a 2-tailed t-test was used to determine statistical significance. One-way ANOVA followed by post-hoc Dunnett's test was used to evaluate the qPCR data, plasma tau-half life data, as well as ISF human tau. 2-way ANOVA with post hoc Dunnett test was used to evaluate plasma tau measurements from mice administered the

neurotoxin 3-NP. Statistical significance was set at $p<0.05$. Outliers were determined by performing Grubbs' test using free web Graph Pad software. There was one outlier each in the 6 and 9 month old groups of mice analyzed in Figure 4 and and55 for plasma and brain tau. All values are presented as mean ± SEM.

4.6 Figures

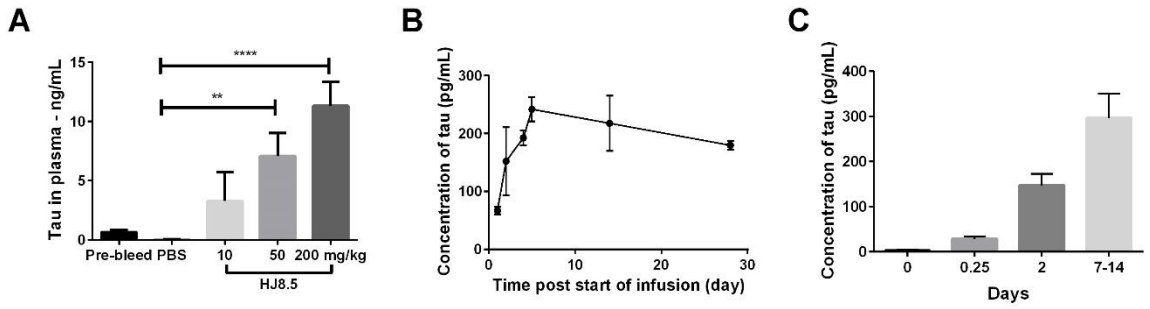

Figure 4.1. Dose dependent increase in plasma tau after anti-tau antibody administration in mice and humans.

(A) 3 month-old P301S Tau Tg mice were administered phosphate-buffered saline (PBS) or anti-tau antibody HJ8.5 at 10, 50 and 200 mg/kg doses (n=5 mice/group) by i.p injection. Plasma tau was measured before the injection (pre-bleed) and 48 hours after antibody injection. Plasma tau level measurements were analyzed using the Simoa HD-1 Analyzer. **$p\leq0.01$, ****$p\leq0.0001$, one-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons. (B) Humanized HJ8.5 at a dose of 7.5 mg/kg was intravenously injected into a single individual and plasma tau was measured before (0 hour time) and after antibody injection at various time intervals (1, 2, 4, 5, 14, 28 days). (C) Humanized HJ8.5 at a dose of 15 mg/kg was intravenously injected into three human subjects and plasma tau was measured before and after antibody injection. Tau was not detected with this assay prior to antibody injection. Human plasma samples were analyzed

with a human plasma tau ELISA assay. N=3 for each time point shown. Values represent mean ± SEM.

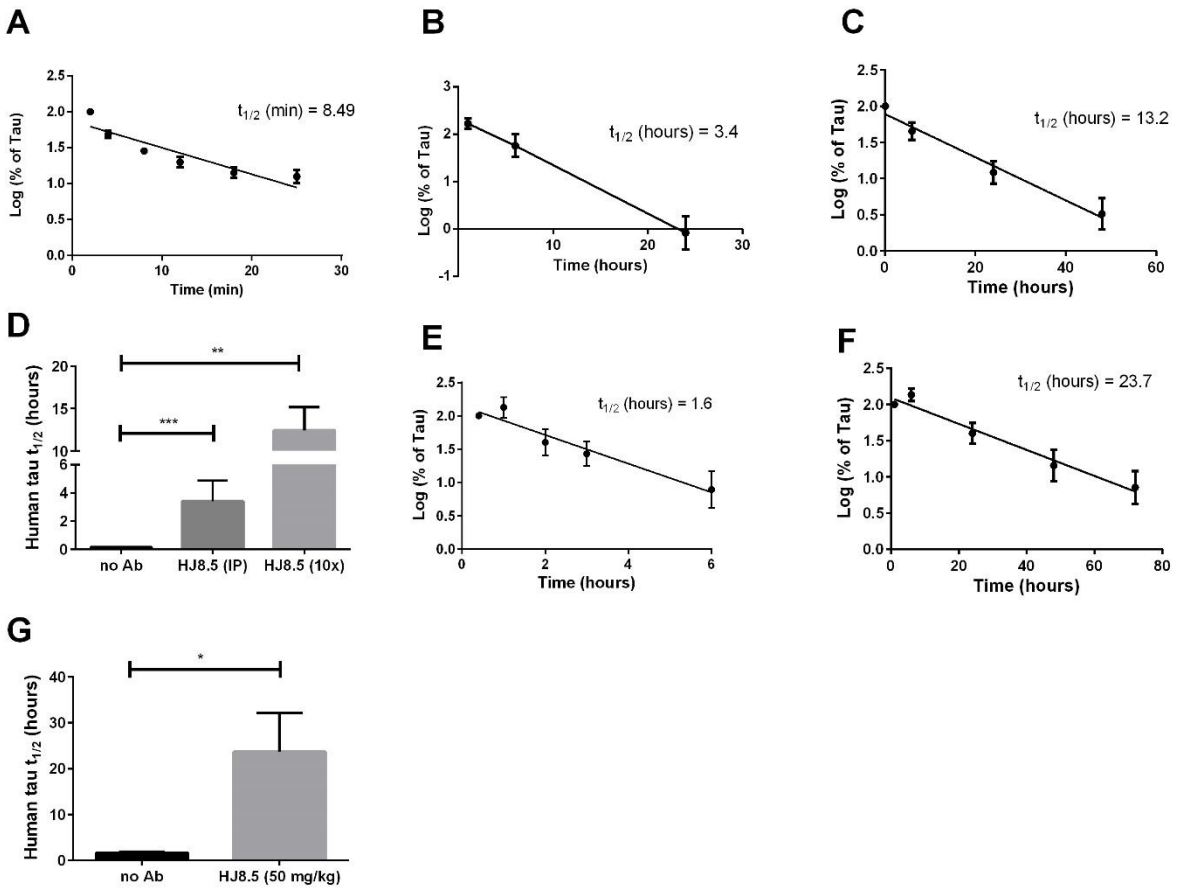

Figure 4.2. Half-life of plasma tau in absence and presence of anti-tau antibody.

(A) Human tau was injected intravenously (i.v.) into wild-type (WT) B6C3 mice (n=6) and plasma tau was analyzed at 2, 4, 8, 12, 18 and 25 minutes (min). (B) Human tau was injected i.v. into WT B6C3 mice (n=9) 1 hour following i.p. administration of HJ8.5 at 50 mg/kg and plasma tau was analyzed after 30 minutes, 6 hours, and 24 hours. (C) Human tau was pre-incubated 1 hour with 10x molar excess HJ8.5 and injected into the jugular vein of WT mice (n=8). Plasma was collected at 10 minutes, 6, 24, 48, and 72 hours. (D) Half-life of plasma tau following

jugular vein injection of tau in the conditions studied in A, B, and C. (E) Human tau was injected into cisterna magna of WT B6C3 mice (n=6) and plasma collected at 25 minutes, 1, 2, 3, and 6 hours. (F) Ten minutes after HJ8.5 injection (50 mg/kg, i.p.), human tau was injected into the cisterna magna of WT mice (n=6) and plasma collected at 10 minutes, 1, 6, 24, 48, 72 hours. (G) Half-life of plasma tau after injection into cisterna magna in the absence (E) and presence of HJ8.5 (F). Tau half-life was calculated by determining the slope from linear regression fit of semi-log plots of concentration vs. time (Cirrito et al. 2003a). Values in D and G represent mean ± SEM. *p≤0.05, **p≤0.01, ***p≤0.001. Values in D analyzed by ANOVA followed by post-hoc Dunnett's test and values in G analyzed by unpaired t test.

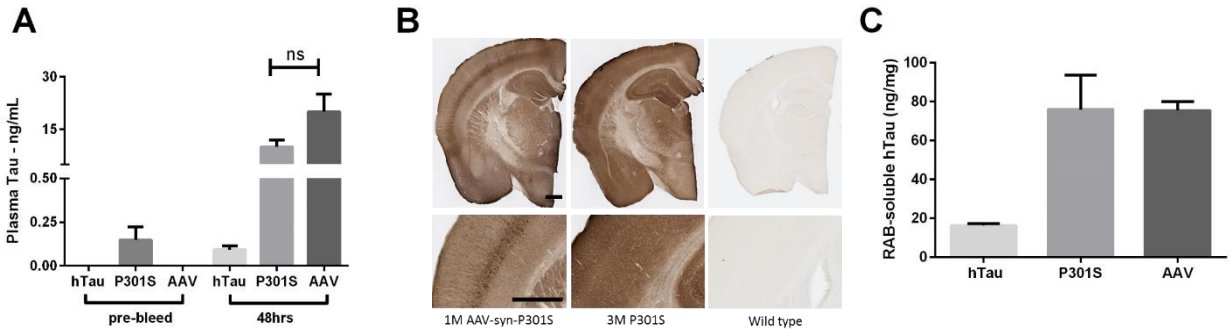

Figure 4.3. Increase in plasma tau after HJ8.5 injection in different models expressing human tau.

(A) Plasma tau was analyzed before antibody injection (pre-bleed) and 48 hours after HJ8.5 injection in 2 month-old hTau mice (n=5), 3 month-old P301S mice (n=8) and 1 month old AAV-Syn-P301S (n=8, labeled as AAV). (B) Representative coronal brain sections of 1 month-old mouse with AAV2/8 mediated expression of P301S human tau under control of synapsin promoter (AAV-Syn-P301S), 3 month-old P301S Tau transgenic mouse (P301S), and a 1 month-old C57BL/6 wild type (WT) mouse stained with biotinylated anti-human tau antibody HJ8.5. Scale bars 600 µm in length. (C) Human tau levels measured by human tau specific ELISA in brain cortical RAB-soluble fractions of 2-month-old hTau mice, 3-month-old P301S mice and 1 month-old AAV-Syn-P301S mice (AAV). Values in A and C represent mean ± SEM.

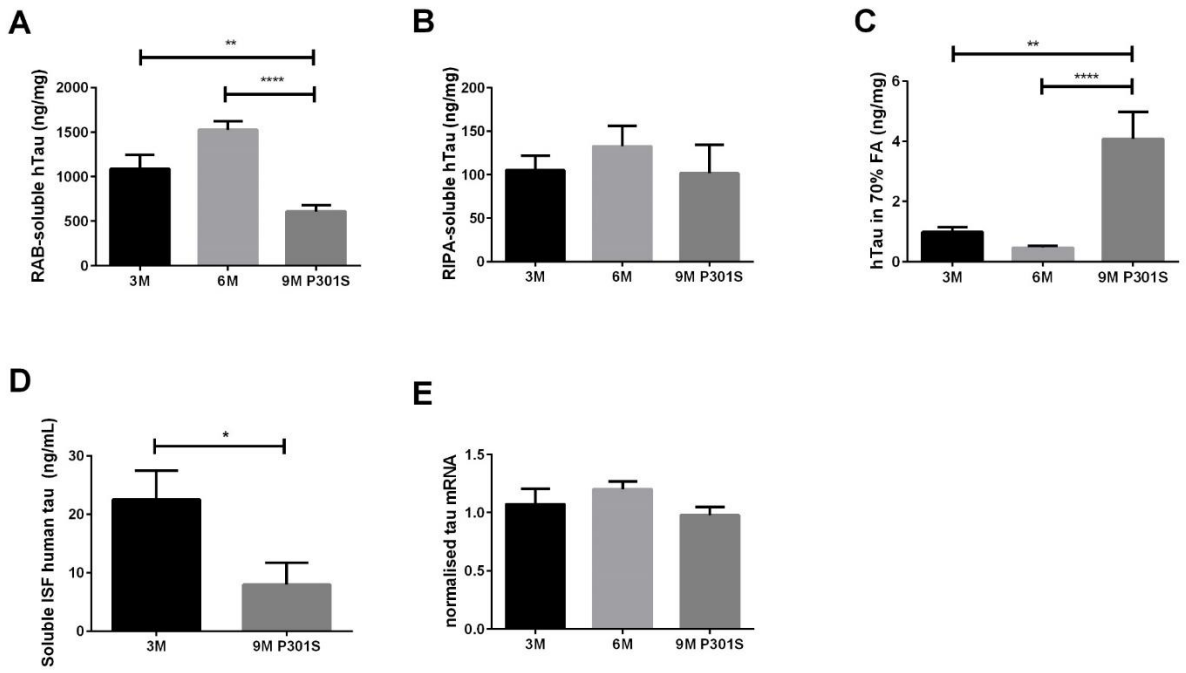

Figure 4.4. Soluble brain tau and ISF tau decrease and insoluble tau increases with the presence of tauopathy in P301S Tau Tg mice.

Levels of brain cortical RAB soluble (A), RIPA soluble (B) and 70% FA soluble tau (C) are shown in 3-month (n=10), 6-month (n=15) and 9-month (n=14) old P301S Tau Tg mice. Nine month-old P301S mice have significantly lower cortical RAB soluble and higher formic acid soluble tau compared to 3- or 6-month old P301S mice. One-way ANOVA followed by post-hoc Dunnett's test, **$p \leq 0.01$, ****$p \leq 0.0001$. (D) Soluble ISF human tau was measured by microdialysis in hippocampi of 3 month old (n=9) and 9 month old (n=7) P301S mice that were not treated with an anti-tau antibody. Nine month old mice have significantly lower ISF tau compared to 3 month old mice. *$p \leq 0.05$, 2-tailed t-test. (E) Levels of human tau mRNA in cortex from 3- (n=10), 6- (n=14) and 9-month (n=9) old P301S Tau Tg mice. Tau mRNA levels were normalized to total cDNA levels. 6 and 9 month-old P301S mice mRNA levels were normalized and compared to 3-month old P301S mice. No difference in human tau mRNA levels between groups were observed (one-way ANOVA post-hoc Dunnett's test, $p > 0.05$). Values represent mean ± SEM.

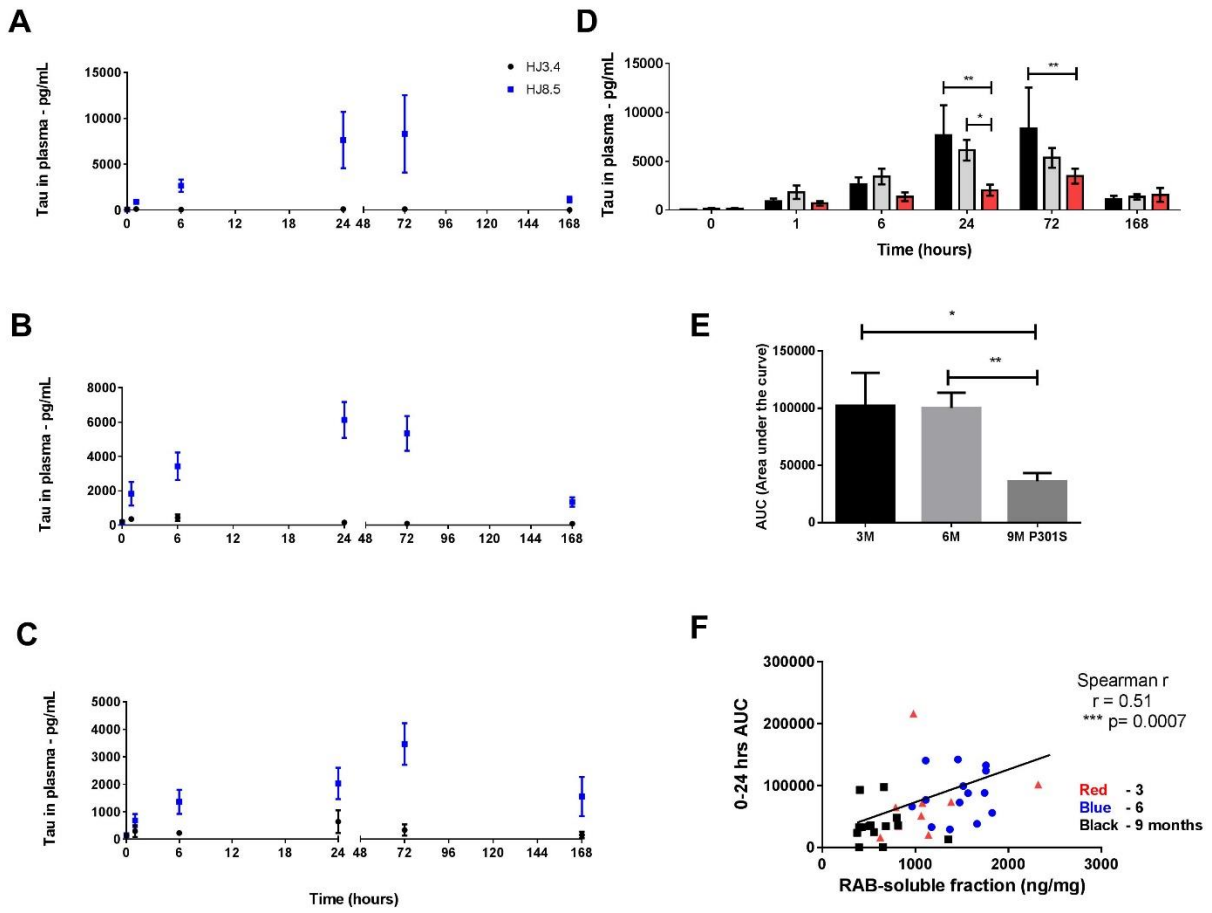

Figure 4.5. Anti-tau antibody mediated increase in plasma tau is significantly lower in 9 month old P301S Tau Tg mice and correlates with levels of soluble brain tau.

Plasma tau was measured before (0 hour time point) and following i.p injection of HJ8.5 (50 mg/kg) or anti-human Aβ antibody HJ3.4 (50 mg/kg) at 1, 6, 24, 72 and 168 hours in 3 (A), 6 (B) and 9 month (C) old P301S Tau Tg mice (n=10-15 mice/group). (D) Plasma tau levels measured in 3 (black bars), 6 (gray bars) and 9 month (red bars)-old P301S mice, before (0 hour time point) and after HJ8.5 i.p injection are presented in the bar graph. *p≤0.05, **p≤0.01, 2-way ANOVA with post hoc Dunnett test. (E) Area under the curve (AUC) of plasma tau (pg/ml x hours) was calculated for the first 24 hours after HJ8.5 injection in 3, 6 and 9 month old P301S mice. *p=0.04, **p=0.004, 2-tailed t-test. Values represent mean ± SEM. All plasma samples were analyzed on a Simoa HD-1 Analyzer. (F) Positive correlation is seen between plasma tau

levels over 24 hours after HJ8.5 injection (y-axis) and brain cortical RAB soluble tau (x-axis). Spearman r, r=0.51, ***p=0.0007. Blue squares, 3 month old mice, Red squares, 6 month old mice, Black squares, 9 month old mice.

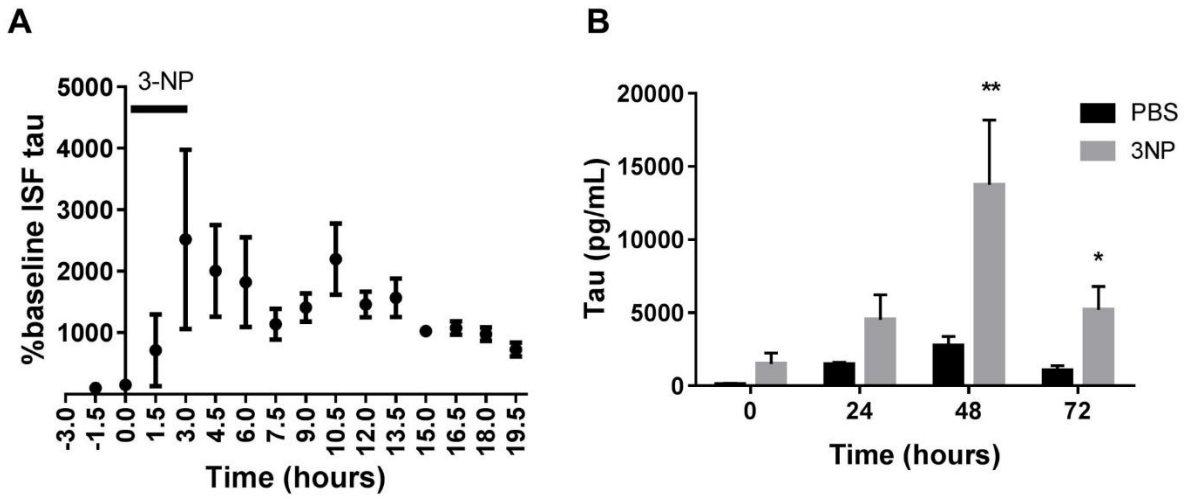

Figure 4.6. Injury to the striatum following administration of the mitochondrial toxin 3-nitropropionic acid (3-NP) leads to increase in human tau levels in the ISF as well as plasma following anti-tau antibody administration.

(A) Soluble ISF human tau was measured by microdialysis in the striatum of 8-9 month old P301S Tau Tg mice (n=4). After stable baseline collection, 3-NP was administered via reverse microdialysis for 3 hours and levels of tau were measured. Injuring the striatum causes significant elevation in ISF tau for 12 hours post 3-NP treatment. One-way ANOVA followed by post-hoc Dunnett's test $p<0.05$. (B) Four to five month old P301S mice were injected bilaterally in the striatum with either PBS (n=5) or 3NP (n=5, 100 nmol/µl). Four hours later, HJ8.5 at 50 mg/kg was i.p administered and plasma tau was assessed before (0 hour) and after 3-NP or PBS injection at 24, 48 and 72 hours. Compared to PBS treated animals, there was significant elevation of plasma tau in animals injured by 3NP treatment at 48 and 72 hours (2-way ANOVA with post-hoc Dunnett test, **$p<0.01$, *$p=0.029$).

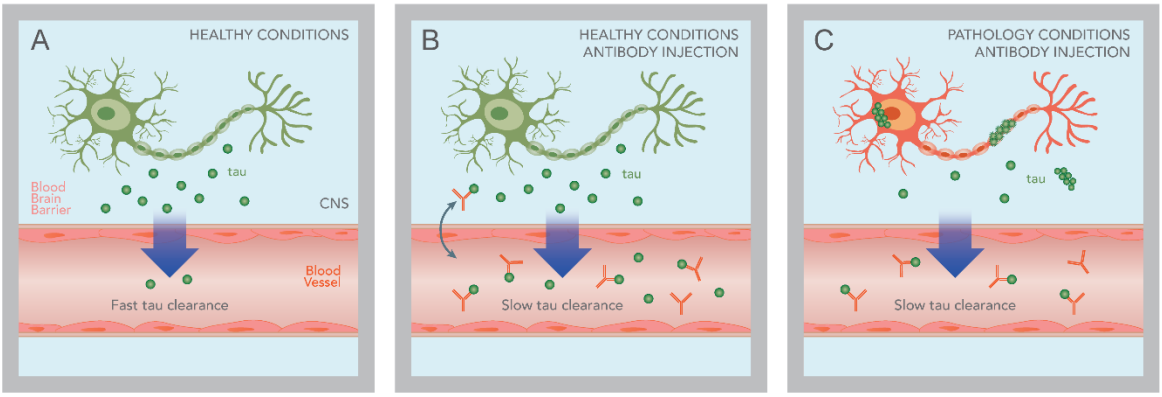

Figure 4.7. Model of plasma tau clearance in the presence and absence of anti-tau antibody.

(A) Tau is released into the ISF by neurons under healthy/physiological conditions. Some extracellular tau in the ISF and CSF reaches the blood where it is rapidly cleared. Green circles represent soluble, monomeric tau and green cell represents neurons under healthy conditions. (B) After peripheral administration of anti-tau antibody, tau exits the CNS where it binds to an anti-tau antibody (orange – Y shaped structures). Anti-tau antibody binding of tau extends the half–life of tau in plasma by slowing its clearance rate. Antibody-tau complexes can form either in plasma after tau exits the CNS or potentially within the CNS prior to efflux to plasma. (C) In pathological conditions such as in a primary tauopathy, tau becomes hyperphosphorylated and aggregates in neurofibrillary tangles and dystrophic neurites (clump of green circles). Under these conditions, levels of soluble ISF tau are decreased and insoluble tau is increased. After administration of an anti-tau antibody, less soluble, extracellular tau exits the CNS. The anti-tau antibody still slows plasma tau clearance but lower plasma tau levels are now detected than in healthy conditions because less extracellular tau is exiting the CNS. Red neuron represents neurons under pathological conditions.

4.7 Acknowledgements

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4.8 Footnotes

Author contributions: KY, TKP, and DMH designed the study. SS, JU, TW, and KY did statistical analysis. HJ, FS, MBF, JK, GR, TKP, and KY performed Simoa, ELISA and immunohistochemistry. PBV developed the formulation for humanized antibody and performed

biochemical characterization, stability and compatibility studies. ALB, DRK, PBV, IF, TW, JB, SSK, and HH collected and analyzed human data. KY and DMH edited and wrote the manuscript with critical revisions from TKP, HJ, GG, BLM, FS, MBF, NC, JU, ALB, DRK, PBV, TK, TW, JB, SSK, HH, and GR.

Competing interests: The other authors declare no competing interests.

Data and material availability: The material in this study generated in the lab of David Holtzman, including anti-tau antibodies, can be requested through the office of technology management at Washington University (https://otm.wustl.edu/).

Chapter 5 Discussion and future directions

5.1 Golden age of tau

A simple pubmed search for keywords ‘tau’ and ‘Alzheimer’ returns about 11000 search results. When the results are graphed by year a striking trend emerges: a near-exponential growth in the number of publications in the last 35 years. While 1984 just had 1 publication – “Immunoglobulin and other proteins in the cerebrospinal fluid of patients with Alzheimer's disease” (Chapel, Esiri, and Wilcock 1984) – 2017 saw an astonishing 1075, more than 3 publications a day. What has driven this intense interest in a once obscure cytosolic protein with an unglamorous binding role?

One big reason is the frustrating lack of disease modifying therapies in AD. Advances in medical science have certainly extended lifespan considerably. As the population ages, the incidence of dementia and neurodegenerative diseases (which, barring the small fraction of familial cases caused by Mendelian mutations, are quintessentially disorders of old age) rises. As a result near-Herculean efforts have been made to decipher the pathogenesis of neurodegeneration (and in particular, AD) in hopes to finding a cure. Setbacks suffered during multiple passive immunotherapies against abeta caused a gradual but firm shift towards finding alternative targets. Tau is an attractive target because of its involvement in many different neurodegenerative conditions. So after relative stagnancy for two decades, the field of tau study has seen a resurgence.

Another factor contributing to this rise in interest is the development and evolution of cutting-edge techniques in imaging, genetics, genomics and molecular biology. Advances in imaging

modalities have led to development and clinical trials of imaging tracers designed for early detection. We are now able to resolve various structural conformations of tau by high-resolution microscopy. Transgenic mouse lines have made modeling tauopathy a straightforward affair as these mice faithfully reproduce human tangle pathology. Mouse lines with regulatable tau expression provided the first *in vivo* evidence of trans-synaptic propagation. Human iPSCs have revolutionized the study of disease. They can be used to screen for drugs and model disease processes in a rapid manner. By mining massive datasets genomics researchers have been able to uncover novel mutations, rare variants and other disease related SNPs that have all spurred even more research. Breakthroughs in single-cell sequencing have made it possible to get a granular view of cell-type and brain region specific expression of not just tau but related genes, providing a better understanding of cellular interactions in disease.

In pathogenesis, one of the biggest unanswered questions about is the identity of tau species mediating prion-like spread of tau pathology. In diagnostics, it is the development of reliable imaging agents to aid in early detection of tau pathology. In therapy, it is the effectiveness of passive immunotherapy. The confluence of factors discussed above has made it very likely that we will have answers to these pertinent questions in the coming years. Early diagnosis is absolutely essential for therapeutic interventions and as such, biomarker efforts carry great importance, possibly more than ever.

Here is a brief summary of progress made in plasma biomarkers (particularly tau) by others in the field:

5.1.1 Tau as a biomarker

Impressive progress has been made in using plasma tau levels as biomarker for diagnosis, disease staging, and potential therapeutic monitoring. Currently approved diagnostics for AD include a PET scan for amyloid plaques as well as a CSF biomarker panel that, among other things, quantifies CSF total tau, phospho-tau (ptau), and the ratio of two amyloid-beta species. CSF studies are by nature invasive and are difficult to use for longitudinal studies. Unsurprisingly, recent efforts have focused on finding and validating a plasma biomarker(H et al. 2017, CC et al. 2018).

One study comparing healthy controls, mild cognitive impairment (MCI) and AD patients found higher levels of total tau and ptau181 in AD patients. Additionally, there was a positive correlation between ptau181 levels and tau tangle and amyloid plaque load in the brain, as measured by PET (Mielke et al. 2018).

Could this be used for other tauopathies? This question was addressed recently by a slew of studies looking at two different ptau species: ptau181 and ptau217. In addition to recapitulating earlier studies, this new work demonstrated that ptau181 can differentiate between different stages of disease pathology in AD. Cognitively normal or mildly impaired patients with high plasma ptau181 were more likely to develop AD in this analysis. Plasma ptau181 was also

shown to be able to differentiate between AD and other tauopathies(Shorena, Mattsson, et al. 2020).

Two other studies performed similar analyses with ptau217 and found that it performed even better than ptau181 at predicting progression to AD dementia as well as distinguishing AD from other tauopathies. A striking feature of these studies is while one of them studies plasma ptau217 with an ELISA(Shorena, Stomrud, et al. 2020), the other studied CSF ptau217 using advanced mass spectrometry techniques(NR et al. 2020), lending credence to the putative status of ptau217 as a superior biomarker.

As always, more work will be needed to validate the clinical applicability of these biomarkers but the fact that plasma levels of these ptau species appear to correlate well with pathology load as measured by multiple different modalities i.e. PET, CSF ELISA indicates high value in this approach.

5.2 Quo vadis?

The work presented here aimed to make a small contribution to understanding tau's role in neurodegeneration. Although we were not able to find evidence of tau oligomers *in vivo* in ISF, our development of high-sensitivity assay as well as preliminary *in vitro* results will hopefully serve as a scaffold for future work in this direction.

Our work on extracellular tau clearance strongly suggests that dural lymphatics play a major role. One notable study refined the anatomical location and distribution of dural lymphatic

veseels, showed that a subsection of these vessels is probably responsible for draining a majority of CSF macromolecules, and that this network is negatively affected in aging (JH et al. 2019).

Finally our published work identifying correlation between plasma tau and CNS soluble tau has important implication for design of therapeutics and for monitoring disease progression. Our approach of using an antibody to increase plasma half-life of tau in tauopathy can be used to study tau levels in other models as well. As mentioned above further work doing direct measurement of plasma tau levels (Shorena, Mattsson, et al. 2020, Shorena, Stomrud, et al. 2020, NR et al. 2020) has shown great promise to be an effective biomarker not just for AD but for other tauopathies.

The life of tau, this seemingly prosaic axonal protein with no definitively established function, is complicated and multi-faceted, and as we unravel more of its life story we are bound to get closer to solving neurodegeneration.

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Curriculum vitae

Tirth K Patel

Education

Washington University in St. Louis school of medicine, Medical Scientist Training Program (2011 – present)

M.D., expected graduation date May 2020

Ph.D in neurosciences, graduation date May 2020

Thesis mentor: Dr. David Holtzman, Department of neurology

University of California, Los Angeles, Bachelor of Science (2007-2011)

Magna cum laude

Major: Microbiology, Immunology, Molecular Genetics

Minor: Biomedical research

Teaching (graduate assistant)

"Principles of the nervous system", Washington University in St. Louis Fall 2014

Publications

Patel TK, Habimana-Griffin L, Gao X, Xu B, Achilefu S, Alitalo K, McKee CA, Sheehan PW, Musiek ES, Xiong C, Coble D, Holtzman DM. 2019. Dural lymphatics regulate clearance of extracellular tau from the CNS. Mol Neurodegener 14:11. PMCID: 30813965

Patel TK, Holtzman DM. 2018 Dual therapy for Aβ amyloidosis in AD: A successful one-two combo. J Exp Med, 215(5):1267-1268. PMCID: PMC5940274

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Holth J, **Patel T**, Holtzman DM. 2017 Sleep in Alzheimer's Disease - Beyond Amyloid. Neurobiol Sleep Circadian Rhythms, 2:4-14. PMCID: PMC5312809

Musiek ES, Xiong DD, **Patel T**, Sasaki Y, Wang Y, Bauer AQ, Singh R, Finn SL, Culver JP, Milbrandt J, Holtzman DM. 2016 Nmnat1 protects neuronal function without altering phospho-tau pathology in a mouse model of tauopathy. Ann Clin Transl Neurol, 3(6):434-442. PMCID: PMC4891997

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Conferences and meetings

American Neurological Association annual meeting 2019

Society for Neuroscience 2014, 2016

Washington University Neuroscience retreat 2013-2018 (Internal)

Hope Center annual retreat 2013-2018 (Internal)

Washington University MSTP retreat 2015 – 2018 (Internal)

Talks

Knight Alzheimer Disease Research Center seminar 2016, 2018 (Internal)

Awards

American Neurological Association travel award recipient 2019

American Neurological Association poster award recipient 2019

Extracurricular activities

Volunteer, Brain Discovery outreach program 2017-2018

WUSTL quiz bowl 2011-2017

Executive board member, Sling health student biotech incubator 2015-2017

Volunteer, Students Teaching AIDS to Students (STATS) 2012-2015

Member, WUSM Arts commission 2011-2014

Editor, Hippocrene literary magazine 2012-2013

Volunteer, Saturday Neighborhood Health Clinic (SNHC) 2011-2013