Extracellular Tau Oligomers Induce Disruption of Endogenous Tau Distribution, Invasion of Tau into the Somatodendritic Compartment and Axonal Transport Dysfunction

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Abstract

Insoluble hyperphosphylated aggregates of the microtubule-associated protein tau define a subset of neurodegenerative disorders known as tauopathies, of which Alzheimer's Disease is the most prevalent. Extracellular tau can induce the accumulation and aggregation of intracellular tau, and tau pathology can be transmitted along neural networks, in a manner that recapitulates the temporal spread of pathology observed upon post-mortem analysis of diseased tissue. There are six splice variants of central nervous system tau, and various oligomeric and fibrillar forms are associated with neurodegeneration *in vivo*. The particular extracellular forms of tau capable of transferring tau pathology from neuron to neuron remain ill-defined as do the consequences of intracellular tau aggregation on neuronal physiology. The work in the dissertation presented here was undertaken to compare the effects of extracellular tau monomers, oligomers and filaments comprising various tau isoforms on the behavior of cultured neurons. This work demonstrates that 2N4R or 2N3R tau oligomers provoked aggregation of endogenous intracellular tau much more effectively than monomers or fibrils, or of oligomers made from other tau isoforms, and that a mixture of all 6 isoforms most potently provoked focal, intracellular tau accumulation. These effects were associated with invasion of tau into the somatodendritic compartment. Preliminary data indicate that this somatodendritic tau accumulation may be due to disruption of ankyrin G and βIV spectrin, key components of the axon initial segment. Finally, this work shows that 2N4R oligomers perturbed fast axonal transport of membranous organelles along microtubules. Intracellular tau accumulation was often accompanied by increases in the

run length, run time and instantaneous velocity of membranous cargo, and these alterations in fast axonal transport were diminished in neurons not expressing tau. This work provides a more physiological model of tau uptake in neurons and indicates that extracellular tau oligomers can disrupt normal neuronal homeostasis by triggering focal tau accumulation and loss of the polarized distribution of tau, and by impairing fast axonal transport. Additionally, by identifying species involved in cellular dysfunction, it provides a target for much needed future therapies in AD and non-AD tauopathies.

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Abbreviations

Aβ – Beta Amyloid

AD – Alzheimer's Disease

AIS – Axon Initial Segment

APP – Amyloid precursor protein

CaMKII - Ca^{2+}-calmodulin dependent kinase II

CCR – Cell Cycle Re-entry

CNS – Central nervous system

CBD – Corticobasal Degeneration

DAPI - 4',6-diamidino-2-phenylindole

FBS – Fetal Bovine Serum

FTD – Fronto-temporal Dementia

FTDP-17 - Frontotemporal dementia and parkinsonism linked to chromosome 17

HBSS – Hank's Balanced Salt Solution

IHC - Immunohistochemistry

IPTG - Isopropyl β-D-1-thiogalactopyranoside

KO – Knockout

MARK - Microtubule affinity-regulating kinase

MCI – Mild Cognitive Impairment

MTBR – Microtubule binding repeat

MAP – Microtubule-associated protein

NFT – Neurofibrillary tangle

PBS – Phosphate buffered Saline

PKA - cyclic-AMP dependent protein kinase

PNS – Peripheral Nervous System

PRD – Proline-rich Domain

PrP – prion protein

PSP – Progressive Supranuclear Palsy

ROI – Region of Interest

TCEP - Tris(2-carboxyethyl)phosphine

WT – Wild-type

Chapter 1 -

Introduction

Chapter 1: Introduction

Insoluble aggregates composed of the microtubule associate protein tau characterize a set of neurodegenerative disorders known as tauopathies, of which the most prevalent and well known is Alzheimer's disease (AD). While these disorders have been researched for more than a hundred years, there is still a great deal to be discovered about how these diseases begin, how they progress throughout the brain and the molecular mechanisms that are eventually responsible for cell death.

1. Historical Context

The recorded history of cognitive deficits we now associate with neurodegenerative disorders such as AD goes back as far as the ancient Egyptians. While they believed that cognition resided in the heart or lungs, they recognized that certain memory deficits were associated with aging [1]. This idea of chronic neurological problems was further expounded upon in the late second century by Aretheus of Cappodocia, who wrote of *leresis,* a dementia affecting old age [2], and may have been the first to distinguish this condition from acute neurological disorders [1]. Galen, a contemporary of Aretheus, wrote that these disorders resulted from cerebral impairments and hypothesized they could be due to primary dysfunction in the brain, or related to systemic problems. Galen's writing would be translated into Arabic and, while still primitive in their understanding of human physiology, his ideas on mind and body would greatly influence thinking on cognition in the Islamic Golden Age [1,3].

Not a great deal is known of the philosophy regarding cognitive dysfunction throughout the subsequent Middle Ages, at least in the western world, possibly due to the prominence of more pressing sweeping health concerns, such as the Black Death [4]. Additionally, the Church had risen in strength during this period and mystical forces were used to explain most natural phenomena, with philosopher and scientist Roger Bacon indicating in the 13th century that "senility is a consequence of the original sin [1]." He did also note however that "old age is the home of forgetfulness [4]."

A more modern view of these disorders began to take hold amid the Age of Enlightenment. During this period, psychiatrist Phillipe Pinel made what is thought to be one of the earliest clinical descriptions of a variety of symptoms he classified as dementia (*demence*) or "the abolition of the thinking faculty" [4-6]. Among these symptoms were loss of memory, loss of judgement, behavioral abnormalities and emotional disconnection, now known to be symptoms of AD and related disorders [5]. It was around this time that anatomical and histopathological observations of these patients were also becoming more common. In 1864, British physician Samuel Wilks provided a definitive description of cerebral atrophy leading to decreased brain weight, a phenomenon we now know is due to profound neuron loss in the brains of these patients [7]. In the early 1890's, French doctors Paul Blocq and Gheorghe Marinescu were the first to describe microscopic plaques in brain tissue, which only a few years later were implicated in senile dementia [8,9].

Another clinician working in the field at the time was a German physician from the Max Plank Institute named Alois Alzheimer [10,11]. At a conference of German

psychiatrists in 1906, Alzheimer reported on the case of a 51-year-old woman named Auguste Deter. Identified as Auguste D., she initially presented with paranoia, followed by sleep disruption, memory loss, aggressiveness, crying and confusion, with these symptoms becoming progressively worse until her death in 1906 [12]. Following her death, Alzheimer analyzed her brain utilizing a silver impregnation technique for staining neural tissue first discovered by Camilo Golgi [13] that was later improved upon by Max Bielchowsky [14]. Upon examination of this stained tissue he identified what are now known as the definitive histopathological hallmarks of the disease that now bears his name: senile plaques and neurofibrillary tangles (NFTs) [15,16] which were subsequently show to be composed of the Beta-Amyloid peptide (Aβ) and the microtubule associated protein, tau, respectively [17].

2. Alzheimer's Disease

While Alzheimer made his initial observations in the early 20th century, it wasn't until 1976 that AD was acknowledged as the most common form of dementia, and a major cause of death [18]. Today AD is still the most common for form of dementia, accounting for anywhere from 60 to 80 percent of all cases [19]. AD is the 6th leading cause of death in the United States and the only disease in the top ten causes of death for which there exists no preventative measures or disease modifying therapies [20]. As of 2013, AD is the costliest disease in the United States, surpassing even heart disease and cancer [21].

While Alzheimer's can only be definitively diagnosed post-mortem, it's clinical presentation is formally defined [22]. The diagnostic criteria for AD were initially based

predominantly on a clinician's judgement, taking into account cognitive tests, general neurological exams and testimony from family and friends [23]. AD has many early symptoms, however most patients present with a gradually memory loss, as cell death in AD has been shown to originate in the hippocampal formation, which governs memory consolidation. This can be accompanied by executive dysfunction, such as alterations in attention span and difficulties in problem solving. As the disease progresses, patients can have language difficulty, loss of insight, difficulty in visuospatial processing and personality changes such as withdrawal, diminished initiative and depression. Eventually patients lose the ability to perform routine tasks such as bathing and dressing one's self, known as Activities of Daily Living (ADL). The most severe cases can progress to a point that individuals are immobile and unable to speak, completely dependent on caregivers [24].

Recent guidelines established by a joint group consisting of the Alzheimer's Association and the National Institute of Aging established three stages of AD. Initially, there exists the designation of a pre-clinical, or pre-symptomatic stage of AD. An early symptomatic stage was labeled "Mild Cognitive Impairment (MCI) due to Alzheimer's", which usually lasts 2-5 years. Finally, as the disease progresses with regards to core diagnostic criteria, the disease is classified as "Dementia due to Alzheimer's" [25]. As the disease process is thought to begin years or even decades prior to the onset of symptoms, those with preclinical AD could greatly benefit from interventions that may come available with the discovery of accurate, non-invasive biomarkers, such as advanced imaging techniques [26].

In recent years, such tests for various biomarkers have been incorporated into the diagnostic process. Structural imaging of the brain utilizing magnetic resonance imagery (MRI) or computed tomography (CT) scans can identify brain atrophy associated with neurodegeneration, and rule out causes such as stroke or focal ischemia [27]. AD has been associated with decreased glucose metabolism in affected regions which can be identified by positron emission tomography (PET) utilizing fluorodexoyglucose [28]. Additionally, there are a number of imaging compounds approved for clinical use that selectively bind to protein aggregates, with still more in clinical trials. The most notable of these is Pittsburgh compound B which binds deposits of Aβ, allowing for PET imaging of pathology in living individuals[29]. Several PET tracers are also in development for tau imaging [30-33]. Levels of total tau and Aβ in the CSF show alteration early in their disease course (increasing and decreasing respectively) [34] and certain tau phosphoepitopes (Thr181 and Thr213) [35-38] in the CSF may be indicative of disease, although these tests require a lumbar puncture. Current studies are focused on identifying measurable changes in pre-symptomatic individuals through less invasive samples, such as blood or urine [26].

As of 2017, about 5.5 million Americans are living with AD, with about 5.3 million of these older than 65 [39]. Age is the highest risk factor, and with the post-World War II baby boom generation beginning to reach age 65, combined with improved health care, social and environmental advantages contributing to increased lifespans [40], the number of people with AD is projected to reach 7.1 million by 2025. These projections may reach anywhere from 13.8 to 16 million by 2050 unless effective therapies are soon developed [39]**.** Care for patients with AD amounted to $259 billion in 2017, representing the largest

financial burden of any disease, and is projected to reach 1.1 trillion per annum, not accounting for inflation [39].

While the root causes of AD remain unknown, several risk factors increase one's susceptibility. As discussed previously, the single highest risk factor for AD is age, with the vast majority of cases occurring in individuals 65 or older. 3 percent of people age 65-74, 17 percent of people age 75-84, and 32 percent over 85 years of age have AD [39]. Having a first-degree relative with AD, even in the absence of known genetic factors, puts an individual at higher risk [41]. This risk is partially, though not entirely due to the effects of the *APOE* gene, which encodes a cholesterol transporting protein [42]. *APOE* is found as three different alleles, ε2, ε3, and ε4. Those with one copy of the ε4 allele have their risk of developing AD tripled versus those with ε3, and two ε4 alleles confers a 12-fold increased risk. Those with one or two ε2 alleles however show a decreased risk. The reasons for these changes in susceptibility remain elusive [42,43].

One cause of AD and other neurological disorders that has received increased coverage in recent years is traumatic brain injury (TBI) [44]. Increased incidences of neurodegenerative disease such as chronic traumatic encephalopathy (CTE) have been identified in populations who suffer repeated blows to the head, such football players, boxers and combat veterans, and as few as one concussion has been demonstrated to increase one's likelihood of developing AD [45-48]. Additionally, factors associated with increased risk of cardiovascular disease such as tobacco use, obesity, diabetes, hypertension and high cholesterol [49-51] are also associated with higher instances of dementia.

In addition to various risk factors, there have been many germline mutations identified in genes for the amyloid precursor protein (APP), presenilin 1 and presenilin 2, discussed in detail later, which effect deposition of Aβ and cause familial early onset AD (fAD) [52]. While patients with these mutations represent a small percentage of total cases, estimated at 1% or less, these mutations are fully penetrant, with an age of onset much earlier in life [53]. People with Down syndrome almost invariably get AD, due to their third copy of chromosome 21, which encodes the protein from which Aβ peptides are derived, APP. Nearly all people with Down syndrome will develop plaques and tangles by the age of 40, and roughly 30 percent in their 50s symptomatic for AD [54]. It is worth noting that no mutations in the gene for tau are associated with fAD, although tau mutations are known to be causative for another category of neurodegenerative conditions [55,56], which will be discussed later (section 1-11).

Along with protein deposition, many neurodegenerative disorders are characterized at both a histological level and a gross anatomical level by profound neuron loss in various regions of the brain, with the region exhibiting neuron death often correlating with clinical presentation [24,57]. At a cellular level, the exact etiology of neuron dysfunction and death is uncertain however many neurophysiological alterations have been characterized. The primary dysfunction in these cells is thought to be synaptic failure. Patients with MCI exhibit a 25% reduction in the amount of synaptophysin, a presynaptic protein [58]. In fact, as the disease progresses the rate of synapse lose is higher than neuron loss and correlates more closely with cognitive decline [59,60]. This synaptic function is associated with disruptions in neurotransmitter release, loss of post-

synaptic ion channels such as NMDA and AMPA receptors [61,62], and impairment of long-term potentiation, a key component of memory formation [63]. It is of interest to note that while no disease modifying therapies are available for AD, there are two classes of FDA approved drugs that work at the synapse with minor clinical efficacy; one class is acetylcholinesterase inhibitors, which block the enzyme responsible for breakdown of the neurotransmitter acetylcholine, allowing for prolonged action at the synapse. The other is an NMDA receptor antagonist, which blocks the post-synaptic action of the neurotransmitter glutamate, a source of excitotoxic effects on neurons [64].

Mitochondrial dysfunction is common in AD neurons, with these neurons experiencing considerable oxidative stress. Models of AD indicate that this oxidative damage may precede pathological changes [65], producing toxic molecules through oxidation of membrane lipids and essential proteins [66]. This leads to impaired glucose transport and imbalances of ions such as calcium, which further strain the neuron [67]. In later stages of the disease, a chronic state of glutamatergic receptor activation may also be responsible for an increase in cytosolic calcium and provoke neuronal damage [68]. Additionally, insulin signaling is disrupted in these neurons leading to metabolic dysfunction, and high serum glucose can upregulate GSK3β, a key tau kinase [69]. The final neuronal defect worth noting here is an impairment in axonal transport [17], which will be discussed in depth in a later section.

Not surprisingly the majority of the research into the cellular and molecular etiology of AD and related disorders has focused on the functions of Aβ and tau, the two proteins responsible for the characteristic pathologies described by Alzheimer more than

a century ago [24]. In the subsequent sections I will give a brief description of Aβ and its role in AD, followed by an in-depth description of tau, which is the focus of the research projects presented herein.

3. Aβ

Aβ is a 36-43 amino acid peptide that results from the sequential cleavage of APP [17]. APP processing can be broken down into two main pathway: the amyloidogenic and the non-amyloidogenic. In the non-amyloidogenic pathway, APP is first cleaved by the enzyme alpha-secretase at amino acid Lys686, followed by an intramembrane cleavage by another proteolytic enzyme, gamma-secretase, at amino acids Val710 or Ala712. These two cleavages produce the N-terminal fragment sAPPα, the APP intracellular domain (AICD) and a peptide called p3. In the amyloidogenic pathway, APP is instead first cleaved by the beta-secretase enzyme, also known as BACE, at amino acid Met670. This cleavage is then followed again by an intramembrane cut by gamma-secretase, resulting in production of N-terminal sAPPβ, the AICD**,** and the Aβ peptide (Fig. 1-1). Variability in the site of gamma secretase cleavage can produce Aβ of assorted lengths, with 40 ($A\beta_{1-40}$) or 42 ($A\beta_{1-42}$) amino acid peptides being the most common[70]. Alterations in the ratio of $A\beta_{1-42}$ to $A\beta_{1-40}$ have been demonstrated to affect the propensity of these peptides to aggregate [71].

Mutations in the APP gene that favor cleavage by β-secretase and lead to increased Aβ production are fully penetrant for familial Alzheimer's Disease (fAD).

Mutations in presenilins 1 and 2, the catalytic subunits gamma secretase, increase the levels of AB_{1-42} relative to AB_{1-40} and also cause fAD [52]. As discussed previously, triplication of the APP gene and the associated increase in Aβ in Down syndrome also cause extensive AD pathology, further implicating overproduction of the Aβ peptide in disease pathogenesis.

This genetic evidence, combined with demonstrated toxicity of Aβ, have led to the proposal of the "amyloid cascade hypothesis," which states that the formation of NFTs, neuronal dysfunction and neuron death seen in AD result from overproduction of Aβ [72,73] However experiments have shown that several effects of toxic Aβ, such as synaptic damage, microtubule loss and excitotoxicity are ameliorated in the absence of tau expression [74], so it is clear that tau, the other protein found aggregated in AD, is necessary for Aβ to enact its most toxic effects. The exact mechanism of their interaction, however, is unknown.

4. Tau

Tau is a microtubule associated protein (MAP) encoded by the MAPT gene which is located on chromosome 17q21.31 [75] and spans approximately 150kb [76]. The primary tau transcript comprises 16 exons, of which 11 encode the majority of tau in the central nervous system (CNS) [56] (Fig 1-2). CNS tau exists as 6 distinct isoforms resulting from the differential splicing of a single MAPT transcript. These isoforms are identified by the presence of 0, 29 or 58 amino acids, encoded by exons 2 and 3, towards the N-

terminus of the protein, and inclusion or exclusion of a fourth microtubule binding repeat (MTBR) encoded by exon 10 [76]. Inclusion of exon 3 is contingent on exon 2 being present as well [77]. For the purposes of this thesis, these isoforms will be identified by a common nomenclature, using the number of N-terminal exons followed by the number of MTBR present: 2N4R, 2N3R, 1N4R, 1N3R, 0N4R and 0N3R (Fig. 1-2). In humans, these CNS isoforms range from 352 to 441 amino acids in length [78]. In the peripheral nervous system (PNS), tau sometimes contains a large N-terminal exon (4A), which is not expressed in CNS tau. This isoform contains 242 extra residues and is referred to as "big tau" [79]. Neither exon 6 nor 8 have been detected in human tau transcripts or protein [80].

5. Tau structure

Tau can be separated into various domains based on amino acid content and ability to bind microtubules. It is commonly divided into four regions: An N-terminal domain; a proline-rich region from amino acids 150-243; a repeat region from residues 244 to 369, containing the microtubule binding repeats; and a C-terminal domain [76,81] (Fig. 1-3).

The N-terminal domain is negatively charged and optimally contains the exons 2 and 3 inserts, which when present add to its acidic character [82]. The proline-rich domain (PRD) is highly positively charged, and contains many phosphorylation sites that will be discussed later [78]. Many functions have been attributed to the PRD. It contains seven PXXP motifs, which is a binding site for proteins containing SRC-homology 3 (SH3)

domains, such as the src-family non-receptor tyrosine kinase, fyn [83], and phospholipase Cγ [84]. The consequences of this binding are discussed later. Additionally, this region is able to bind spectrin and actin, pointing to a role in cytoskeletal function [85,86], and has been shown to indirectly regulate the binding of the MTBRs to microtubules [87].

Combined, the N-terminal domain and the PRD are referred to as the projection domain, as they project off the microtubule surface [81]. The projection domain may additionally interact with the neuronal plasma membrane, with tau acting as a mediator between the cytoskeleton and the membrane [82].

The three or four MTBRs in the next domain are composed of 18–amino-acid repeats that are highly conserved, separated by 13 or 14 amino acid inter-repeat domains that are less conserved [88]. These repeats share ~70% homology with two other MAPs, MAP4 and MAP2 [89]. Following the MTBRs is the C-terminal tail, which has both basic and acidic subregions, and like the PRD indirectly regulates microtubule binding [76].

Small-angle X-ray scattering and nuclear magnetic resonance (NMR) have shown that monomeric tau is an intrinsically unstructured protein, lacking any stable secondary or tertiary structure [90]. On its own, tau has little tendency for aggregation. It is highly soluble due to its hydrophilicity [78], stable under acidic conditions, and also very heat stable [91]. Some small structural features have been attributed to tau however. By NMR it has transient short elements of α-helical and β-strands, and poly-proline helices in the PRD [90] (Fig 1-3). Additionally, tau has been shown to preferentially adopt what is referred to as a ”hairpin” or “paperclip fold” formation [92,93], in which the N- and C-termini are in close proximity to each other and the MTBRs.

6. Tau function

Binding and stabilizing of microtubules

Tau is enriched in axons of the central nervous system and peripheral nervous system where, as previously discussed, it interacts with microtubules through the MTBR and flanking regions [78]. Of tau's many functions in neurons and particularly the axon, the first to be widely characterized is its role in the binding and stabilizing of microtubules [91,94,95] Tau was first identified in 1975 by Weingarten and colleagues [91] as a "heat-stable protein essential for microtubule assembly" and was initially isolated associated with tubulin in porcine brain. This study demonstrated that tau was able to stimulate tubulin polymerization, and it was in fact called ***t****au* because of its association with **t**ubulin. Tau is present at roughly 2 μM in healthy normal neurons and binds microtubules with a K_d of 100 nM, implying that under normal physiological conditions most tau is bound to microtubules [96]. In the presence of tau, tubulin demonstrates an increase in its assembly rate and a decrease in its depolymerization rate [97]. The ability of tau to bind microtubules is negatively regulated by phosphorylation. Studies using NMR and mass spectrometry have shown that tau binds tubulin in a hydrophobic region between the αβ tubulin dimers. Covalent crosslinking showed interactions between lysine residues 225, 240, 257, 311 and 383 on tau and the side chains of lysines 336 and 338 in α-tubulin, with no cross-links observed with β-tubulin [98]. When tau is bound to microtubules, two hexapeptide motifs in the second and third MTBR that are important for the assembly of tau into aggregates, ^{275}VQIINK^{280} and ^{306}VQIVYK^{311} can form a local hairpin structure in 4R

isoforms [98]. Levels of tau correlate with levels of assembled tubulin during neurite outgrowth and extension in PC12 cells, a pheochromocytoma cell line with neuroblast lineage that can be induced to produce tau [82,99,100]. One study also indicates that neurons from a specific tau-null mouse line have a deficit in neurite extension [101], and a decrease in tau expression by siRNA can decrease the length of axons [102].

Regulation of axonal transport

In neurons, due to their extreme polarity, molecular motors are required to transport cargo form their origins in the soma along the length of the axon and to the synapse [103]. Tau has been shown to play an integral role in the regulation of axonal transport by altering the function of molecular motors responsible for anterograde and retrograde transport respectively.

Microtubules in the axon are polarized, with their plus ends directed towards the synapse and their minus ends toward the soma. Traffic toward the plus end is powered by kinesins [103]. The superfamily of mammalian kinesin proteins comprises 45 genes, of which 38 are expressed in the brain [104]. Kinesin is grouped into multiple subfamilies based structural and functional homology, with subfamilies kinesin-1, kinesin-2 and kinesin-3 contributing to axonal transport [105].

Kinesin-1 family proteins transport a number of cargoes, such as RNAs, proteins, vesicles and organelles. These motors are composed of a dimer of Kinesin heavy chain molecules, KIF5A, B, or C and a dimer of kinesin light chains [106]. Kinesin-2 proteins are made up of homodimer or heterodimer motor complexes, and drive the transport of

plasma membrane precursors, N-cadherin, β-catenin, and choline acetyl-transferase. Kinesin-2 has also been demonstrated to associate with Rab7-positive vesicles [107]. Kinesin-3 complexes transport synaptic vesicle precursors and dense core vesicles [108], with the exception that dense-core vesicles transporting the neurotrophic factor, BDNF (brain derived neurotrophic factor), are transported by Kinesin-1 [109,110].

Traffic towards the minus end of microtubules is achieved by cytoplasmic dynein. Whereas kinesins are encoded by a large family of genes, the motor domain of dynein is the product of a single gene [111]. This motor domain comprises two dynein heavy chains which form a dimer through their n-terminal tails, while numerous light, intermediate and light-intermediate chains associate with these tails to form various cargo binding domains. The variance in these extra subunits may allow for organelle specific recruitment to the complex [111]. Most functions of dynein are facilitated by dynactin, a multiprotein complex of which the Arp1, p25 and p27 subunits are implicated in cargo binding. A dimer of the subunit $p150^{Glued}$ projects from Arp1 and binds both dynein and microtubules [112].

Tau has been demonstrated to affect trafficking by both kinesin and dynein to varying extents. Overexpression of tau in non-neuronal cells inhibits traffic of membrane bound vesicles, mitochondria and ER [113]. Kinesin in this case demonstrated higher inhibition by tau, the result of which was cargo accumulating at the minus end of microtubules. Tau can also regulate the attachment and detachment of motors transporting organelles and vesicles by competing for microtubule binding [114]. Tau was further shown to effect the number of motors engaged at any time, which affected the

rate of transport in these cases [115]. Finally, a study demonstrated that individual fluorescently-labeled kinesin and dynein motors, when coming into contact with individually labeled molecules of tau bound to microtubules, showed an increased propensity to detach or pause, in the case of kinesin, and reverse direction in the case of dynein [116]. This effect is dependent on the concentration of tau, providing a model by which detachment of motors is regulated by an increasing tau gradient towards the distal axon [116,117].

Nuclear Tau and Dendritic tau

There have been some reports of tau located in the nucleus of both neurons and non-neuronal cells [118,119]. While the exactly role of nuclear tau has yet to be established, it may provide a protective role with regards to the integrity of genomic DNA, and both cytoplasmic and nuclear RNA. For example, mild heat stress of neurons resulted in accumulation of dephosphorylated tau in the nucleus, where tau bound DNA and protected it from heat shock related damage, effects absent in tau knockout neurons [120]. Small nuclear tau inclusions have been identified postmortem in AD, and recently in Huntington's Disease as well [121]. Reports of tau in dendrites under physiological conditions are contentious, though it has been hypothesized to play a part in synaptic plasticity, as activation of synapses by pharmacological means has been shown to induce translocation of endogenous tau to the synapse in both cultured mouse neurons and brain slices [122].

Roles in signaling pathways

Tau has been shown to play a part in some signaling pathways within the neuron. As mentioned previously, tau contains several binding motifs in the PRD that bind SH3 domains. Tau has been shown to interact through these motifs with the src-family tyrosine kinase, fyn. Tau immunoprecipitated with both fyn and PSD95, a post synaptic scaffolding protein, and absence of tau suppresses fyn translocation to postsynaptic sites. Even though tau expression in the dendrites is very low, it is hypothesized that tau delivers fyn to dendrites where the latter phosphorylates the NMDA-receptor subunit 2 at the post-synapse, which stabilizes its interaction with PSD95 and augments glutamate signaling.

This same region interacts with phospholipase C-γ (PLC-γ) enzymes. PLC-γ is normally activated by phosphorylation, but in a cell free environment was shown to be activated independently of phosphorylation in the presence of tau. This activity was enhanced by unsaturated fatty acids such as arachidonic acid. Tau may interact with the enzyme's substrate in addition to PLC-γ to facilitate cleavage of the phospholipid [84]. Tau has been shown to associate with PLC-γ in SH-SY5Y cells, a neuron-like cell line [123].

7. Tau Knockout mice

Mice lacking the tau gene have been generated to identify other functions of tau. The first such tau knockout (TauKO) model showed that no overt pathology existed in tau-null mice, although they exhibit increased expression of microtubule associated protein 1A (MAP1A) which may compensate for some tau functions [124]. Crossing of these mice

with MAP1B-null mice is lethal within one postnatal month, pointing to compensation by MAP1B as well [125]. While these mice demonstrate normal life span and fecundity, several phenotypes have been described. Characterized by a battery of behavioral tests at 10 to 11 weeks of age, these mice exhibited impaired motor function by multiple metrics, impaired learning memory, and hyperactivity. Other tests such as fear conditioning were performed, but the results were confounded by the hyperactive phenotype [126]. Early studies on another line [101], indicate no behavioral phenotypes up to 7-10 months of age. Several characterizations of these mice indicate they exhibit normal behavior by a number of behavioral tests for metrics such as anxiety and exploration, learning and memory, and motor function [127]. It is worth noting however that these mice [101] are the line obtained by our lab and used in the following studies, and anecdotally they exhibit hyperactive behavior from an early age. They often continuously run in circles, although the exact cause of this behavior is undetermined. Two more mouse lines have been developed as tools for neurons specific expression of cre recombinase or EGFP, neither of which have identified behavioral deficits [81,128].

At a neuronal level, some phenotypes have been described in these animals. Adult neurogenesis in the hippocampus is impaired in the GFP expressing knockouts [129], while the Dawson mice show no changes in adult neurogenesis and instead exhibit deficits in migration of these newborn neurons from the subgranular zone into the granular neuron layers, both pointing to some role for tau in neurogenesis. At 12 months, the Dawson tau knockout mice exhibit intraneuronal iron accumulation [130] and neuron loss in regions of the brainstem [131] which were associated with severe locomotor issues,

counter to what had been previously described in these animals [132]. Finally, using electrophysiological recordings from hippocampal slices, tau knockout mice from Dawson et al. have similar NMDA receptor currents, synaptic transmission strength and both long- and short- term synaptic plasticity, though they exhibited a resistance to seizure activity induced by a variety of means, including disinhibition and excitotoxins [127]. Additionally, knockdown of tau in the rat hippocampus led to a deficit in long term depression, but not long term potentiation (LTP), also pointing to a role in synaptic plasticity.

It is worth mentioning that in the absence of tau many of the toxic effects seen in AD disease models are shown to be diminished or eliminated. It had long been debated whether the deficits in Alzheimer's disease were primarily due to Aβ or tau, a conflict often referred to as the βaptists versus the *Tau*ists [133] A pair of papers published together in 2001 were the first to show a connection between Aβ or tau. These studies demonstrated that tau pathology in a mouse overexpressing mutant tau was exacerbated by both intracerebral injection of Aβ [134] and crossing the mutant tau mouse to a transgenic animal that overexpresses mutant APP [135]. This phenomenon was expounded upon by Roberson and colleagues, who showed that learning and memory deficits present in a mouse line overexpressing mutant human APP were mitigated by crossing the animals to a tau null background [127]. Further studies have shown that a variety of toxic effects and cellular phenotypes due to Aβ, such as NMDA receptor related excitotoxicity [122], Aβ–induced microtubule loss [136], inhibition of mitochondrial transport [137], impairment of LTP [138], synaptic damage [139], aberrant cell-cycle re-

entry [140] and overall cell toxicity [141] could be ameliorated through loss of tau expression. This wealth of data identifying tau-dependent phenotypes has led many to propose tau depletion as a possible therapy in tau-related disorders [142].

There does exist a condition with a chromosome 17q21.3 microdeletion, Koolen-de Vries syndrome, in which patients have lower expression of tau. The syndrome is characterized by intellectual disability, hypotonia (a state of low muscle tone) and distinctive facial features which result from haploinsuffinciency of another gene at this locus, KANSL1, which encodes a protein that influences gene expression by histone modification. The 50% reduction in tau has not been associated with any issues in brain development in these cases, indicating reduction of tau may be viable as a therapy [143,144].

8. Post-translational modification

Many of tau's functional properties are regulated by post-translational modifications. Tau is known to be post-translationally modified by a number of different methods, the most well characterized of which is phosphorylation. There are 85 potential phosphorylation sites on tau, 80 serines or threonines and 5 tyrosines, and phosphorylation at ~50 of these sites has been observed *in vivo* [76]. Phosphorylation plays a key role in regulating the interaction of tau with microtubules, and thus the assembly and stabilization of the microtubules. Phosphorylation of KXGS motifs within or close to the MTBRs, most importantly Ser262 has been demonstrated to reduce tau affinity for microtubules. Within the PRD, phosphorylation at Ser214 and Thr231 potently

decreases the binding of tau to microtubules [145,146]. Other phosphorylated residues in the PRD demonstrate only a weak effect on microtubule binding. Phosphorylation at Thr231 can cause tau to undergo a *trans* to *cis* isomerization, which further reduces its affinity for microtubules. This loss of affinity can be reversed by the peptidyl-prolyl-*cis-trans* isomerase, PIN1, which converts tau back to the *trans* conformation [147]. An increased level of *cis* tau has been identified at both early and late stages of AD pathogenesis [148]. Phosphorylation of tau at Tyr18, Ser409 and Ser416 have been shown to be required for ectopic cell cycle re-entry (CCR), in which normally post-mitotic neurons begin expressing proteins involved in the cell cycle, which research in our lab and others has shown may be involved in the neuron loss associated with AD [140].

Several kinases are responsible for phosphorylating tau. The PRD contains several serine-proline and threonine-proline sites that are phosphorylated by the proline-directed kinases glycogen synthase kinase 3β (GSK3β), cyclin-dependent kinase 5 (CDK5), mitogen-activated protein kinase (MAPK) and JUN n-terminal kinase [78]. Microtubule affinity-regulating kinase (MARK), cyclic-AMP dependent protein kinase (PKA) and Ca^{2+}-calmodulin dependent kinase II (CaMKII) phosphorylate sites around the MTBRs that decrease microtubule affinity [78]. Additionally, fyn, CaMKII, and PKA phosphorylate sites responsible for CCR [140].

The main phosphatase of tau is protein phosphatase 2A (PP2A), which accounts for roughly 70% of all tau phosphatase activity. Its activity can be reduced by as much as 40 percent in AD brain, although it is unknown whether this decrease is due primarily to post-translational modifications in the PP2A catalytic domain, decreases in PP2A

expression or increase in endogenous inhibitor expression [149]. While most dephosphorylation is done by PP2A, the phosphatases PP1, PP2B, PP2C and PP5 have also been demonstrated to dephosphorylate tau [145].

While tau is known to be hyperphosphorylated under disease conditions, there are also some physiological conditions in which tau has a high level of phosphorylation. Fetal tau is highly phosphorylated, a condition that is not associated with any pathology [150]. Tau also becomes reversibly hyperphosphorylated in hibernating animals and in cases of hypothermia [151]. It thought this temperature sensitive increase in phosphorylation is due to tau phosphatases being more inhibited by temperature decreases than tau kinases.

Another post-translational modification of tau that has only recently been identified is acetylation. Tau can be acetylated by the P300 acetyltransferase and CREB-binding protein at lysines in or around the MTBR, and tau also has weak acetyltransferase activity of its own [152,153]. It is deacetylated by sirtuin 1 and histone deacetylase 6 (HDAC6). Acetylation at various sites has been proposed both to inhibit degradation and to facilitate degradation, along with inhibiting phosphorylation and aggregation. Acetylation at various residues in KXGS motifs has been identified in normal tau, and is decreased in human AD and transgenic models of tau pathology. Elevated acetylation at sites such as Lys280 and Lys174 has been shown to be elevated in a number of diseases with tau pathology, AD included [154,155]. The exact function of acetylation at all of these sites however is still under investigation.

Numerous other post-translational modifications of tau include glycosylation, glycation, deamidation, nitration, methylation, ubiquitination, and sumoylation. An increase in N-glycosylation has been demonstrated postmortem in AD brain, which is thought to stabilize tau aggregation and increase phosphorylation by alterations in local tau conformation. O-glycosylation contrastingly is reduced in AD brain, and has been demonstrated to decrease aggregation and phosphorylation of tau by modifying the serine-proline and threonine-proline phosphoepitopes in the PRD. Finally, non-enzymatic modifications of tau such as glycation and deamidation are elevated in pathological tau, and various sites of nitration have been found preferentially in either normal or AD tau [78].

Tau can be degraded by the ubiquitin proteasome system, which is impaired in neurodegenerative disorders, resulting in an increase in ubiquitin-positive inclusions in tauopathies [156]. Studies have also shown that tau is sumoylated at Lys340 *in vitro* [157], although studies of AD brain have yielded conflicting results, with one demonstrating no sumoylation of tau aggregates [158] and another showing sumoylation of aggregates in AD brain, but none in normal brains [159]. In this case sumoylation was tied to hyperphosphorylation, which led to further sumoylation, and thus inhibition of ubiquitination and degradation.

9. Tau Isoforms

In the brains of healthy individuals, tau is found at a roughly 1:1 ratio of 3R to 4R isoforms [160], while in mice, adult tau is almost all 4R [161]. The major exception to this

is during development, when predominantly 3R tau is present in both mice and humans. The N-terminal inserts are found at levels such that 1N>0N>2N. No specific role has been demonstrated for these various isoforms, though certain experimental evidence indicates there may be some unique functions. As mentioned, in both mice and humans, fetal tau is primarily 3R [78]. 4R tau has a three-fold higher binding affinity for microtubules than 3R tau [162,163], and having predominantly 3R tau during development likely allows for a more dynamic microtubule network needed for axonal extension and synaptogenesis [164]. 4R isoforms promote the initiation of microtubule assembly better than 3R tau, and while both inhibit the extension of microtubules to a certain extent, 4R isoforms inhibit extension 3 fold more than 3R [162]**.** 4R can inhibit the shortening of microtubules, while 3R exhibits no shortening inhibition. Finally, the inhibitory effect that tau has on fast axonal transport has also been demonstrated to be more potent for 4R than 3R Tau. [115]

There are also some regional differences in isoform expression. For example, the isoform ratios change from 3R to 4R at different rates in the cortex versus the hippocampus in the developing brain. Higher ratios of 3R tau are also present in areas of adult neurogenesis such as the subgranular zone of the hippocampus [161]. As the specificity of isoform specific antibodies increases, it may be possible to further characterize regional differences in isoform expression tied to specific functions.

More is known about how the various isoforms behave *in vitro*. Tau isoforms exhibit distinct differences in their capacity to self-assemble based on the number of both N-terminal exons and MTBRs. In tau that has been induced to aggregate, inclusion of an

extra MTBR increases the rate of both nucleation and extension in tau fibrils [165]. Assembly rates of pure 4R or 3R tau are also inhibited by aggregating in the presence of tau with a different number of MTBRs [166].

1N tau has an increased propensity to extend fibrils after nucleation vs 0N tau, while addition of the second n-terminal exon inhibits this effect, though not to 0N levels. These effects may result from the aforementioned paperclip formation tau is known to take, with addition of exons 2 and 3 disrupting this conformation to varying extents and making the MTBR regions available for self-assembly [165]. Based on these data functional differences are likely to exist among the various tau isoforms.

10. Tau Aggregation

Insoluble, hyperphosphorylated inclusions of various morphologies composed of tau characterize a spectrum of neurodegenerative disorders known as tauopathies, of which Alzheimer's Disease is by far the most prevalent. Less prevalent tauopathies include Pick's Disease (PiD), a frontotemporal lobe disorder, characterized by language and cognitive difficulties; progressive supranuclear palsy (PSP), which normally first presents with motor difficulties, such as loss of balance; and corticobasal degeneration (CBD), which also presents with motor issues such as parkinsonism (a syndrome characterized by tremor, slowness of movement, and rigidity) [56,160]. These diseases, among others (Table 1-1) all exhibit tau pathology and together represent the non-Alzheimer tauopathies. It should be noted that there is a great deal of heterogeneity in these disorders and that symptoms and underlying pathology may overlap [167]. Additionally,

while the majority of the inclusions in these disorders are in neurons, or become extracellular after the neuron has died (a feature known as ghost tangles), some patients also exhibit varying levels of glial tau pathology [168].

The hyperphosphorylated state of tau in these cases may prime tau for aggregation. There are 17 serine-proline or threonine-proline residues that exhibit an abnormally high level of phosphorylation in AD and related tauopathies. Increases in phosphorylation at these sites due to kinase and phosphatase dysregulation [96] may inhibit tau binding to microtubules and prime it for intermolecular aggregation through the MTBR. While there is some variance in the specific sites, normal tau has roughly 2-3 phosphates per molecule, while “hyperphosphorylated” tau has about 8-9 per molecule [169,170]. In fact, one of the key histological tools in examining post-mortem brain tissue are antibodies for specific phosphorylation sites known to be elevated in AD brain tissue. Major phosphoepititopes in AD tau include Ser396/404, recognized by the PHF1 monoclonal antibody [171] and Ser202/205, recognized by the AT8 monoclonal antibody [172], both of which are commonly used in diagnostic staining. The monoclonal antibodies AT100, AT180 and AT270 can also be used to identify phosphorylation at Ser212/214, Thr231 and Thr181 respectively in NFTs [173,174]. There is some debate however on how accurately postmortem examination of tau phosphorylation levels recapitulates physiological phosphorylation, as kinases require ATP and thus lose activity following death, while tau phosphatase activity persists post-mortem [175]. This could result in the level of phosphorylation being greatly underestimated. Tau phosphorylation levels have in fact been shown to be dependent on post mortem interval [176].

At the ultrastructural level tau inclusions appear as paired helical filaments (PHFs) and straight filaments, which vary in width from 8-20 nm [177]. In these filaments, tau assembles through the ^{275}VQIINK^{280} and ^{306}VQIVYK^{311} motifs in the 2nd and 3rd MTBR. The VQIVYK motif is necessary and sufficient to form fibrils, creating a "steric zipper" formed by tightly interdigitated β-pleated sheets [178]. It is thought that exposure of these motifs, possibly by disruption of the paperclip formation, may be key in tau fibril formation. In fact, disruption of the motifs by proline mutations decreases tau's propensity for aggregation, and mutations such as P301L and ΔK280 increase the strength of the β-sheets and speed up aggregation both *in vivo* and *in vitro* [179].

Filaments of tau can also be produced *in vitro*. Given tau's low propensity to aggregate on its own, recombinant tau polymerized *in vitro* requires a cofactor to initiate assembly. Filaments can be formed from full-length unphosphorylated tau of any isoform by using polyanionic cofactors such as heparin or RNA, or free fatty acids such as arachidonic acid, a method commonly used to produce tau aggregates *in vitro* [180-182]. The morphologies of these recombinant tau filaments are similar, but not identical to those found in tauopathies [75]. Still, tau may interact with some of these cofactors within the cell, such as RNA and arachidonic acid, pointing to other possible ways in which tau aggregation may initiate intraneuronally [78].

Truncated forms of tau consisting of only the MTBR have a very high capacity for self-assembly and represent another method of producing tau filaments *in vitro* [183]. It is possible that this propensity for truncated tau to aggregate is again due to loss of the masking of this region by the N- and C-terminal tails in full-length tau. Many studies on

the effects of tau aggregation have utilized these truncated forms of tau to replicate tau pathology [184-188]

11. Tau in Disease

While the Amyloid-Cascade hypothesis still holds a great deal of sway, the role of tau in pathogenesis has gained increased focus, both in light of the tau dependence of many of Aβ's toxic effects and the identification of several mutations in the tau gene associated with neurodegenerative disorders. As mentioned previously, some familial cases of AD have a genetic component, with a multitude of mutations identified in both the APP gene and in presenilins 1 and 2 that cause Aβ accumulation and eventually lead to tau deposition. The connection between Aβ and tau hyperphosphorylation and aggregation in these cases, however, is unknown. While no tau mutations are implicated in AD, more than 80 [78] (Fig 1-4) that cause tauopathies have been identified in the *MAPT* gene, demonstrating a direct causative role for tau dysfunction in the onset and progression of neurodegenerative disorders [167].

The first disease causing mutations were identified in tau in 1998 in several different families with frontotemporal dementia (FTD) inherited in an autosomal dominant fashion. The disorders associated with this mutation are known collectively as frontotemporal dementia and parkinsonism linked to chromosome 17, or FTDP-17 [189]. Since then many pathogenic mutations all along the tau gene have been identified. Most of the missense mutation that have been identified are in the or near the MTBR domains, such as P301L, R406W and ΔK280. P301L is the most common FTDP-17 mutation, and

transgenic mice expressing this mutant have a robust neurodegenerative phenotype [190,191]. These mutant forms of tau exhibit a decreased affinity for microtubules and an increased propensity to aggregate. The A152T mutation, while not near the MTBR, is also associated with lower microtubule binding affinity. Two mutations of an N-terminal arginine residue affect the binding of tau to the p150 subunit of dynactin and may result in retrograde axonal transport dysfunction [192].

Another set of mutations are located near or even within intron 10. Most of these mutations alter the splicing of tau and lead to an increased amount of 4R vs 3R tau. There are some however, notably ΔK280, that increase 3R vs 4R. In the case of the intronic mutations, it is currently unknown why simply altering the ratios of tau isoforms leads to neuron dysfunction and tau aggregation, though it may result from differences in the capacity of various isoforms to both stabilize microtubules and to self-assemble through the MTBR [167].

For reason also not quite understood, inclusions in various disorders are often composed of isoforms with the same number of MTBRs. While NFTs in AD have 3R and 4R isoforms in a roughly 1:1 ratio, inclusions in Pick's Disease, known as Pick bodies, comprise 3R tau. Conversely, inclusions in CBD and PSP are composed primarily of 4R tau [56] (Fig. 1-5). 4R tau is additionally the principle component of insoluble tau in cases of mutations that alter splicing and increase the ratio of 4R to 3R tau, as well as in rod-like nuclear inclusions recently identified in Huntington's Disease [121].

Isoform effects similar to these have been identified in mouse models as well. Unlike human isoforms, mice transition exclusively to 4R tau by P90. Interestingly, mice

that express all six isoforms of wild type human tau in the absence of endogenous mouse tau develop tau aggregates, neuron death and learning and memory deficits [193]. While the mechanisms may be unknown, it is clear that isoform plays an important role in tau dysfunction.

Curiously, while several tau mutations cause a consistent phenotype, many mutations can cause a great deal of phenotypic variability, even in the same family. P301L, the most common mutation, is associated with a broad spectrum of clinical phenotypes including AD, CBD, PSP and FTD [194]. For example, in the case of two brothers with P301L mutations, one presented with parkinsonism and relatively intact cognition, while the other had frontotemporal dementia [167]. Patients with P301S mutations can exhibit epileptic seizures, parkinsonism, FTD, or a combination thereof. Additionally, while the majority of patients with the +16 intronic mutation present with FTD with disinhibition, one patient harboring a +16 mutation exhibited symptoms of PSP [195]. Moreover, post-mortem examination of two siblings, both with the +16 mutation, showed frontotemporal degeneration in one sibling, while the other had little frontal and temporal lobe loss but extensive pathology in the brainstem and subcortical regions [167]. The underlying causes of these disparities remain unknown.

It is worth noting there exist two major tau haplotypes, H1 and H2, resulting from a ~900 kb inversion polymorphism and a resulting linkage disequilibrium that may play some role in susceptibility. The H1 haplotype appearing to confer an increased probability of AD, PSP or Parkinson's [196-198], however, the source of this increased risk is unknown.

Both loss of function and toxic gain of function of tau have been described under pathological conditions. As discussed previously, tau plays an important role in axonal transport regulation. Axonal transport deficits are common in both patients with neurodegenerative disorders and in mouse models of neurodegeneration [199,200]. Cargoes such as cytoskeletal components and mitochondria can accumulate in the cell body, the proximal axon, or the distal axon [78,115]. It is possible that some of these deficits result from a loss of tau's role in microtubule stability, leading to an increased disruption and irreversible damage to the cytoskeleton over time. Reductions in tubulin acetylation and microtubule stability have in fact been identified in AD patients [201]. As discussed previously, other tau functions such as regulation of iron transport, neurogenesis, and DNA protection could be adversely affected by tau dysfunction.

Certain toxic effects of tau may result from the translocation of tau to the somatodendritic compartment. Under normal conditions, tau has a polarized distribution in the axon, while diseased neurons exhibit extensive accumulation of tau, both soluble and inclusions, within the somatodendritic compartment. Somatodendritic tau has been demonstrated to result from tau mutation, hyperphosphorylation, and overexpression [202]. A major source of tau toxicity in neurons seems to be through mediating Aβ neurotoxicity in dendrites. As previously discussed, tau can target the kinase, fyn, to dendrites, where fyn modulates the interaction of PSD95 with the NMDA receptor. The excess dendritic fyn resulting from tau invading the somatodendritic compartment can cause glutamate toxicity [122]. Tau has also been demonstrated to localize tubulin

tyrosine ligase-like enzyme 6 to dendrites, which eventually leads to microtubule cleavage by spastin [102,203].

It is important to note that the toxic effects of tau do not require tau to be aggregated. Initial research on the role for tau in neurodegeneration focused on the role of hyperphosphorylated insoluble tau as the causative factor in disease etiology [167]. However, neuronal dysfunction has been demonstrated to precede tangle formation. Additionally, in mice expressing a mutant form of tau under an inducible promoter, inactivation of tau expression rescues behavioral phenotypes while insoluble tau remains. Increased attention has accordingly been paid to soluble tau oligomers, low-n tau species that have been shown to be elevated in the brain and CSF of patients with tauopathies. Tau oligomers produce many deleterious effects in neurons [204-206], such as axonal transport dysfunction (utilizing a squid axoplasm assay), synaptic dysfunction and LTP-inhibition. Indeed, levels of oligomeric tau correlate with memory loss in the rTg4510 conditional mouse model of tauopathy [207]. Tau oligomers also mediate synaptotoxicity, neuronal dysfunction and neuron death in murine and cultured neuron models of AD [206,208-211]. Given this data, it is noteworthy that there exists a familial tau mutation that in addition to decreasing the ability of tau to bind microtubules, enhances the formation of oligomers but not fibrils.

12. The Prion-like hypothesis

The prion theory of pathogenesis has been around for a while in the field of neurodegeneration. The first description of prions appears in Nobel prize winning work

from Stanley Prusiner [212]. This work demonstrated that by treating an infectious preparation of scrapie with a variety of nucleic acid degrading treatments that the infectious agent was in fact a cellular prion protein (PrP), and required no DNA or RNA to transfer its pathogenic phenotype from cell to cell [213]. In the pathogenesis of these disorders, PrP gains a misfolded conformation by mechanisms not yet known, comes into contact with a naïve monomer of PrP, and converts the naïve monomer to the pathogenic phenotype. Then by mechanisms that are also poorly understood, the protein is able to leave the cell, get taken up by a nearby cell, gain access to a new intracellular pool of monomer, and thus starting the process over again [214].

Several prion disorders involving the prion protein have been well described. Bovine spongiform encephalopathy (Mad Cow Disease) in cattle, chronic wasting disease in deer, scrapie in sheep, and several human diseases including Creutzfelt-Jacob Disease, Kuru, and fatal familial insomnia have all been shown to result from pathogenic prion protein transmission [215]. Recently however this hypothesis has increasingly been applied to proteins in other neurodegenerative disorders, as a “prion-like” mechanism of disease pathogenesis. In this hypothesis pathological aggregates of a protein originate in one area of the brain and are spread along defined synaptically connected neuroanatomical pathways [216]. This hypothesis arises from the observation that several neurodegenerative disorders, such as AD or Parkinson’s Disease, demonstrate a temporally ordered spread of pathology throughout the brain upon post-mortem examination of brains at defined stages of disease [217]. Distinguishing this “prion–like” phenomena from other prion cases is the fact that while true prion disorders have been

shown to be “infectious”, with transmissibility possible between organisms often following consumption of contaminated tissue, no other neurodegenerative disorders are known to be infectious from organism to organism [218].

13. Prion-like characteristics of tau

Tau is one of the proteins known to exhibit this prion-like behavior. It had been hypothesized as early as Prusiner’s initial 1982 publication that prions may play a role in the etiology of diseases such as AD [212,219]. The first studies to demonstrate the transmissibility of tau aggregates showed that tau pathology could be induced in mice that expressed human tau and normally lacked tau inclusions by intracranially injecting brain homogenates from a mouse expressing a mutant form of tau [220]. *In vitro* studies soon thereafter showed that non-neuronal cells expressing fluorescently-tagged tau exhibit intracellular inclusions of the transfected tau when exposed to extracellular aggregates of the truncated tau MTBR, and that these cells could then be replated and transmit the misfolded tau to a naïve group of cells [184]. Experiments using this same paradigm, non-neuronal cells treated with truncated tau aggregates, have gone on to show that intracellular tau accumulations of varying morphologies will recapitulate these morphologies when naïve cells are exposed to homogenates containing these aggregates, indicating that various species act as seeding templates for the pool of monomeric tau [221-223]. Intracellular tau aggregation has been replicated in neurons that overexpress a mutant form of tau [224], and in neurons induced from human pluripotent stem cells

[225], however until the work described in this thesis was completed, no previous studies have demonstrated accumulation of endogenous, wild-type tau in neurons.

Furthermore, two mouse lines have been developed that demonstrate the cell to cell transmissibility of tau [226,227]. Both of these lines restrict mutant human tau expression to the entorhinal cortex (EC), a region where early tau pathology is found in AD. There is a time dependent accumulation of tau inclusions in these animals, as identified by several conformation-dependent and phosphorylation-dependent antibodies associated with pathological tau. Remarkably, other than in the EC itself, those inclusions comprise exclusively endogenous mouse tau. Taken together, these models show that misfolded tau can transmit its pathological phenotype from cell to cell in vivo.

As with PrP, this template mediated, cell-to-cell transmission of pathogenesis requires protein-protein contact, indicating misfolded proteins need to leave one cell after which the now extracellular protein needs to gain access to another intracellular space by some mechanism [214]. While little mechanistic evidence exits, several hypotheses have been proposed as to how this takes place. Tau has been demonstrated to be released at the synapse during neuronal activity [228]. Tau may also be released through exocytosis at the cell body or synapse, or from exosomes derived from multivesicular bodies [229,230]. Heparan sulfate proteoglycans (HSPGs), initially shown to play a role in PrP gaining access to cells can mediate the update of extracellular tau aggregates by receptor mediated endocytosis [231]. Other studies have shown uptake of extracellular tau by fluid-phase endocytosis, although it is still undetermined how tau enters the cytosol from the endosome or lysosome. Tunneling nanotubes, microscopic

plasma membrane connections between cells that have been identified in neurons, have also been hypothesized as possible transfer points [232].

Tau does play a role in some signaling cascades, and the possibility that this well-defined, time-dependent spread of pathology is due at least in part to a tau signaling function cannot be dismissed out of hand [233]. For example, in mice over expressing tau on a microglial fractalkine receptor Cxcr1-knockout background, microglial activation occurs prior to tau pathology along these defined neural pathways [234], indicating the possibility that inflammation may be playing a role in tau progression.

While these studies demonstrate fairly definitively that under certain conditions, extracellular tau can induce the accumulation of intracellular tau, the exact tau species responsible for cell-to-cell transmission in vivo have yet to be determined. The majority of these experiments have been done utilizing truncated mutant tau to facilitate the aggregation of tau [184,231,235]. However, as previously described, a great number of the toxic effects attributed to tau result from soluble oligomers. In fact, in cases of true prion disease, soluble oligomeric PrP aggregates show a higher capacity to transmit the prion phenotype than insoluble species [236]. One study has shown that low-*n* soluble tau and short fibrils are preferentially taken up by neurons both at the synapse and at the cell body and localize to late endosomes, whereas monomers and large fibrils were not taken up [238]. That tau species as low as trimers have been definitively demonstrated to propagate misfolding fortifies a role for oligomers in disease progression [237].

14. Summary

While many significant advances in the field of neurodegenerative disease research have been made since Alois Alzheimer first described the pathology of AD, there is still a great deal that is unknown about these disorders. The initial events leading to Aβ or tau deposition remain largely uncertain, and while cell-to-cell transmission of pathogenic proteins as a possible source of disease progression has been described by several experimental paradigms, it is still unknown what affects this accumulation of tau may have on key biological processes within neurons.

The background presented here provides the basis for the original work presented in this dissertation. These studies set out to further define the effects of extracellular tau on intracellular tau, such as identifying what extracellular tau species may be responsible for intracellular tau accumulation and mislocalization. Furthermore, they describe the effects that intracellular tau accumulation has on neuronal physiology, pointing to a mechanism by which the cell-to-cell transmission of toxic tau causes neuronal dysfunction.

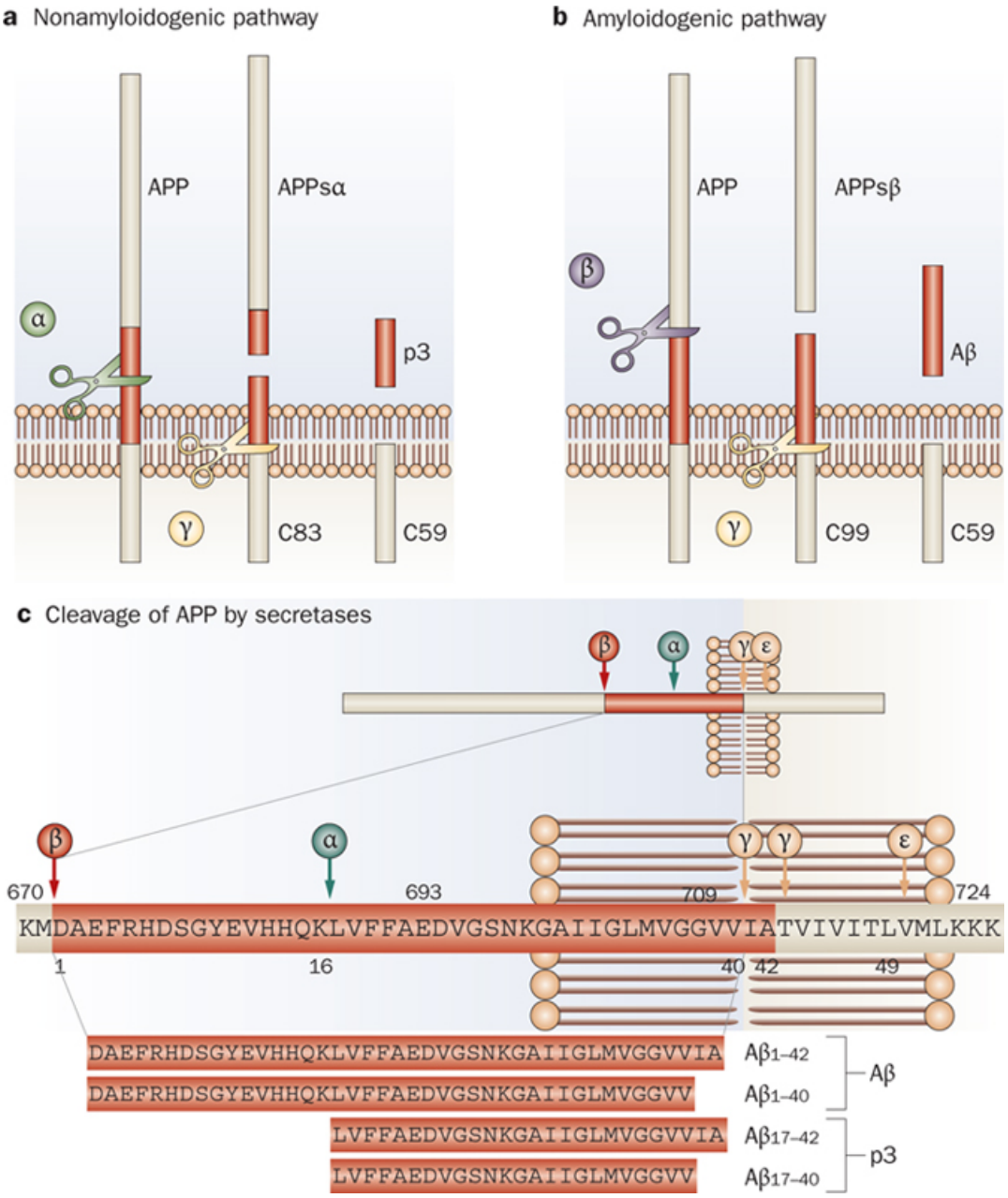


Figure 1-1: Schematic of APP processing (adapted from De Strooper B, Vassar R, Golde T., The secretases: enzymes with therapeutic potential in Alzheimer disease [70]) APP processing can occur by either the non-amyloidogenic pathway, in which α-secretase makes a cut within sequence of Aβ, releasing the APPsα fragment, after which the remaining C-terminal tail is processed by γ-secretase, releasing the p3 fragment and the APP intracellular domain (AICD, also referred to as C57 or C59, depending on where γ-secretase cuts.)In the amyloidogenic pathway, β-secretase first cuts APP, releasing APPsβ, after which γ-secretase cleavage produces Aβ and the AICD.

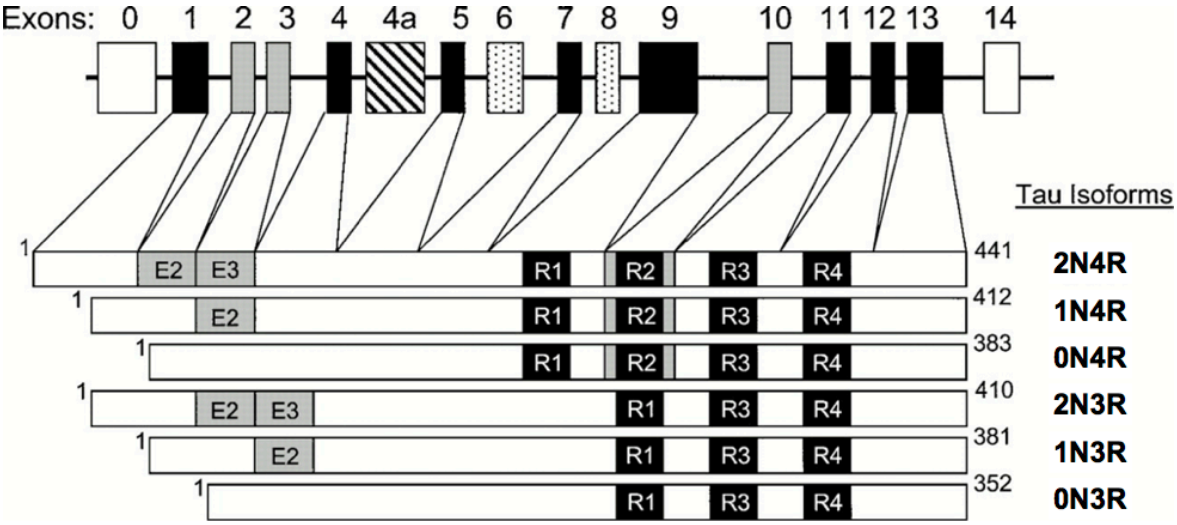
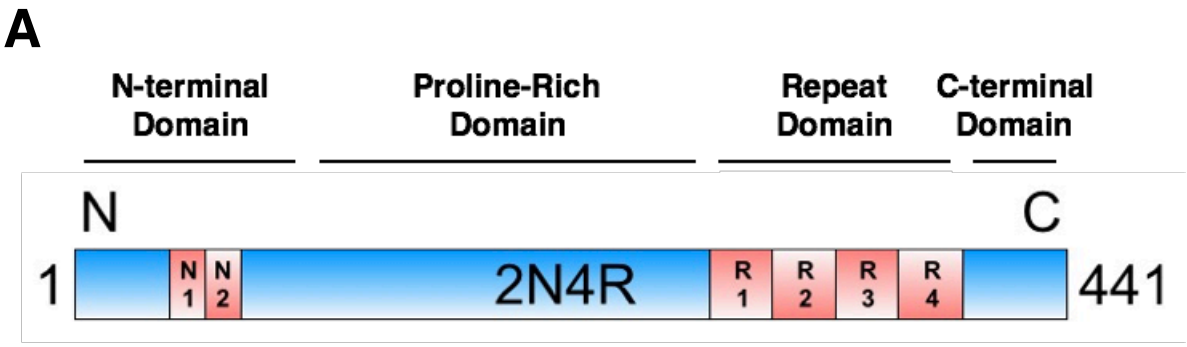


Figure 1-2: Schematic of MAPT gene alternative splicing and tau protein isoforms (adapted from Lee et al., Neurodegenerative Tauopathies [56]) The tau gene contains 16 exons. Exons represented in grey undergo alternative splicing to produce 6 CNS isoforms, characterized by the inclusion of 0, 1, or 2 N-terminal inserts, and 3 or 4 C-terminal MTBR. (Relative sizes of exons and introns not to scale.)



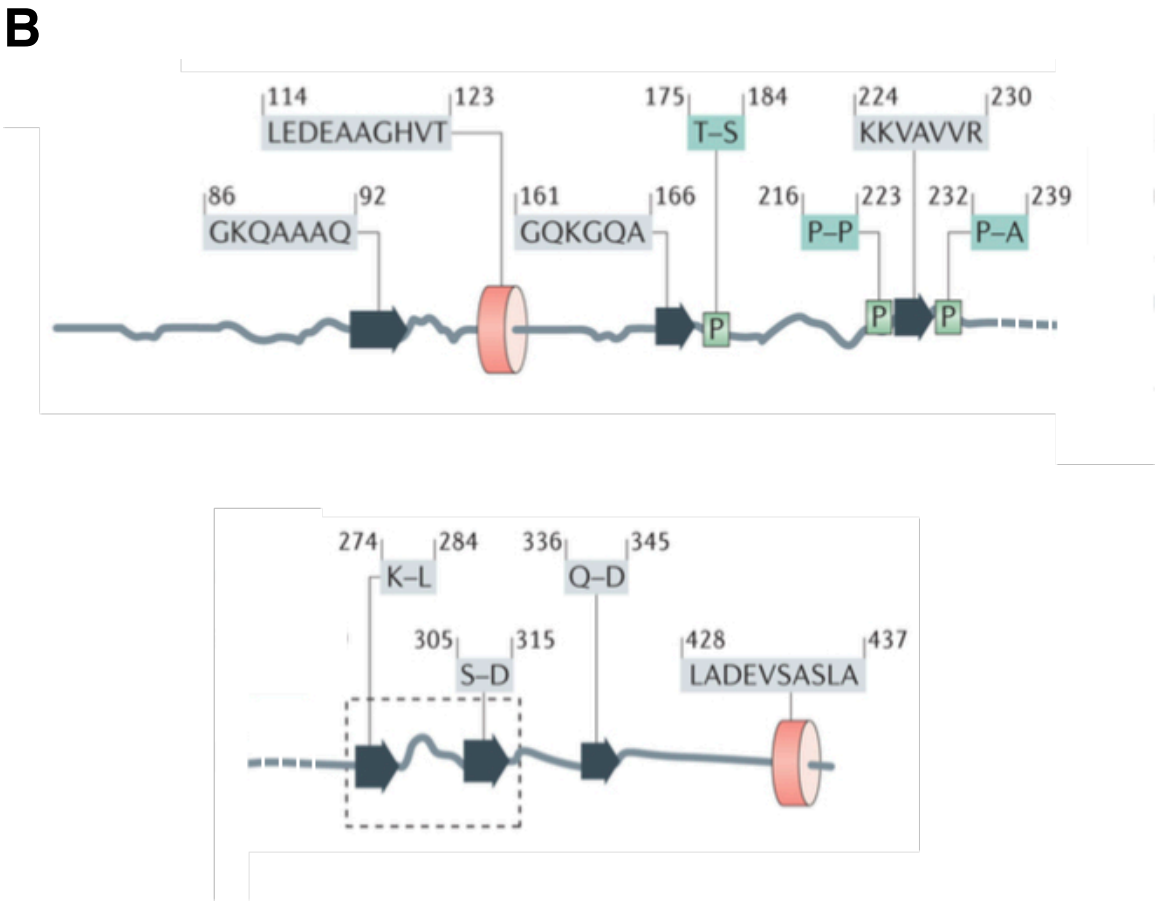


Figure 1-3: Domains and structural features of tau (B adapted from Wang et al., Tau in Physiology and Pathology [78]) A) Tau is commonly divided into four regions: An N-terminal domain; a proline-rich region from amino acids 150-243; a repeat region from residues 244 to 369, containing the microtubule binding repeats; and a C-terminal domain B) Various structural features have been reveal by NMR. The majority of the protein is unfolded, as identified by grey lines. Some transient secondary structure exists; such as α-helices (red cylinders), β-strands (black arrows) and poly-proline helices (green boxes). The dashed-line box indicates the two hexapeptide motifs in MTBRs 3 and 4 that are responsible for tau aggregation.

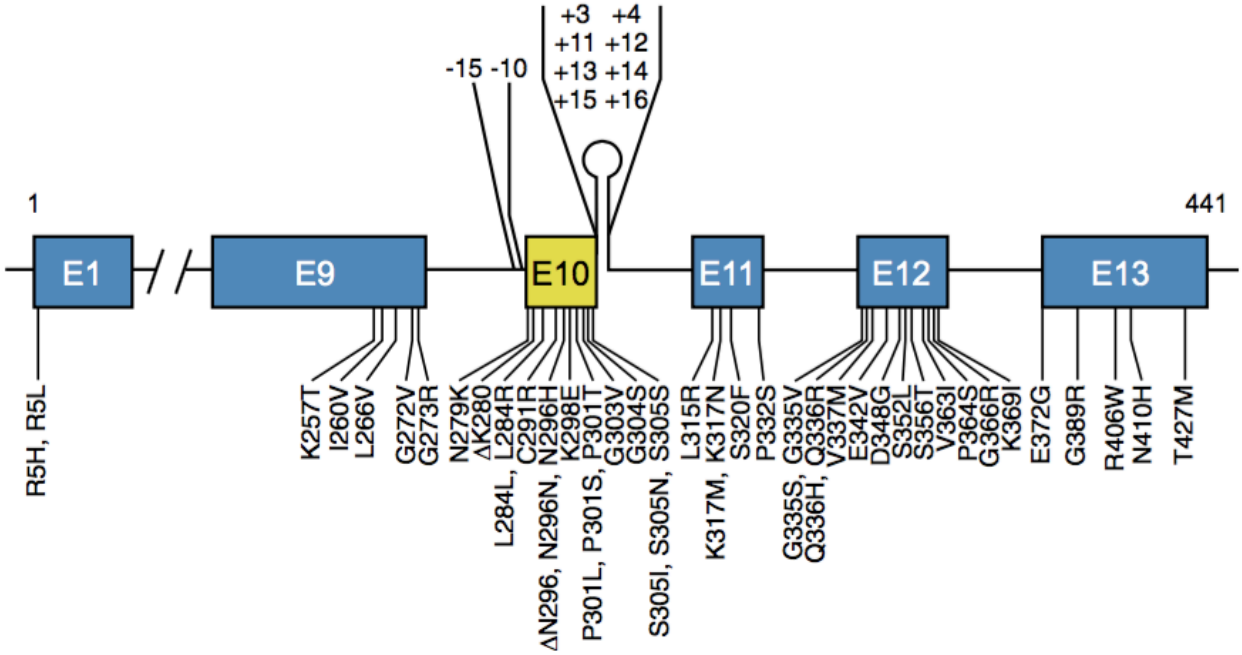


Figure 1-4: Mutations identified in MAPT gene (adapted from Goedert M, The ordered assembly of tau is the gain-of-toxic function that causes human tauopathies [75]) Mutation in MAPT causes cases of FTDP-17. The 47 mutations below represent mutations in the coding region, while the 10 mutations above are intronic mutations that affect exon 10 splicing.

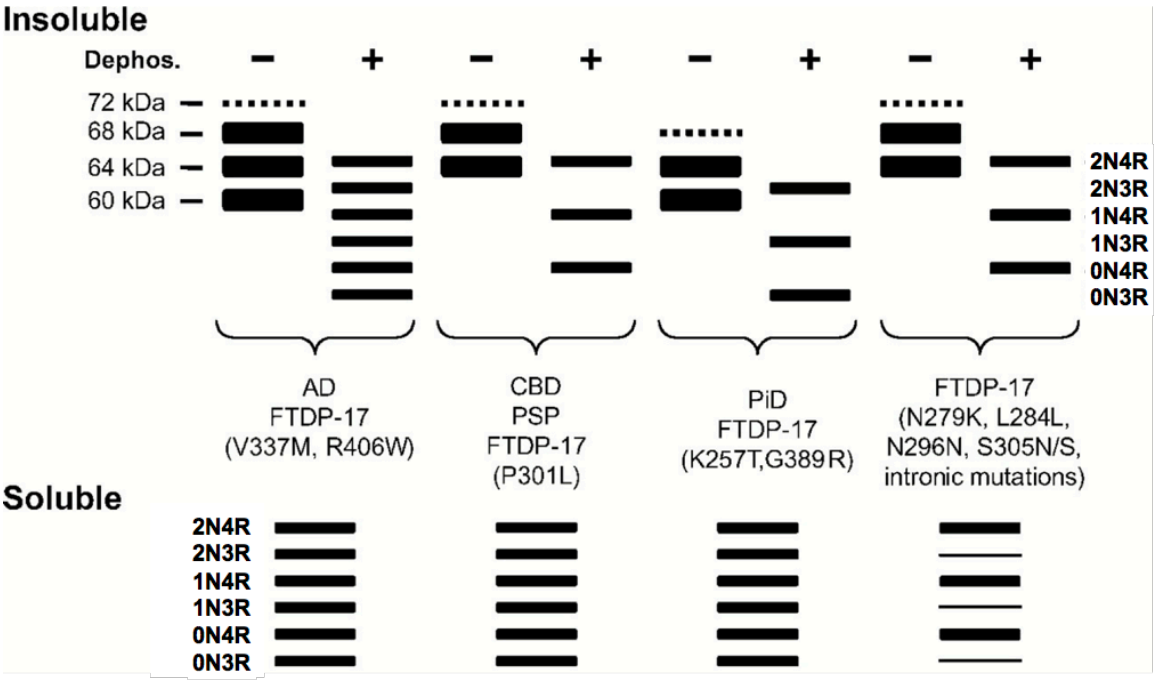


Figure 1-5: Cartoon representation of western blot patterns of insoluble and soluble tau isoforms from various tauopathies (adapted from Lee VMY, Goedert M, Trojanowski J, Neurodegenerative Tauopathies [56]) Western blot of nondephosphorylated insoluble tau from AD or FTDP-17 (V337M and R06W mutations) shows 3 major bands at 60, 64, and 68 kD, with a minor band at 72. Upon dephosphorylation, these insoluble aggregate can be resolved into individual isoforms. Tau from CBD, PSP, and FTDP-17 (P301L mutation) has only two major and one minor nondephosphorylated bands, that upon dephosphorylation are shown to be composed of only 4R tau. Tau from PiD and FTDP-17 (K257T and G389R mutations) also have two major nondephosphorylated bands, though these are composed of only 3R tau. Soluble tau in all these cases comprises all six isoforms, indicating that tau in these cases undergoes selective aggregation. FTDP-17 mutations which affect mRNA splicing however have predominantly 4R soluble tau. In these cases, only 4R tau is found in inclusions. The thickness of each band correlates with relative abundance.

Alzheimer's disease
Amyotrophic lateral sclerosis/parkinsonism-dementia complex
Anti-IgLON5-related tauopathy
Argyrophilic grain disease
Chronic traumatic encephalopathy
Corticobasal degeneration
Diffuse neurofibrillary tangles with calcification
Down's syndrome
Familial British dementia
Familial Danish dementia
Familial frontotemporal dementia and parkinsonism
Gerstmann-Sträussler-Scheinker disease
Guadeloupean parkinsonism
Huntington's disease
Meningio-angiomatosis
Myotonic dystrophy
Neurodegeneration with brain iron accumulation
Niemann-Pick disease, type C
Non-Guamanian motor neuron disease with neurofibrillary tangles
Pick's disease
Postencephalitic parkinsonism
Progressive supranuclear palsy
SLC9A6-related mental retardation
Subacute sclerosing panencephalitis
Tangle-only dementia
White-matter tauopathy with globular glial inclusions

Table 1-1: Diseases with tau inclusions ((adapted from Goedert M, The ordered assembly of tau is the gain-of-toxic function that causes human tauopathies [75]) A number of neurodegenerative orders exhibit tau pathology as a defining feature. Notably, CBD, PiD, PSP and FTDP-17 can all result from mutations in the tau gene, evidence for a causative role of tau in neurodegeneration.

Chapter 2 -

Materials and Methods

Chapter 2: **MATERIALS AND METHODS**

Recombinant tau

Expression vectors for all 6 wild-type human tau isoforms with a 5'-His-tag (kindly provided by the late Lester "Skip" Binder) were transformed into BL21 cells. Cells were selected for transformation on an ampicillin plate, and individual colonies grown in starter cultures overnight. These starter cultures were used to inoculate flasks containing 200 mL L-broth with 100 µg/ml ampicillin (Sigma.) Cells were grown to an optical density of 0.6 after which protein expression was induced with 0.5 M Isopropyl β-D-1-thiogalactopyranoside (IPTG.) Cells were pelleted by centrifugation at 5000 x *g* and resuspended in lysis buffer (0.3M phosphate pH 8.0, 0.3M NaCl, 50 mM imidazole), supplemented with protease inhibitor cocktail, 1mM PMSF(Sigma), 5mg/ml lysozyme (Sigma) and 50 U DNAse (Clonetech), after which the cells were incubated on ice for 20 minutes, and sonicated 5 times for 20 seconds at 25 watts. Cell lysate was then centrifuged at 40K x *g* for 40 minutes and the supernatant passed through a 0.45 µM filter. The resulting lysate was purified by affinity chromatography utilizing a HiTrap Ni^{2+} column (GE Healthcare) and imidazole gradient from 20 mM to 500 mM, according to the manufacturer's instructions. Recombinant protein was then concentrated by centrifugation for 10 minutes in 10K molecular weight cut off spin columns (Millipore) and dialyzed into phosphate buffer, pH 7.4.

Oligomer production

Oligomeric tau was prepared as described previously by another group [205]. Tau was brought to 4 µM in 100 mM Tris, 0.1 mM EDTA, 150 µM Tris(2-carboxy-ethyl)phosphine (TCEP; Life Technologies) and treated overnight with 50 µM benzophenone-4-maleimide (B4M; Sigma-Aldrich). The protein was then treated with 5 mM dithiothreitol (DTT; Roche) to inactivate B4M and dialyzed in the dark overnight into 100 mM Tris, 0.1 mM EDTA and 5 mM DTT. A portion was removed for monomer treatments, and the protein was then allowed to aggregate overnight in the presence of 150 µM arachidonic acid (AA; Invitrogen), after which the protein was treated for 5 minutes with UV light at 254 nm (Spectroline model EF-180). Oligomers were used within 2 weeks of preparation. B4M- and AA-treated protein not exposed to UV was used as the fibrillar tau preparation.

Antibodies

The following antibodies were utilized in this study: Alz50 and MC1 (conformation-sensitive mouse monoclonals to tau, provided by Dr. Peter Davies of the Feinstein Institute for Medical Research); chicken anti-MAP2 (Abcam, catalog # ab5392); PHF1 (mouse monoclonal to tau phosphorylated at S396/S404; provided by Dr. Peter Davies; Tau 5 (mouse monoclonal pan-tau, recognizing mouse and human tau ; provided by the late Dr. Lester “Skip” Binder of Michigan State University); T14 (mouse monoclonal to human tau; provided by Drs. Virginia Lee and John Trojanowski of the University of Pennsylvania); Tuj1 (mouse monoclonal to βIII-tubulin, a neuron-specific

tubulin; provided by Drs. Tony Spano and Tony Frankfurter of the University of Virginia); rabbit anti-ankyrin G and rabbit anti-βIV Spectrin, both kindly provided by Tatyana Svitkina of the University of Pennsylvania; AlexaFluor 488 goat anti-mouse IgG (Invitrogen catalog # A-11001); AlexaFluor 594 goat anti-mouse IgG (Invitrogen catalog # A-11005); AlexaFluor 488 goat anti-chicken IgG (Invitrogen catalog # A-11039).

Western blots

Protein samples were diluted in 1X LDS sample buffer (Invitrogen) and separated on 4-12% gradient Bis-Tris SDSPAGE gels (Invitrogen) for 2 hours and transferred to a nitrocellulose membrane for 2 hours at 100V. Membranes were blocked in Licor tris buffered saline (TBS) blocking buffer (Licor) and treated with primary antibodies overnight (see above.) Membranes washed 3 times for 5 minutes in .2% PBS/Tween 20 and were then incubated with infrared-tagged secondary antibodies (Licor) at room temperature (RT) for 1 hr, again washed 3 times in PBST, washed once in TBS and dried overnight. Blots were analyzed using a Licor Odyssey imaging system.

Electron microscopy

Samples of monomeric, oligomeric and fibrillar tau at 4 μM total tau each were passed through a .22 μm filter and adsorbed overnight to 300 mesh copper EM grids (Electron Microscopy Services), counter-stained with uranyl acetate and imaged on a JEOL 1010 transmission electron microscope equipped with a 16 megapixel cooled CCD (model SAI-12c, Scientific Instruments and Applications, Inc.).

Primary neurons

Cortical neurons were isolated from wild type (C57/Bl6) and tau-knockout [101] embryos aged 16.5-18.5 days as previously described [141]. Brains were removed from the embryos and placed in Hank's balanced salt solution (HBSS; Invitrogen) in a sterile environment, after which the cortical tissue was isolated following removal of the meninges. The tissue was then digested at 37° C in 0.25% Trypsin lacking EDTA for 45 minutes. The trypsin protease activity was quenched with and equal volume of heat-inactivated fetal bovine serum (FBS; HyClone/GE Healthcare), and the cells were washed 3 times with warm HBSS and treated with DNAse II (Worthington) diluted in HBSS at 100 units per brain. The cells were then mechanically dissociated with flame polished Pasteur pipettes, diluted in Neurobasal media containing serum-free neuron supplement B27, l-glutamine, glucose, penicillin/streptomycin (all from Invitrogen) and 5% FBS, and plated at roughly 62500 cells/cm^2 on 50 µg/ml poly-D-lysine (Sigma-Aldrich) coated plates. Neurons were stored in an incubator in a humidified atmosphere of 5% CO_2/95% air. Plating media was removed 3 hours later and replaced with plating media lacking FBS to inhibit growth of glia in the culture.

Extracellular tau treatment

Primary cortical neurons were treated at 10 days *in vitro* with the indicated monomeric, oligomeric or fibrillar tau species at a final concentration of 50 nM total tau for 18 hours. Cells were washed with PBS prior to immunofluorescence staining.

Immunofluorescence labeling

Primary neuron cultures were washed briefly at RT in PBS, fixed at RT for 15 minutes in 3.7% paraformaldehyde, washed 3 times in cold PBS, and permeabillized in 0.2% TritonX-100 (Sigma) in PBS for 10 minutes. Cells were again washed in PBS, followed by blocking in 2% bovine serum albumin (Sigma)/0.1% Tween-20 (Fisher) in PBS for 30 minutes and incubation with the primary antibodies overnight. Cells were then treated with secondary antibodies labeled with Alexafluor-488 or Alexafluor-568; (Life Technologies) for 1 hour at RT. After a final series of brief washes in PBS, the coverslips were mounted on glass slides using Fluormount G (Fisher).

CVN mice, which overexpress a mutant form of APP on a NOS2 null background and have tau pathology around 10-12 months of age, [239] were deeply anesthetized and perfused transcranial with PBS for 5 minutes followed by 4% paraformaldehyde for 5 minutes, after which the brain was removed. Tissue was dehydrated, embedded in paraffin and cut into 5 µm thick sections that were adsorbed onto glass slides. The sections were deparaffinized using xylenes, rehydrated in a graded series of ethanol:water washes consisting of 100% ethanol, 95% ethanol, 70% ethanol and 100% water, blocked for 30 minutes in 5% FBS/0.2% Tween 20 and incubated overnight with primary antibody (Alz50 or MC1). Following 3 PBS washes to remove excess primary antibody the sections were then incubated with secondary antibodies labeled with Alexafluor-488 for 1 hour at RT. Finally, the tissue sections were washed twice with PBS, incubated for 5 minutes with 200 nM DAPI in PBS to stain nuclei, washed once more with

PBS, and then overlaid with glass coverslips that were secured to the glass slides with Fluormount G.

Quantitative light microscopy of tau aggregation

Neurons labeled with Tau-5, a monoclonal antibody that recognizes all tau isoforms independently of post-translational modifications [172], were imaged using a 20X 0.4 NA objective on an EVOS FL cell imaging system (ThermoFisher Scientific) at a constant level of illumination. Immunofluorescence micrographs were analyzed using FIJI software (also known as ImageJ2: http://imagej.net/ImageJ2) with an algorithm adapted from a previously described protocol [42]. Images were thresholded at the mean setting ([maximum fluorescence-minimum fluorescence]/2) and pixel groups were then segregated into contiguous groups larger or smaller than 10,000 utilizing the "analyze particles" function in FIJI [43]. The area of groups smaller than 10,000 contiguous pixels divided by the total area above the threshold intensity x 100 gives the value for percent accumulation/disruption of the protein of interest. Groups smaller that 10,000 are pseudo-colored red and groups larger than 10, 000 are pseudo-colored green to assist with visualization. Staining for the dendritic microtubule associated protein, MAP2, and the neuron specific βIII tubulin, a neuron specific tubulin, were analyzed by the same algorithm for control experiments.

Axon Initial segment analysis

Primary cortical neurons were treated with monomeric and oligomeric tau as described above. Two preparations of tau oligomers were utilized for these experiments, one allowed to oligomerize in the presence of 75 µM AA, and one oligomerized in 150 µM AA to accelerate the level of aggregation. Final tau concentrations in the tissue culture media were again 50 nM. After 18 hours, cells were immunofluorescently labeled for tau (Tau5), MAP2, (chicken anti-MAP2) and either ankyrin G or βIV Spectrin (rabbit anti-ankyrin G and rabbit anti-βIV Spectrin), two main components of the axon initial segment (AIS) (discussed in the chapter 4 introduction.) After staining cells were imaged utilizing a Leica SP5 white light laser confocal microscope. Utilizing the Leica image analysis software, regions of interest (ROI) were defined as two pixel by two pixel squares in the ankyrin-G or βIV Spectrin channel. Values were then taken for the relative fluorescence of all three proteins in these ROIs, with fluorescence ratios indicating the level of colocalization for each set of proteins. Fluorescence ratios for every ROI were then graphed in a frequency distribution.

Fast axonal transport analysis

Neurons were transfected with fluorescent expression vectors for BDNF-mRFP1, NeuropeptideY-mCherry (both kindly provided by Dr. Michael Silverman of Simon Fraser University) or BACE1mRFP1 (kindly provided by Dr. Huaye Zhang of Rutgers Robert Wood Johnson Medical School) utilizing Lipofectamine 2000 transfection reagent (Invitrogen). 800 ng vector DNA and 2 µl Lipofectamine 2000 were used for each transfection in 1.6 mL

of serum-free neurobasal media (Invitrogen). Cells were transfected 3 days prior to imaging, then vesicle movement was visualized upon excitation at 568 nm with a Nikon 60X planapo objective mounted on Nikon Eclipse Ti inverted microscope equipped with a Yokogawa spinning disc confocal head, and a Plexiglas chamber that maintained an atmosphere of 37° C and 5% CO_2. Images were taken at 2 frames per second. Axons were identified as neurites of uniform length with minimal to no branching, and directionality was determined by tracing them to a synapse or neuronal perikaryon of origin. The movement of fluorescent vesicles was analyzed as kymographs utilizing the ImageJ plug-in "Multiple Kymograph" (European Molecular Biology, Heidelberg, Germany). With this plug-in, an image of the maximal fluorescence for each point during the video was used to identify the tracks of imaged axons. The axon was then traced by plotting points along its length, after which the axon trace was straightened and a kymograph compiled from each frame. Run length and run time were derived from the kymographs by measuring particle movement in the kymographs x-axis and y-axis, respectively, as was "instantaneous velocity", which we define as the average velocity of the vesicle while in apparently continuous motion, and is calculated from the slope of each kymograph line. Velocity was taken for all non-vertical slopes in the kymograph, while run time and run length were reported only for organelles whose motility began and ended during the imaging timeframe.

Statistical Analysis

Data were analyzed using GraphPad Prism software, version 6. All comparisons were done by one-way analysis of variance (1-way ANOVA), with a Bonferroni post-test utilized to analyze variance between each set of treatments. P-values are annotated * for $p<.05$, ** for $p<.01$ and *** for $p<.001$.

Chapter 3 -

Effects of extracellular tau oligomer treatment on endogenous tau distribution and axonal transport

Chapter 3: Effects of extracellular tau oligomer treatment on endogenous tau distribution and axonal transport

This chapter has been modified and expanded from a published manuscript: Swanson E, Breckenridge L, McMahon L, Som S, McConnell I, and Bloom GS. Extracellular Tau Oligomers Induce Invasion of *Endogenous Tau into the Somatodendritic Compartment and Axonal Transport Dysfunction. Journal of Alzheimer's Disease* (2017, accepted).

INTRODUCTION

The role of extracellular tau in the initiation and spread of pathology within neurons has received a great deal of attention in recent years. Recent studies have demonstrated that extracellular tau can be internalized by cells and propagate along synaptically connected neuronal networks *in vivo* [220,226,227]. *In vitro* studies have also demonstrated that extracellular tau can induce the aggregation of intracellular tau, which can then be released from the cell and taken up by other cells [184,223,224]. Taken as a whole, these data have led to the hypothesis that the propagation of tau pathology is mediated by a prion-like process in which extracellular pathological tau enters a naive cell, whereupon it seeds pathogenic conformational changes in the pool of monomeric internal tau that can then be transmitted again to interconnected neurons [74,214,229,240,241] While the evidence for this seeded-recruitment phenomena has expanded greatly in recent years, selective vulnerability of certain neuronal populations has also been postulated as an important contributing factor for this well-defined disease progression [233].

Several important issues remain unresolved, however, including which forms of extracellular tau induce intracellular tau aggregation, and how that aggregation alters neuronal behavior. Because neurons do not divide and are more difficult to culture than constitutively dividing non-neuronal cell lines, and because wild type (WT) tau is not prone to aggregate on its own, most prior *in vitro* studies of the effects of extracellular tau on intracellular tau have utilized immortalized, non-neuronal cells overexpressing truncated or otherwise mutated forms of aggregation-prone tau [184,223,231]. Additionally, while it was initially thought that the poorly soluble filaments of hyperphosphorylated tau found in tauopathies represent the most toxic forms of tau, there is growing evidence that prefibrillar, soluble tau oligomers [204-206] mediate synaptotoxicity, cellular dysfunction and neuron death in murine and cultured neuron models of AD [206,208-211]. Soluble tau oligomers may therefore resemble oligomers of the prion protein (PrP), which have been shown to exhibit a greater capacity than fibrillar PrP species to seed misfolding of monomeric PrP [28].

Tau is found in the CNS in six alternatively spliced isoforms, and interestingly, the isoform composition of tau aggregates varies among different tauopathies, such as corticobasal degeneration (predominantly 4R) [31], Pick's Disease (predominantly 3R) [32], and AD (approximately equal amounts of 3R and 4R [33]). Moreover, intronic mutations resulting in the alteration of tau isoform ratios, but not protein levels, can be fully penetrant for neurological disorders, indicating that isoforms play an important role in tau pathology [56].

While cell-to-cell transmission of pathological tau is well established, the functional consequences of intracellular tau aggregation to neuronal physiology have attracted little attention. Beyond its role in stabilizing microtubules, tau has also been shown to play an important role in the regulation of fast axonal transport. Microtubule-bound tau is known to act as a "speed bump" for kinesin-based transport, and there is an increased propensity for microtubule motors to detach or pause in the case of kinesin, or reverse direction in the case of cytoplasmic dynein, upon contact with tau-enriched regions of microtubules [113,116,242]. It has further been demonstrated that kinesin motors *in vitro* make longer runs and have a higher motive force in the absence of tau [115], but little is known about how a disruption or loss of tau may alter this microtubule motor transport inside living neurons.

With this background in mind, the study presented in this chapter was undertaken to test whether extracellular tau oligomers affect the endogenous intracellular tau distribution and the behavior of cultured mouse cortical neurons. This study demonstrate that the ability of extracellular tau oligomers to drive accumulation of intracellular tau varies by the isoform composition of the oligomers, that oligomers can cause dramatic invasion of endogenous intracellular tau into the somatodendritic compartment, and finally, that these effects are associated with the dysregulation of microtubule-based fast axonal transport. Altogether, the results imply that extracellular tau oligomers induce a breakdown of normal neuronal homeostasis that represents an early stage in neurodegeneration.

RESULTS

Production of tau oligomers

Formation of oligomers from recombinant versions of each of the 6 human CNS tau isoforms was confirmed by western blotting (Fig. 3-1, 3-2, B) Tau oligomerized by this protocol (see Materials and Methods) has previously been shown to migrate on western blots similarly to soluble multiple-*n* tau species isolated from AD brain [205]. Negative stain, transmission electron micrographs of monomeric, oligomeric and fibrillar 2N4R tau used throughout this study is shown in Fig. 3-2, B.

Extracellular Tau oligomers induce endogenous tau redistribution and accumulation

Previous studies have established that extracellular tau filaments can cause intracellular tau fragments corresponding to the MTBR domains to accumulate as puncta in non-neuronal cells stably transfected to express the tau fragments [184,223]. Extracellular tau filaments have also been demonstrated to cause tau aggregation in neurons expressing mutant tau [224,243]. To determine if extracellular tau oligomers can similarly drive accumulation of endogenous full length tau in cultured mouse neurons, we utilized a quantitative assay for accumulation of axonal tau (see Materials and Methods). Immunofluorescence micrographs were first thresholded by intensity to eliminate background fluorescence, after which each set of contiguous pixels above the threshold value was assigned to a group that was either larger or smaller than a pre-defined number of contiguous pixels. As the immunofluorescence staining becomes discontinuous there is an increase in smaller pixel groups, indicative of tau accumulation (Fig. 3-3, A). After

initially testing cutoffs between 1000 and 20,000 contiguous pixels (Fig. 3-3, B), we chose 10,000 pixels of contiguous labeling as the cutoff between continuous and discontinuous tau staining, which are pseudo-labeled green and red, respectively, throughout this report. Antibodies specific for human tau did not stain the cells above background levels (Fig. 3-4), indicating that the accumulated and missorted intracellular tau was predominantly or exclusively endogenous mouse tau. As can be seen in Fig. 3-5, extracellular tau oligomers also caused extensive accumulation of intracellular tau and its conspicuous invasion into the somatodendritic compartment.

Using this quantification method, we found that untreated neurons, and neurons treated for 18 hours with monomeric or fibrillar 2N4R tau at 50 nM total tau had 20%, 19%, and 17% endogenous tau accumulation, respectively. In contrast, cells treated comparably with oligomeric tau showed an average of 34% tau accumulation, an ~1.75-fold increase over the other conditions tested (Fig. 3-6, Table 3-1).

To determine whether intracellular tau accumulation represented a global disruption of microtubules, neurons exposed to extracellular 2N4R tau under the same conditions were labeled with antibodies to MAP2, a somatodendritic protein [244,245] that shares a highly conserved MTBR region with tau [246], and to βIII-tubulin, a neuron-specific tubulin isoform [247]. Neither MAP2 (Fig. 3-7, A) nor βIII-tubulin (Fig. 3-7, B) showed signs of accumulation in response to extracellular monomeric, oligomeric or fibrillar tau. These data imply that extracellular tau oligomers alter the distribution of endogenous tau without affecting microtubules in any other ways that might have been detected by our methods.

Extracellular tau oligomers do not rapidly induce AD-like conformational change or phosphorylation of endogenous intracellular tau

Cells treated with 2N4R tau oligomers were also analyzed by immunofluorescence using a panel of anti-tau antibodies that detect conformational or phosphorylation-dependent changes characteristic of pathological tau in AD neurons. Cells were stained with the conformation-specific antibodies, Alz50 [248,249] and MC1 [250], which detect early pathology-associated changes in tau misfolding, and PHF1, which recognizes tau that is phosphorylated at S396/S404 [171] and is enriched in late stage tau inclusions [251]. No immunoreactivity was observed with Alz50 or MC1 in untreated neurons or in neurons treated with tau monomers or oligomers (Fig. 3-8), although as shown in Fig. 3-9, both antibodies labeled misfolded tau in brain sections from the CVN strain of AD model mice [239]. In contrast to what was observed for Alz50 and MC1, a low, constant level of PHF1 immunoreactivity was observed in cultured neurons regardless of whether or not they were exposed extracellular tau monomers or oligomers (Fig. 3-8).

Isoform composition of tau oligomers affects the extent of intracellular tau accumulation

To determine whether accumulation of endogenous tau depends on the isoform composition of the externally applied tau oligomers, we repeated these experiments with the remaining 5 isoforms of CNS tau. Oligomeric 2N3R tau was able to induce statistically significant accumulation of endogenous tau, albeit not quite as effectively as 2N4R tau (Fig. 3-10). Oligomeric 0N4R tau induced accumulation of endogenous to a small, though

significant extent, while oligomeric 0N3R, 1N3R and 1N4R tau did not significantly stimulate accumulation of intracellular tau (Figs. 3-11 and 3-12).

Finally, neurons were exposed to extracellular tau of 2 mixed isoform compositions, a 1:1 mixture of 2N3R and 2N4R tau, and all 6 isoforms in equimolar ratios. Total tau concentrations for each experiment were held constant at 50 nM and isoform mixtures were allowed to oligomerize together. The 1:1 mixture of 2N3R:2N4R did not alter the tau distribution when presented to neurons as monomers or fibrils. In contrast, when presented as oligomers, the 1:1 mixture did cause a significant increase in tau accumulation, although to a lesser extent than observed for either 2N3R or 2N4R oligomers alone (Fig. 3-13, A, Table 3-1). Interestingly, the most potent response was seen upon treatment with all 6 isoforms, as both monomeric and fibrillar treatments caused an increase in tau mislocalization relative to no tau treatment, and oligomeric tau had an even larger, statistically significant effect (Fig. 3-13, B, Table 3-1). It is also noteworthy that oligomers made from all 6 isoforms caused disruption of MAP2 as well (Fig. 3-13, B), indicating that this treatment represents a particularly toxic insult to neurons.

Accumulation of endogenous axonal tau is associated with dysregulation of fast axonal transport

In prior studies of cell-to-cell propagation of tau, little analysis has been performed regarding effects of tau accumulation on neuronal physiology. As a predominantly axonal protein, tau is known to regulate the attachment and movement of various motors involved in axonal transport, and this effect has been shown to affect kinesin-1 more

potently than cytoplasmic dynein [116]. More specifically, tau appears to act as an obstacle, or "speed bump", that can reduce cargo run lengths [113,116,242] and increase the likelihood of the motor disengaging from microtubules [115,116]. To analyze the effect, if any, that the mislocalization of tau we describe here has on axonal transport, primary neurons were transfected with fluorescently-tagged proteins to label vesicles undergoing microtubule-directed fast axonal transport. Specifically, BDNF-mRFP1 and BACE1-mRFP1 were utilized to analyze effects on kinesin-1-dependent transport [103,252], and NeuropeptideY-mCherry to observe kinesin-3-dependent transport [103] (see Supplementary movie 1). Because 2N4R tau oligomers produced a robust response in previous experiments and data analysis for these experiments was extremely time consuming, we focused only on 2N4R tau oligomers. Trafficking of the various fluorescently labeled vesicles was analyzed by live cell, time lapse imaging. 1-2 % of neurons expressed the fluorescent fusion proteins, allowing for the imaging of isolated neurons. Axons were identified by finding regions of consistent thickness and low branching, which were then traced back to their cell body of origin to discriminate anterograde versus retrograde transport. Vesicle transport was analyzed by kymograph analysis (Fig 3-14), with values obtained for run length, run time and velocity while in motion (Fig. 3-15 and Table 3-2).

BDNF-mRFP1 labeled vesicles showed no significant changes in run length, run time or instantaneous velocity in either the anterograde or retrograde direction upon treatment with monomeric full-length tau, as compared to a non-treated control. Neurons treated with oligomeric tau showed an ~2.5-fold increase in anterograde and

retrograde run length, an ~30% increase in anterograde run time and an ~2-fold increase in retrograde time, and an ~1.5-fold increase in anterograde instantaneous velocity (Fig. 3-15, A). Similar results were obtained for neurons transfected with BACE1-mRFP1 (Fig. 3-15, B), a protease that helps produce amyloid-β (Aβ) from the amyloid precursor protein (AβPP), and has been shown to be trafficked along with APP by kinesin-1 [252]. BACE1-mRFP1 expressing neurons treated with oligomeric tau showed an ~2-fold increase in anterograde run length and a ~40% increase in anterograde instantaneous velocity, suggesting that oligomeric 2N4R tau treatments affect multiple kinesin-1 cargoes in the same general manner.

Otherwise identical experiments were also performed using mCherry-tagged NeuropeptideY, which is trafficked anterogradely by a different motor, kinesin-3 [103]. No significant alterations were seen for anterograde run length, run time, or velocity when comparing untreated, 2N4R monomer-treated, and 2N4R oligomer-treated cells. 2N4R oligomers did not affect retrograde run time either, but they did increase retrograde run length and velocity by ~2.3-fold and ~20%, respectively (Fig. 3-15, C). Taken together, these results indicate that tau mislocalization following oligomeric tau treatment of neurons leads to dysregulation of axonal transport for multiple types of cargoes.

Robust disruption of fast axonal transport disruption by extracellular tau oligomers requires endogenous tau

To determine whether these effects of extracellular tau oligomers on axonal transport depend on intracellular tau, transport experiments were repeated using neurons isolated from tau knockout mice [101]. While the values for run time, run length and instantaneous velocity in untreated tau knockout neurons were slightly elevated relative to the control values in WT neurons, with minor exceptions, no changes were seen in these parameters for any of the 3 cargos in the anterograde or retrograde direction after cellular exposure to 2N4R tau monomers or oligomers (Fig 3-16). The only exceptions were small, but significant changes in the retrograde run length and run time for BDNF-transfected cells and retrograde velocity for NeuropeptideY transfected cells when treated with monomeric tau (Fig 3-16C, Table 3-2). The collective results of the organelle transport experiments in wild type and tau knockout neurons support the hypothesis that extracellular tau oligomers disrupt anterograde and retrograde fast axonal transport by causing accumulation of endogenous intracellular tau.

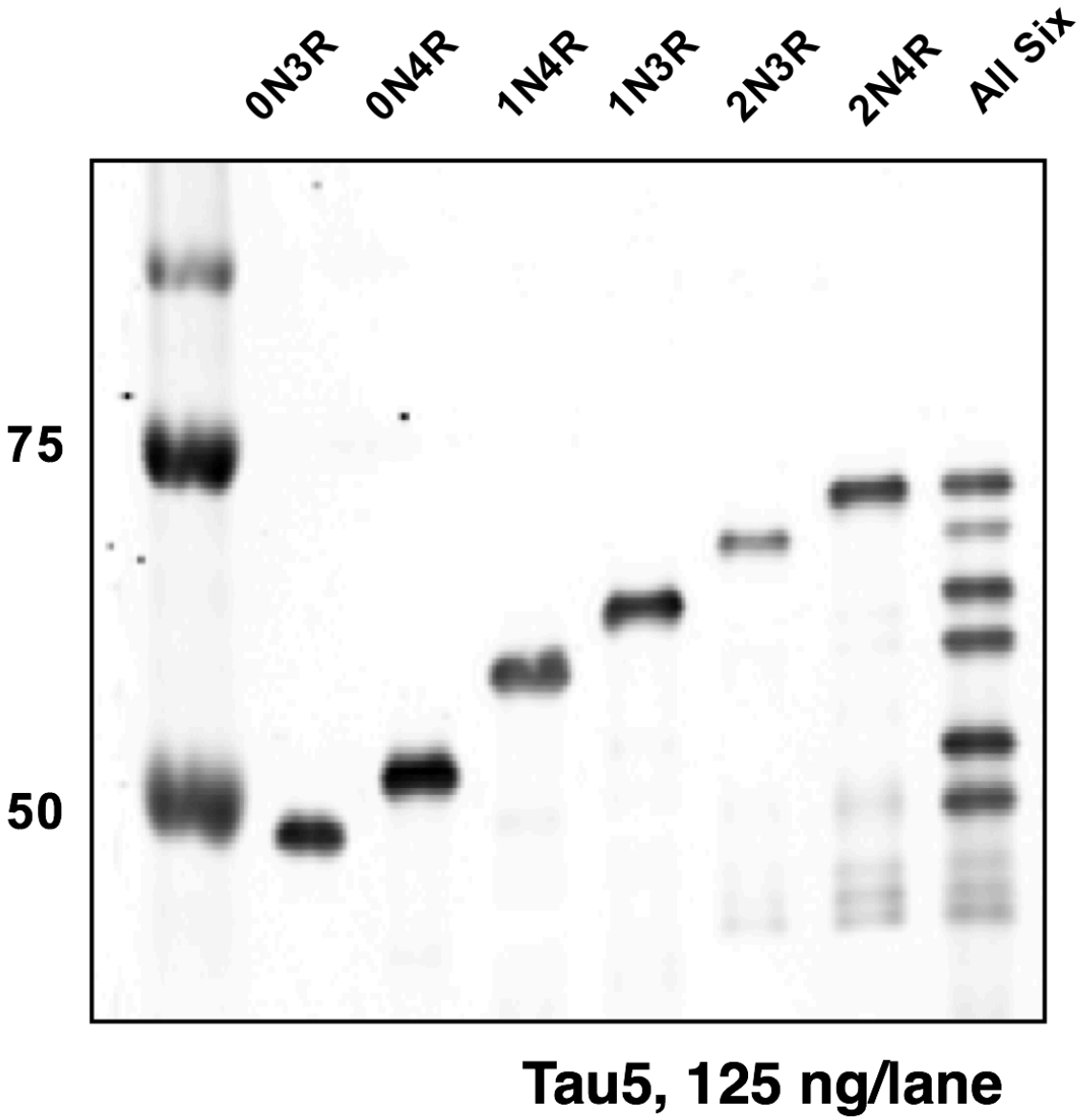


Figure 3-1: **Western blot of recombinant tau isoforms utilized in this study.** Expression vectors for all six isoforms with 5'-his-tags were transformed into BL21 and purified by metal affinity chromatography. Purified proteins were concentrated in 10K molecular weight cutoff spin columns (Millipore) and dialyzed into PBS pH 7.4. Protein concentration was determined by BCA assay. Samples above were diluted in 1X LDS sample buffer (Invitrogen) and 125 ng of each isoform was loaded per lane. The all six lane represents 125 ng of each individual isoform.

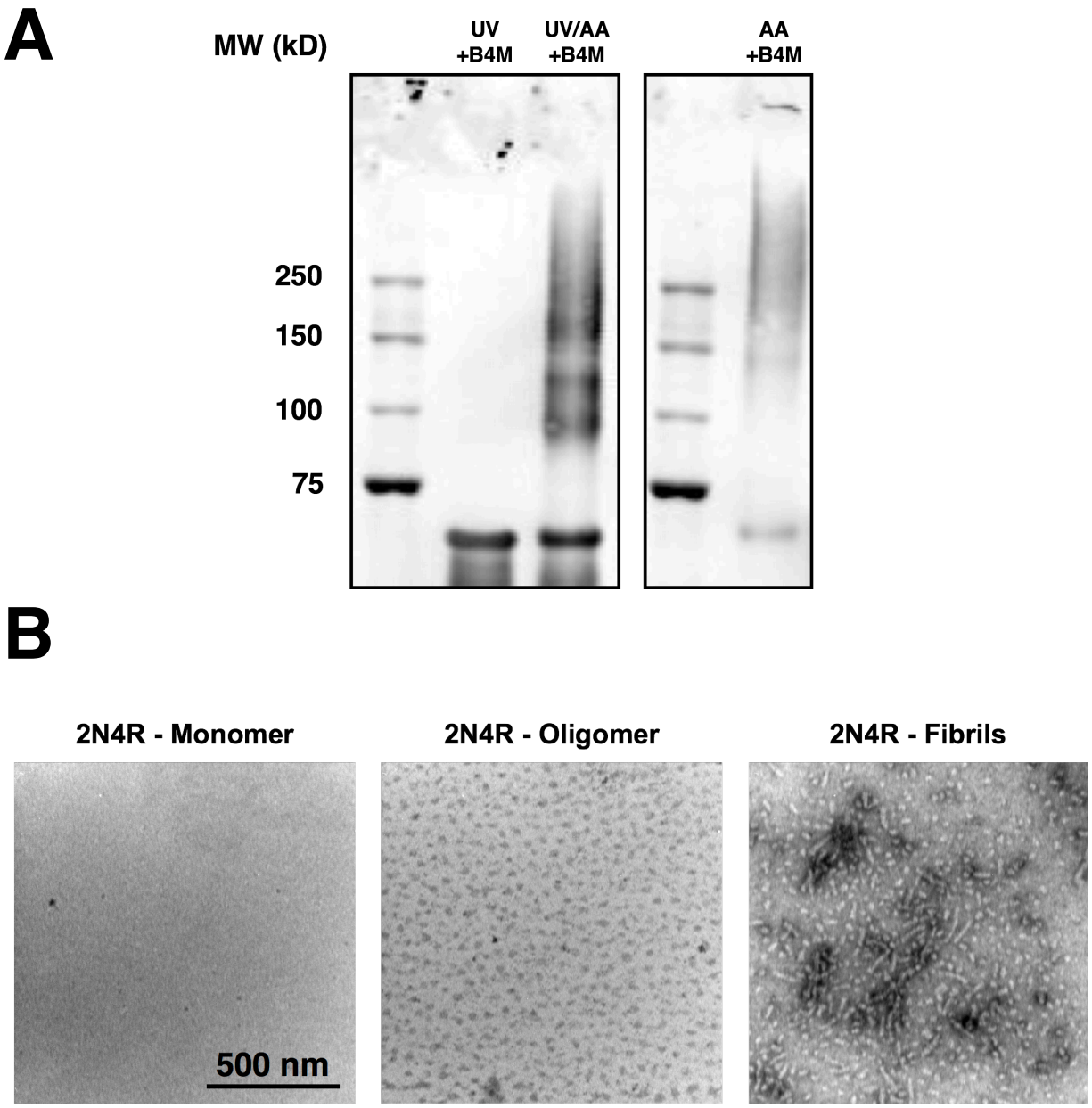


Figure 3-2: **Characterization of tau oligomers** A) Tau 5 western blot of monomeric, oligomeric, and fibrillar 2N4R tau. Monomeric tau represents tau treated with a covalent crosslinker (B4M) and exposed only to UV light (UV +B4M). Oligomeric tau represents B4M-treated tau induced to aggregate with AA overnight, then exposed to UV light (UV/AA + B4M). Fibrillar tau represents B4M-treated tau induced to aggregate with AA for 3 days, with no UV light exposure (AA + B4M) B) Negative stain transmission electron micrographs of 2N4R monomeric, oligomeric and fibrillar tau.

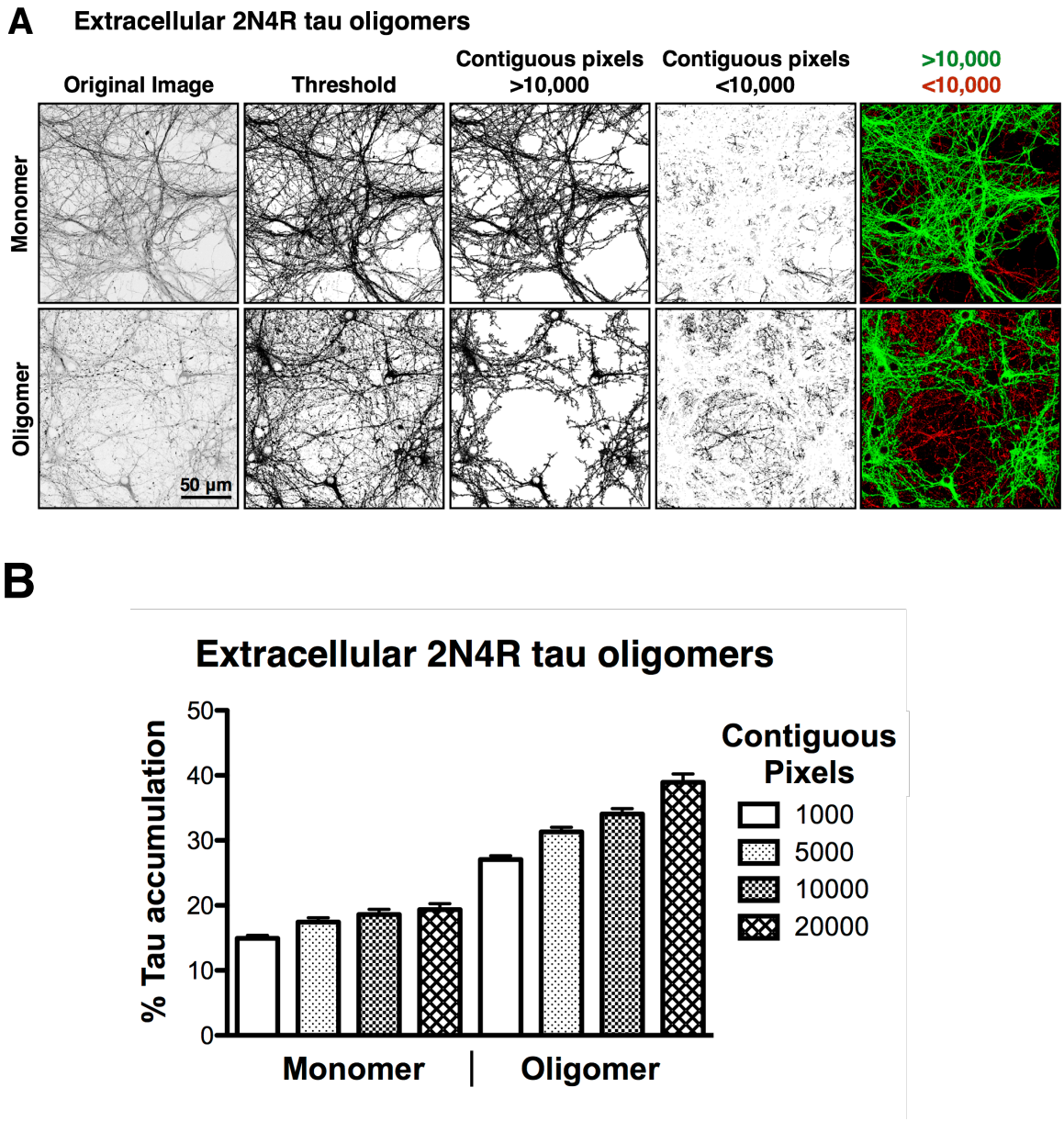


Figure 3-3: Image analysis of tau-treated neurons. **A**) Representative immunofluorescence images of primary neurons stained for total tau with Tau 5 after treatment with monomeric or oligomeric extracellular tau are shown on the left. Fluorescence micrographs were thresholded to indicate pixels above background intensity, segregated into groups of >10,000 or <10,000 contiguous pixels above the threshold intensity, then pseudo-colored green to indicate intact staining (>10,000 contiguous pixels) or red to indicate disrupted staining (<10,000 contiguous pixels). **B**) Test of various pixel size cutoffs in monomer or oligomer treated neurons. 10,000 contiguous pixels was chosen as the cutoff between intact and disrupted tau for all subsequent assays.

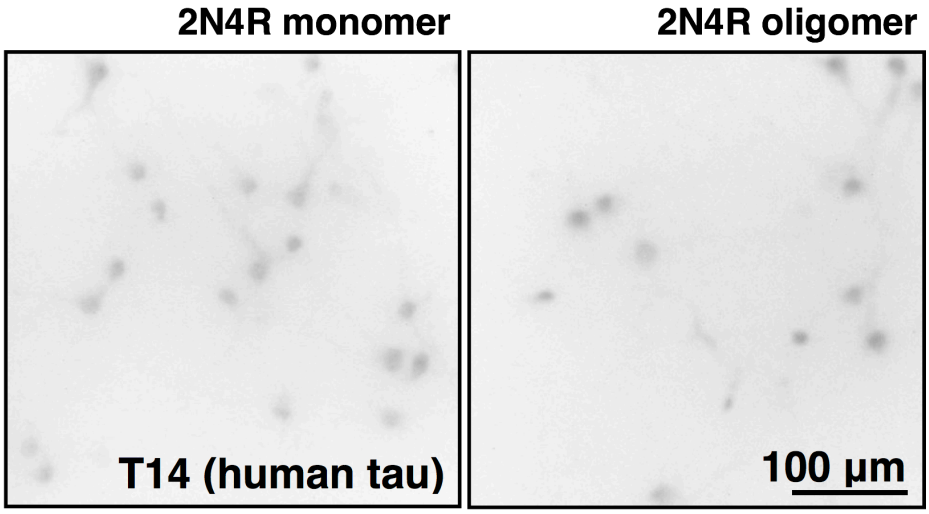


Figure 3-4: Monomer and oligomer treated cells probed with antibodies for human tau (T14) exhibit only background staining Low background staining with a human specific tau antibody indicates the localized tau accumulations observed upon 2NR oligomer treatment are composed off endogenous mouse tau.

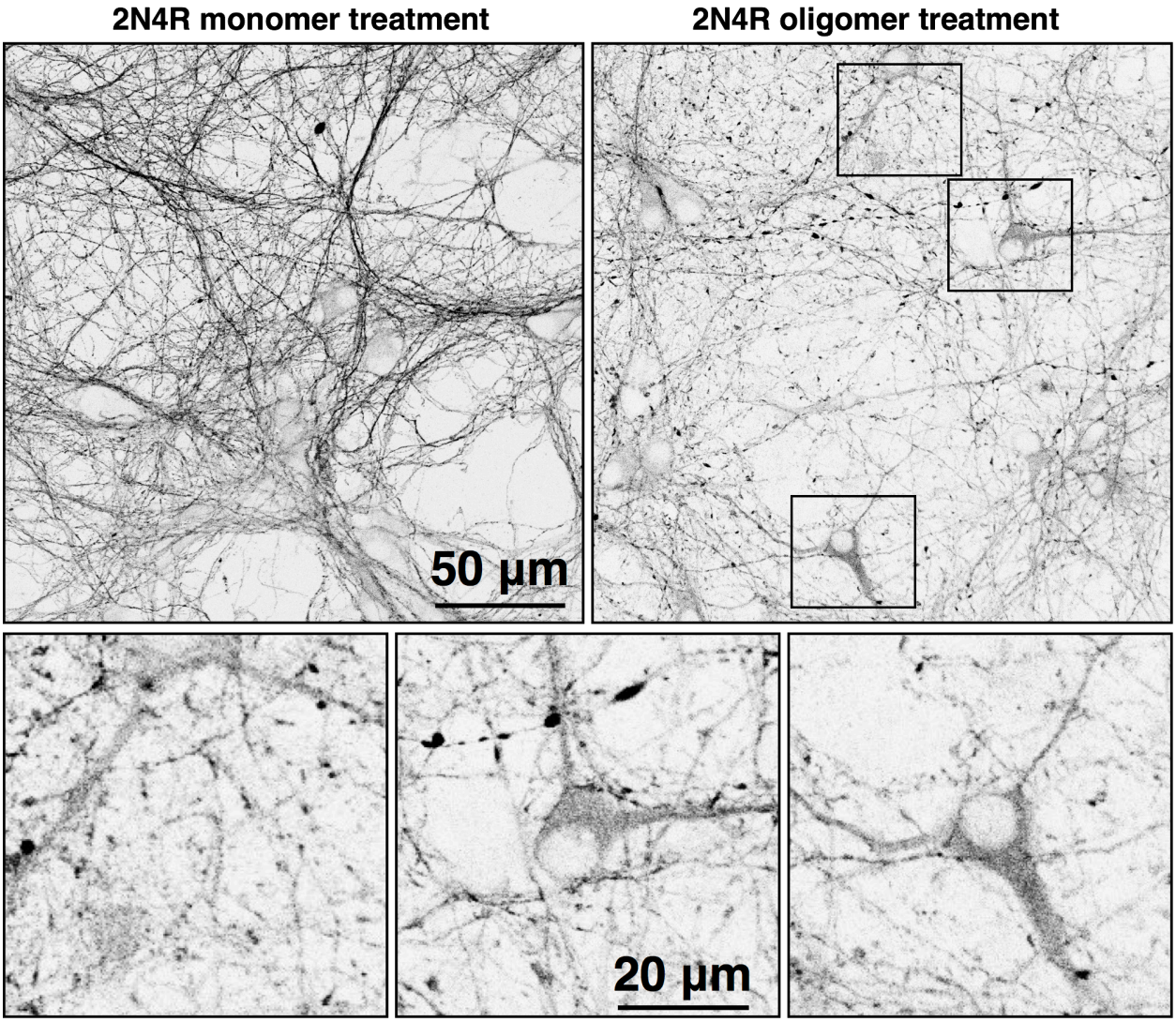


Figure 3-5: Extracellular tau oligomers induce endogenous tau to aggregate in axons and invade the somatodendritic compartment. High-resolution immunofluorescence micrographs of primary mouse cortical neurons are shown here. Relatively continuous axonal tau staining and minimal somatodendritic tau staining was observed following exposure to 2N4R monomers, while 2N4R oligomer-treated cells developed tau puncta and varicosities in axons, and striking tau accumulation in the somatodendritic compartment.

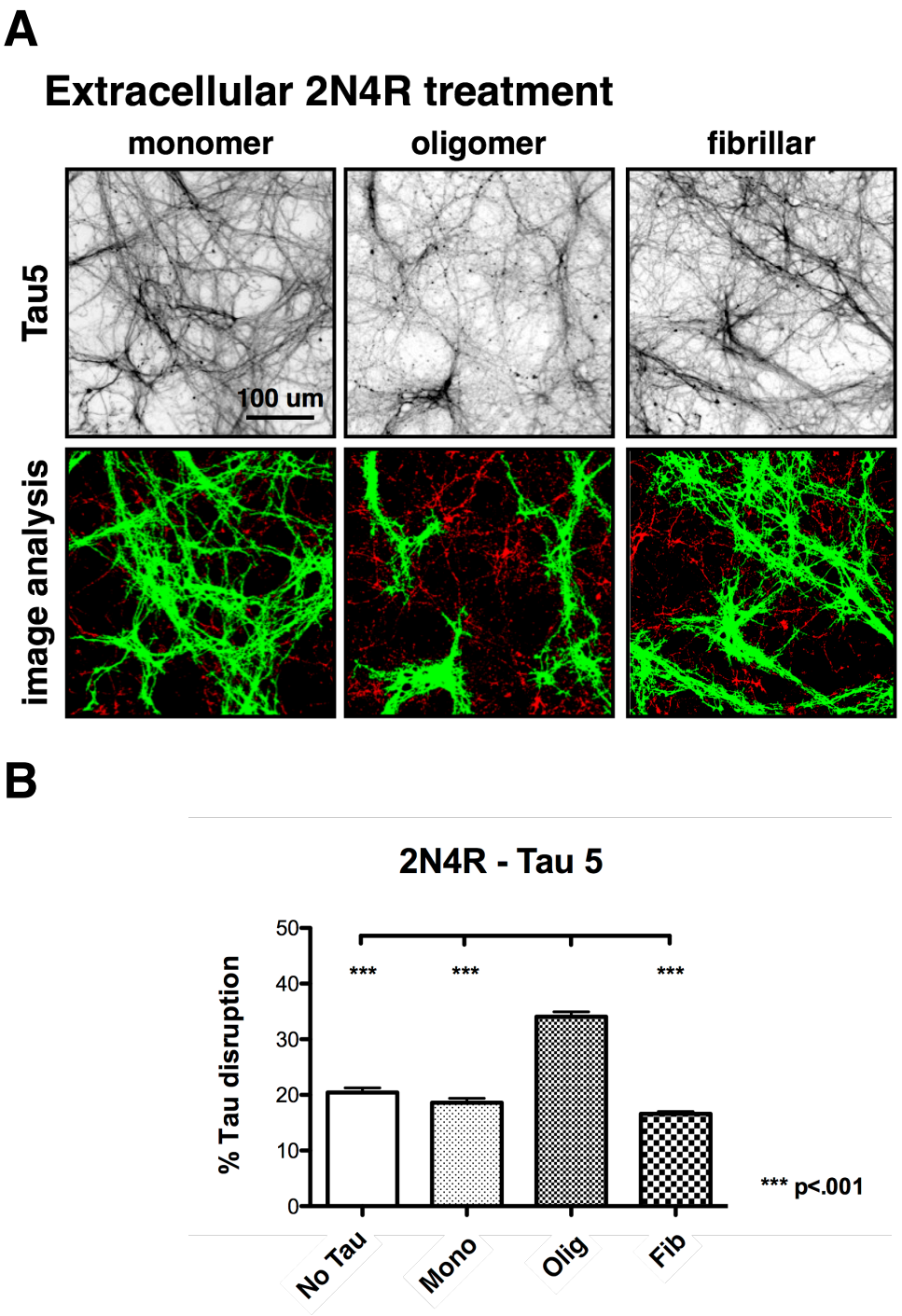


Figure 3-6: Extracellular 2N4R tau oligomers disrupt endogenous wild-type tau. A,B) Extracellular 2N4R oligomers, but not monomers or fibrils, disrupt endogenous tau, as visualized by Tau 5 staining. Error bars represent SEM.

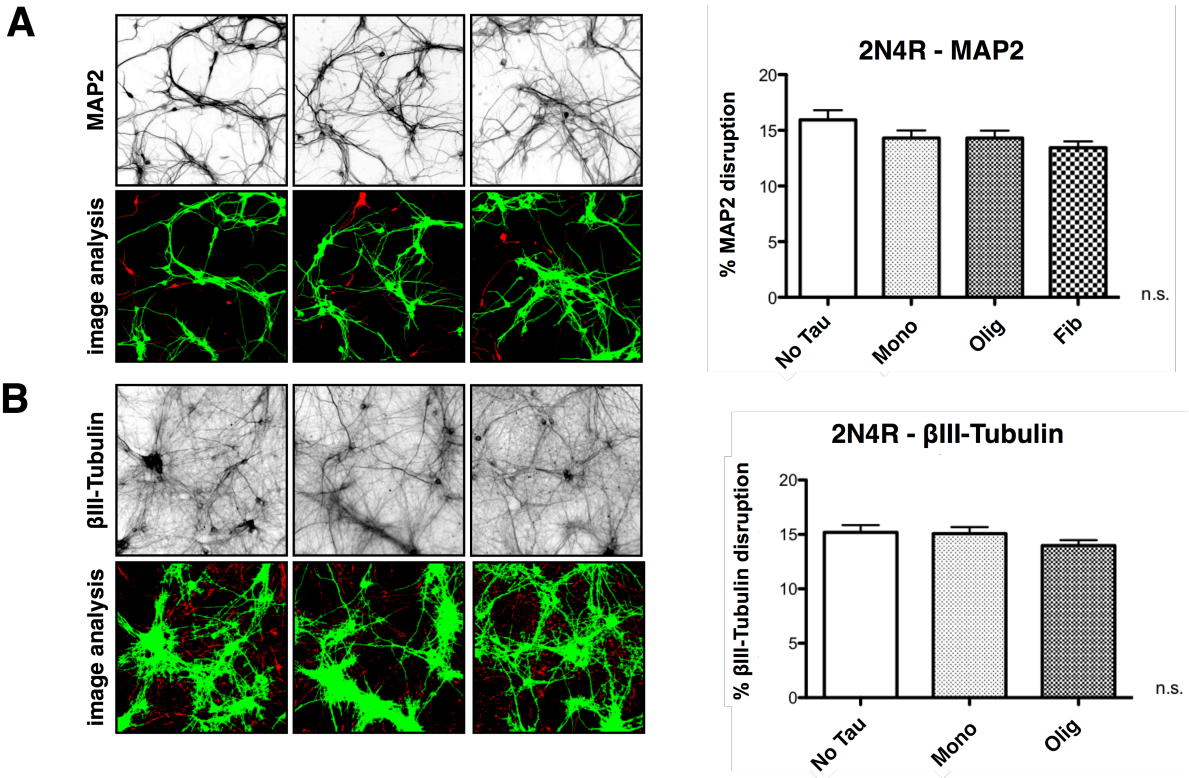


Figure 3-7: **Extracellular tau oligomers alter the distribution of endogenous tau without affecting microtubules in other ways detectable by this analysis.** No disruption of MAP2 or BIII tubulin was observed after treatment of neurons with monomeric, oligomeric or fibrillar 2N4R tau. Error bars represent SEM.

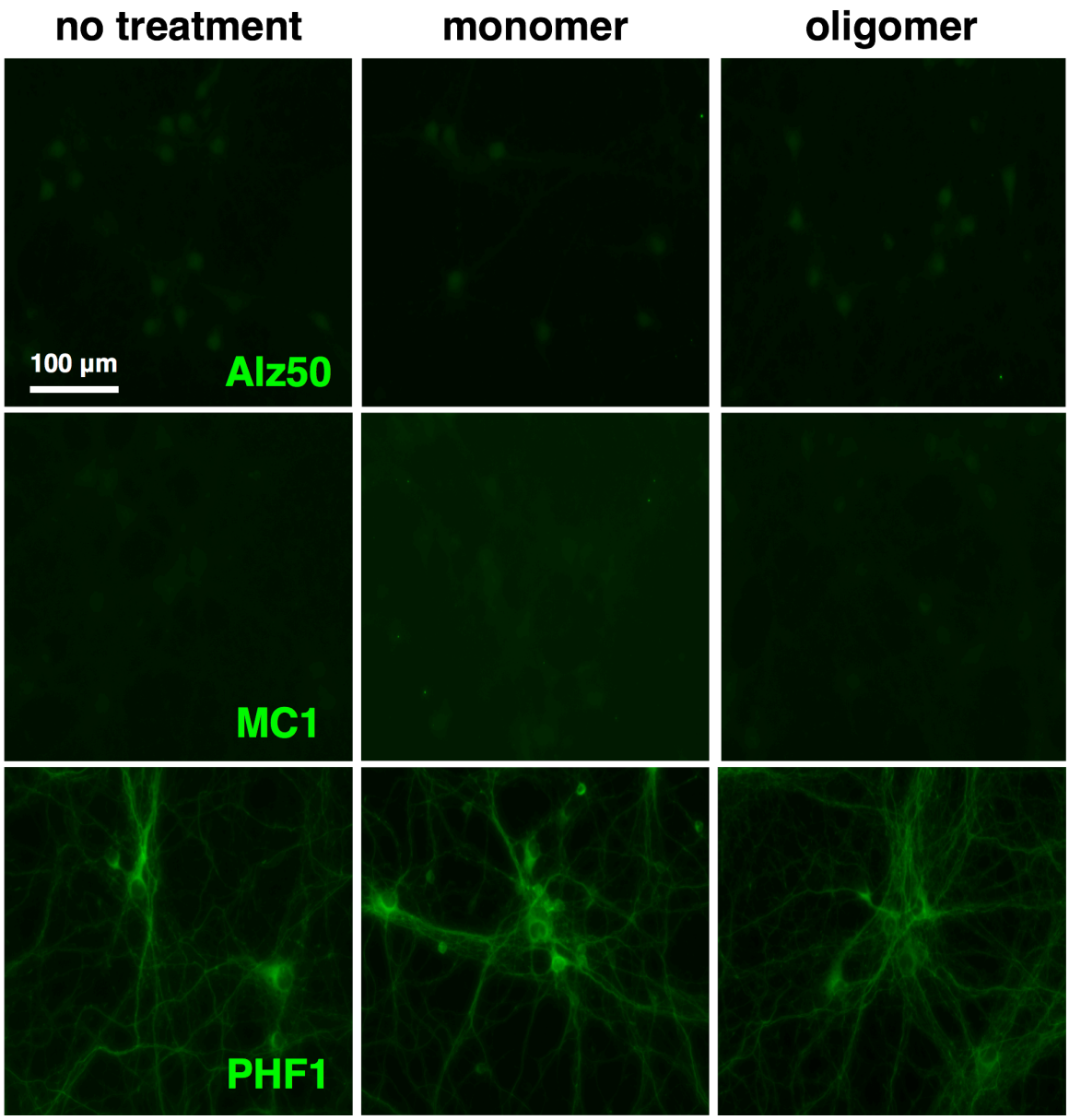


Figure 3-8: Extracellular tau oligomers do no rapidly induce selective AD-like conformational changes or phosphorylation of endogenous intracellular tau. No immunoreactivity was observed for the conformation-specific antibodies, Alz50 or MC1, in untreated neurons or those exposed to extracellular 2N4R tau monomers or oligomers. Additionally, a low constant level of PHF-1 was observed for untreated, monomer-treated and oligomer-treated cells.

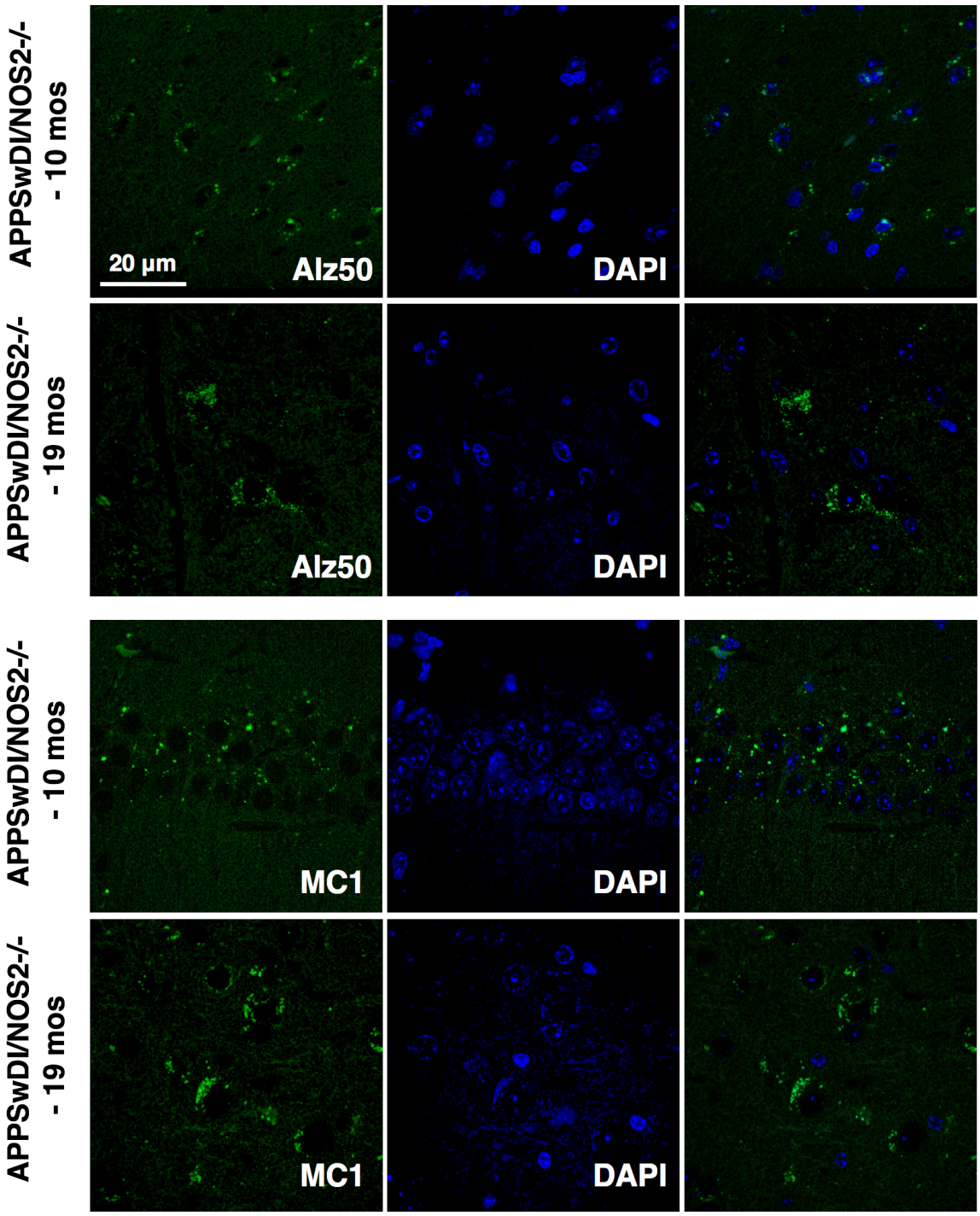


Figure 3-9: Staining of aged mouse tissue confirms activity of Alz50 and MC1 antibodies
CVN mice, which overexpress a triple mutant human APP gene, exhibit an age dependent accumulation of pathogenic intraneuronal tau that is detect by both Alz50 and MC1 antibodies, indicating that these antibodies effectively identify abnormally misfolded tau.

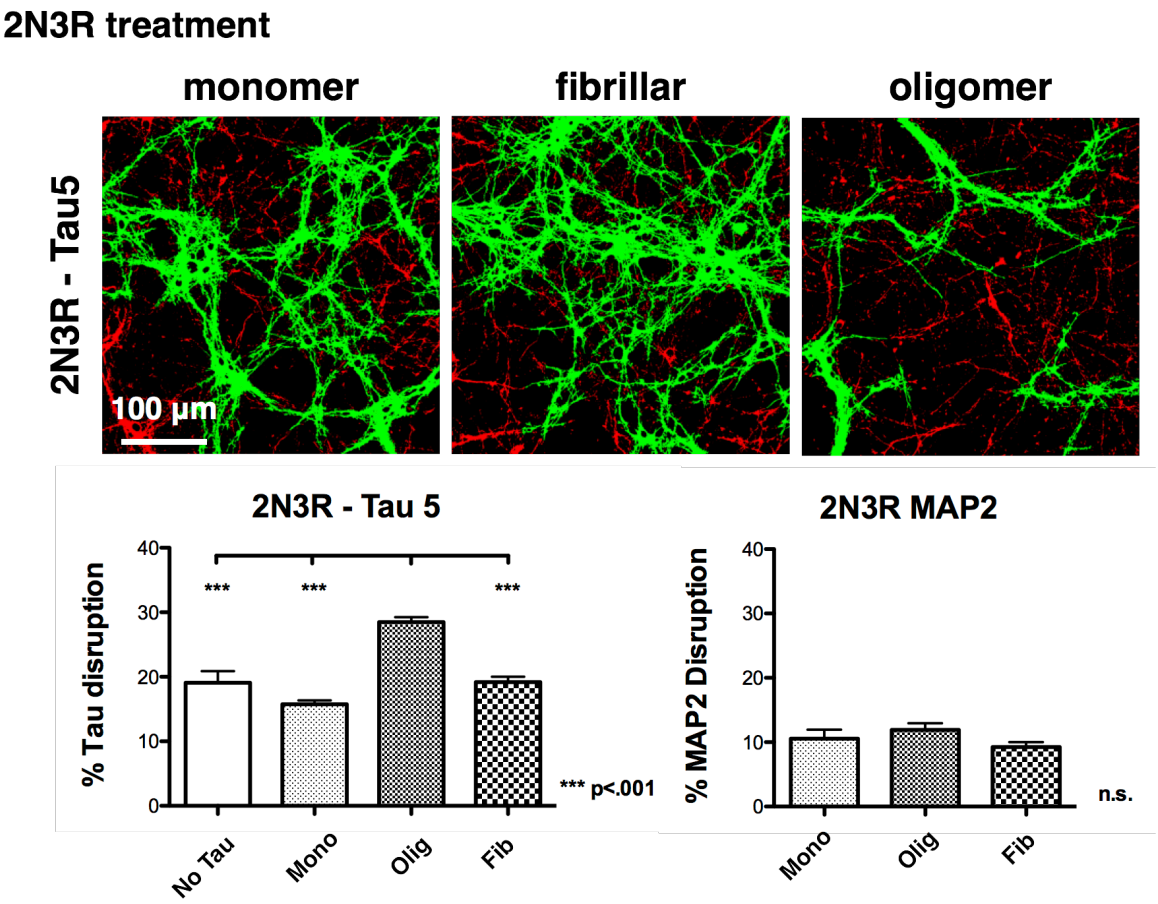


Figure 3-10: The extent of intracellular tau disruption in tau oligomer treated-cells varies by the tau isoform composition of the oligomers. A) Neurons treated with oligomeric, but not monomeric or fibrillar 2N3R tau, show aggregation of tau, but not of MAP2. Error bars represent SEM.

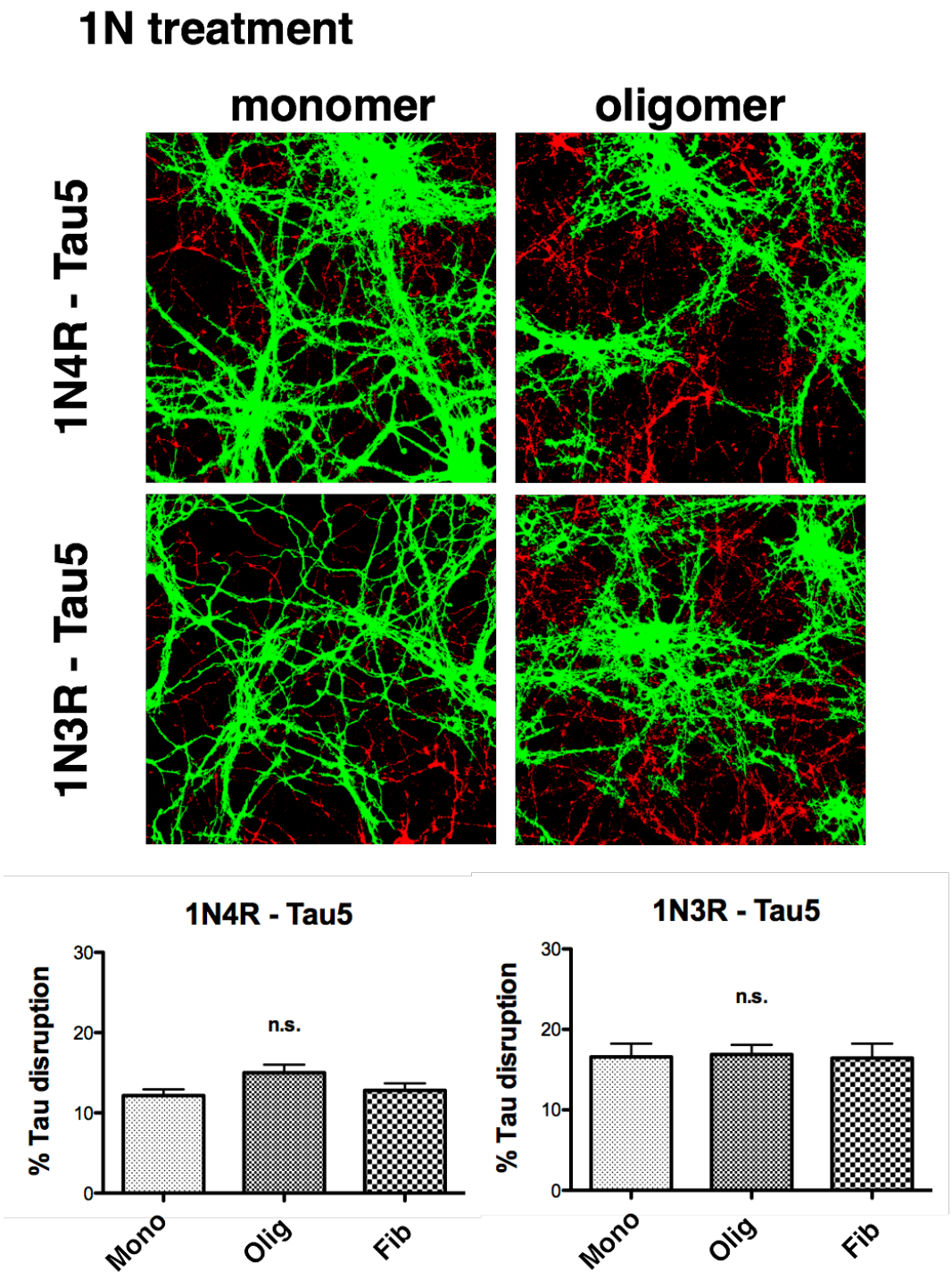


Figure 3-11: Treat of neurons with 1N isoforms causes no significant tau disruption. Neurons treated with extracellular monomers, oligomers, of fibrils composed of either 1N tau isoforms exhibit no significant disruption of endogenous tau. Error bars represent SEM.

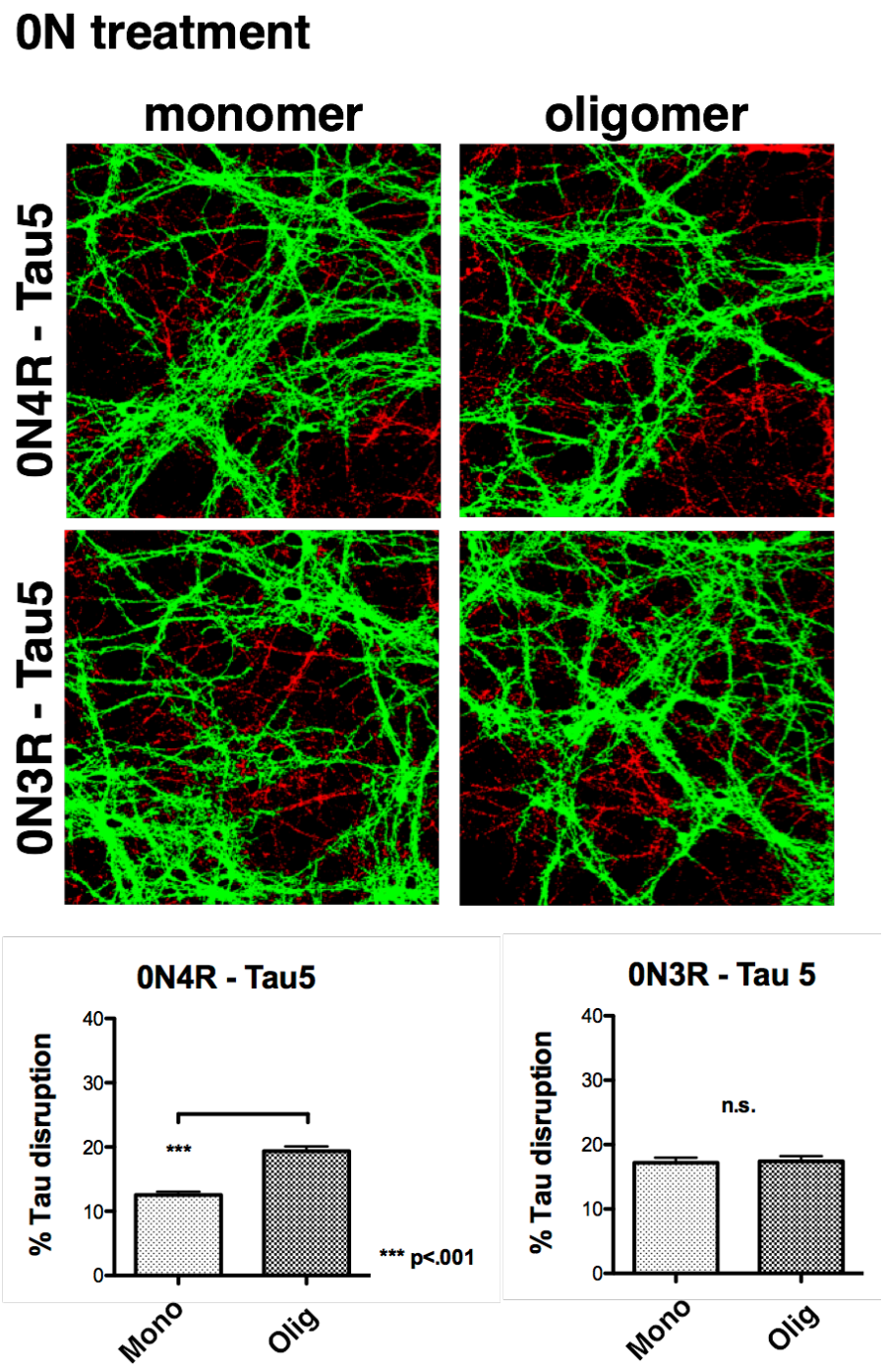


Figure 3-12: Extracellular oligomers composed of 0N4R, but not 0N3R tau caused aggregation of endogenous axonal tau. Treatment with extracellular oligomeric 0N4R tau significantly increased endogenous tau disruption, compared to monomeric 0N4R. Oligomeric or monomeric 0N3R tau treated cells exhibit no significant disruption of endogenous tau. Error bars represent SEM.

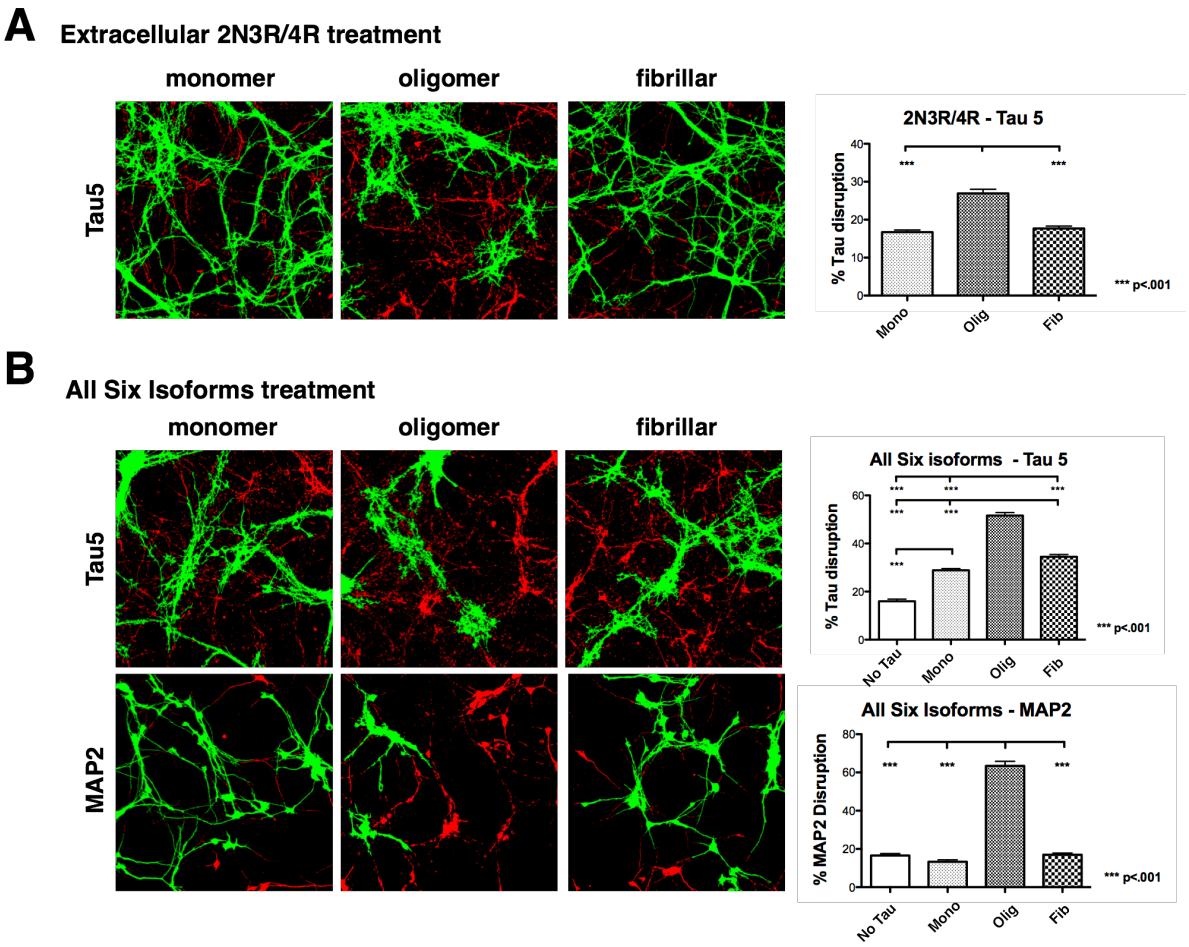


Figure 3-13: Extracellular oligomers of mixed isoform composition cause endogenous tau aggregation. A) Neurons treated with oligomers, but not monomers or fibrils, of a 1:1 mixture of 2N3R and 2N4R tau caused aggregation of endogenous axonal tau. B) Neurons exposed to an equimolar mix of all 6 tau isoforms developed endogenous tau aggregates, especially when the extracellular tau was oligomeric. Treatment with oligomers of all 6 isoforms additionally caused extensive aggregation of MAP2. Error bars represent SEM.

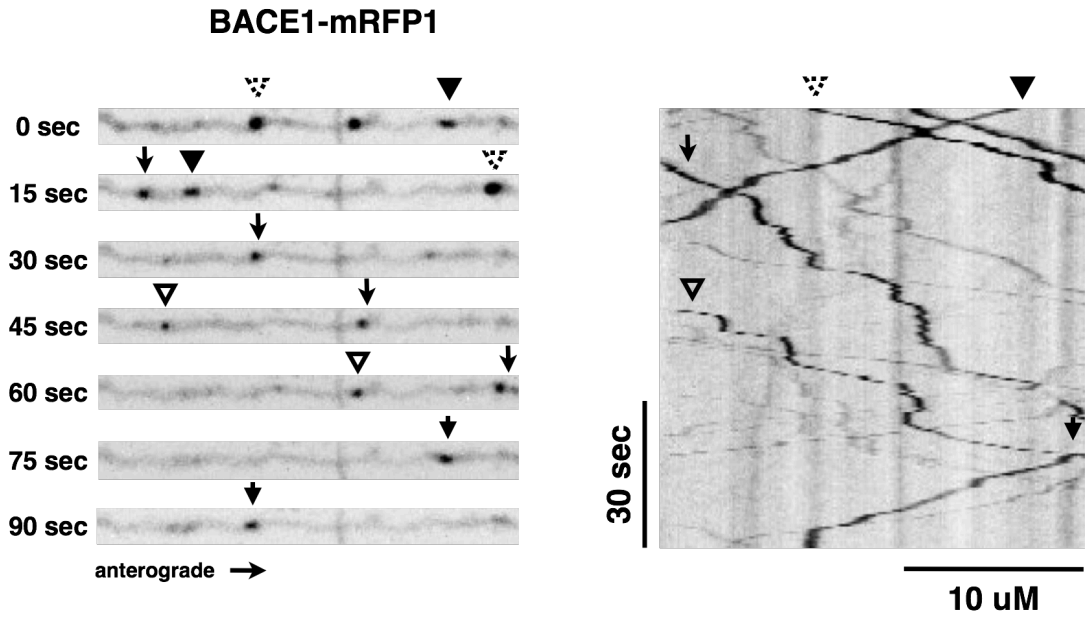


Figure 3-14: Analysis of axonal trafficking by kymograph analysis. Transport parameters were analyzed by live cell, time lapse fluorescence microscopy (left) followed by kymograph analysis (right). Axons are identified from a projection of the maximum fluorescence of each frame and traced in ImageJ after which the kymograph is generated from the multiple kymograph plug-in in Image-J

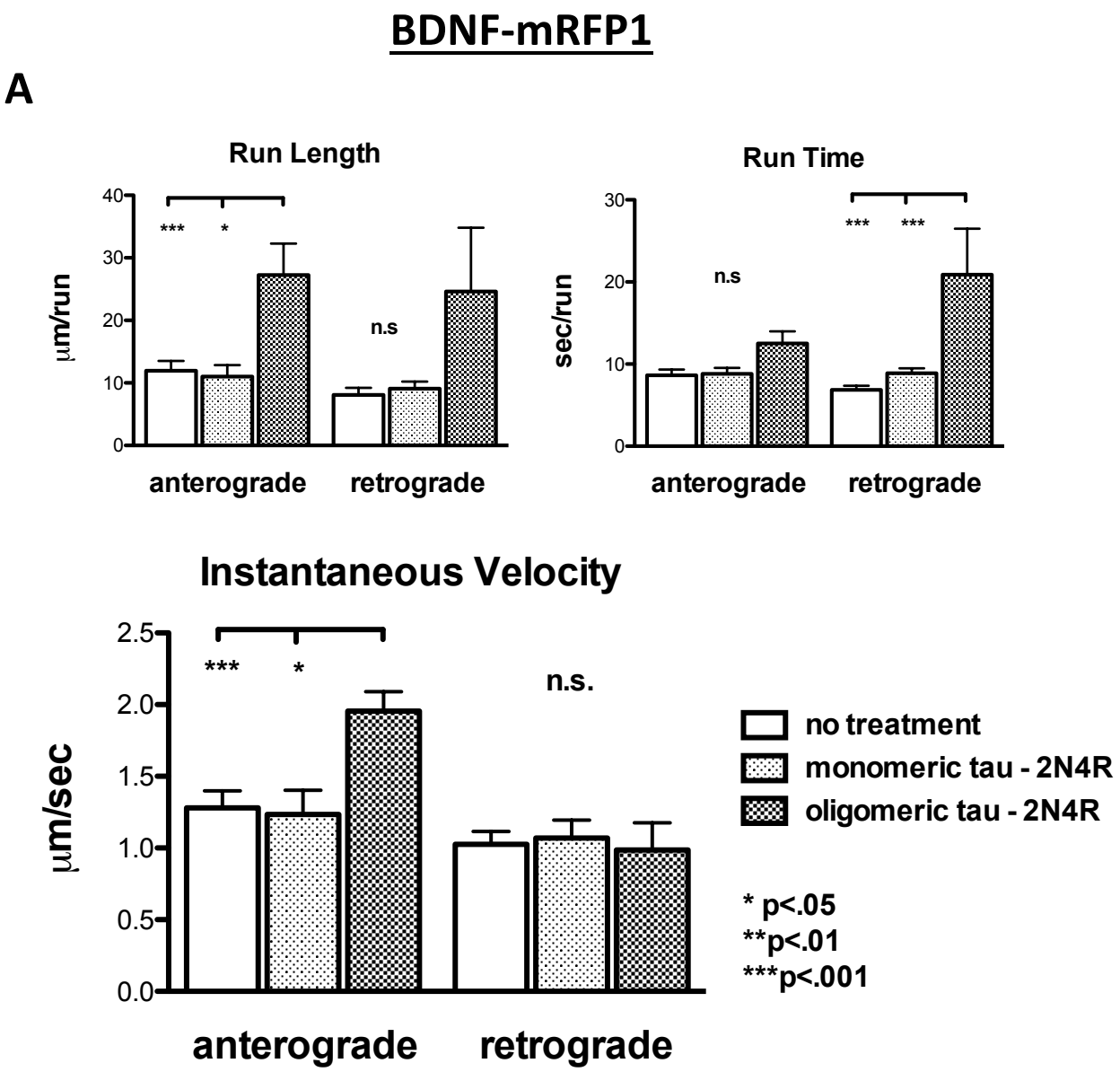
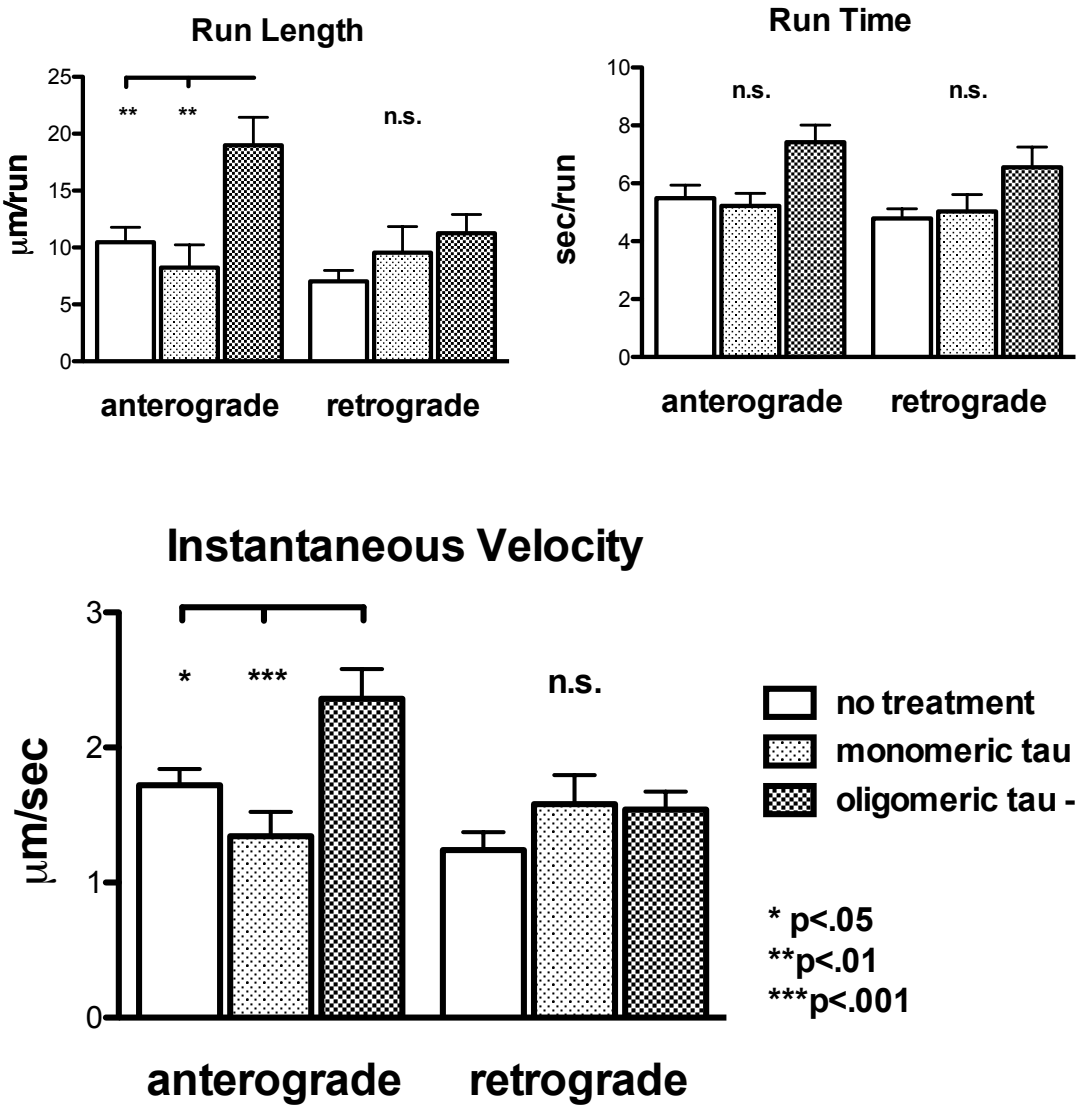


Fig. 3-15. **Tau oligomer exposure induces disruption of fast axonal transport.** Run length, run time and instantaneous velocity were quantified for neurons transfected with expression vectors for **A**) BDNF-mRFP1 (pg. 79), **B**) BACE1-mRFP1 (pg. 80) and **C**) NeuropeptideY-mCherry (pg. 81). Error bars represent SEM.

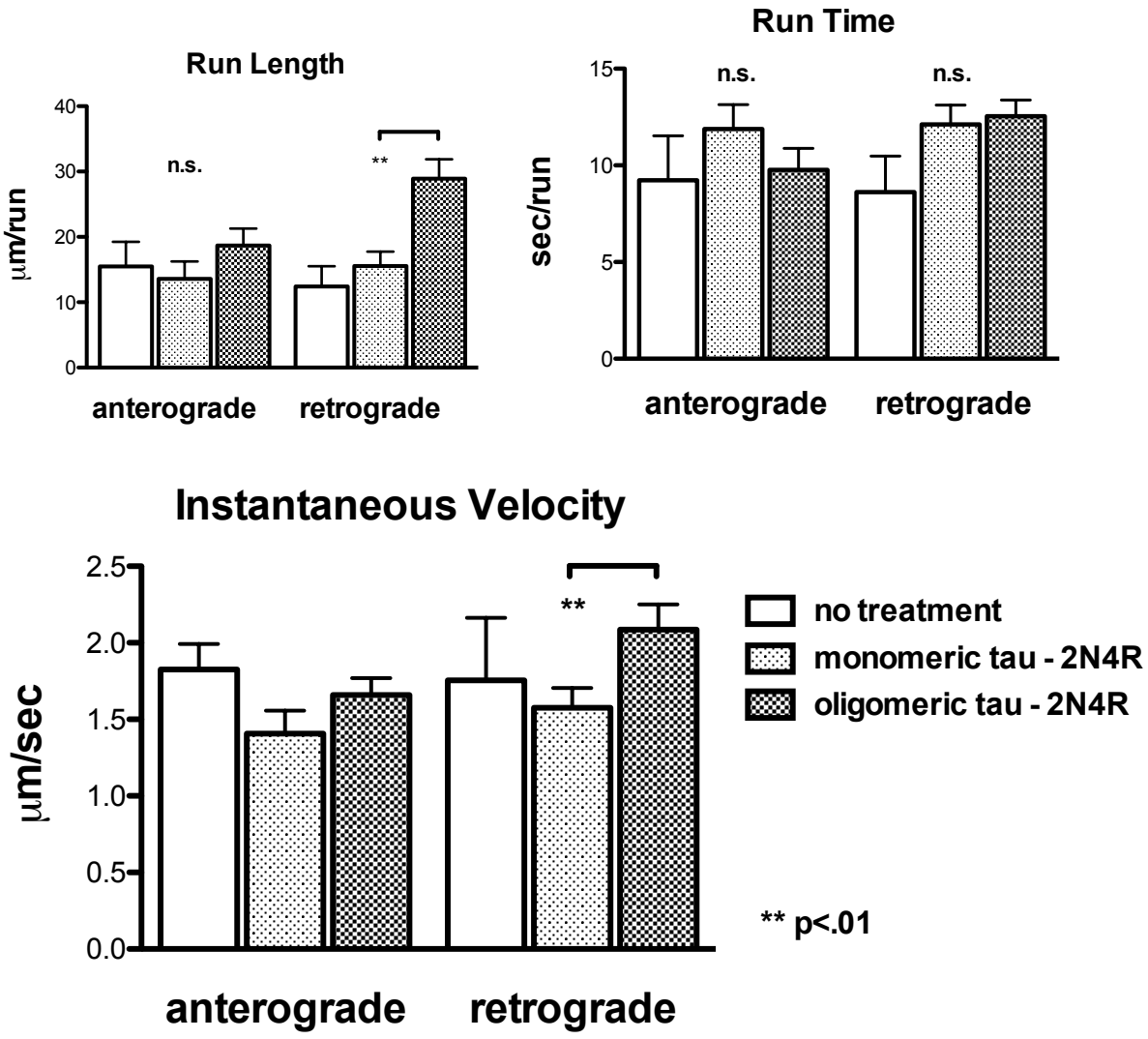
BACE1-mRFP1

B



NeuropeptideY-mCherry

C



BDNF-mRFP1
Tau Knockout

A

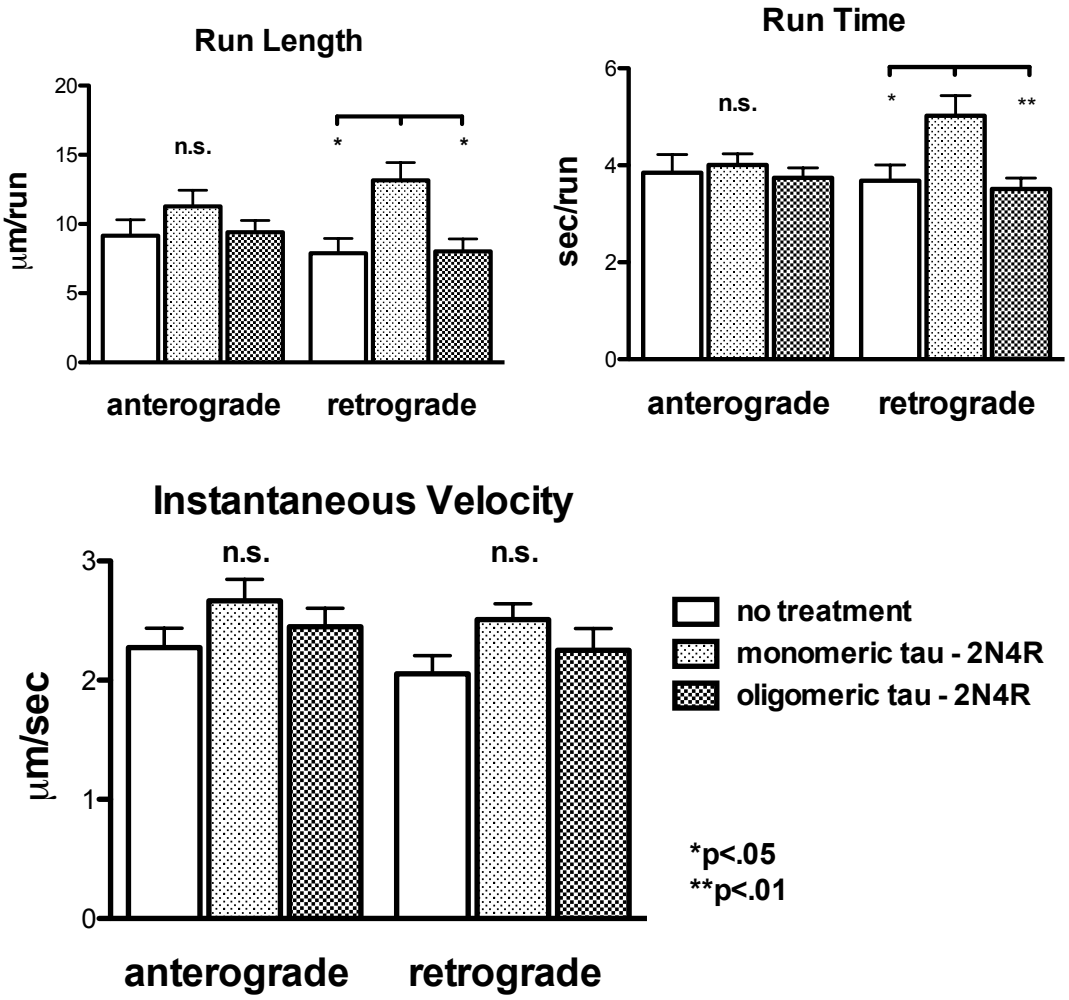
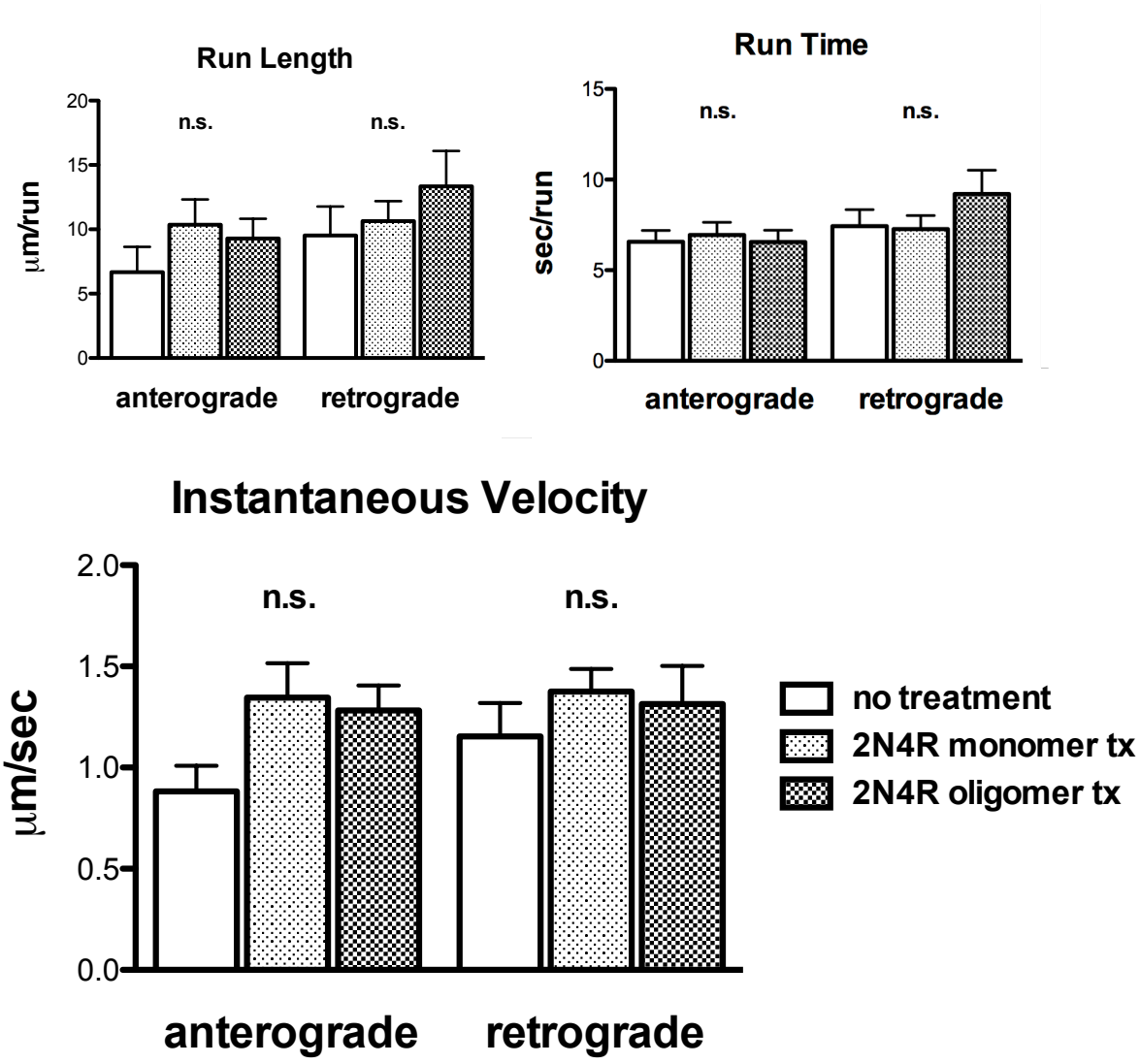


Figure 3-16. **Robust disruption of fast axonal transport disruption by extracellular tau oligomers requires endogenous tau.** Transport parameters were analyzed in tau knockout neurons by live cell, time lapse fluorescence microscopy followed by kymograph analysis, as for 3-15. Run length, run time and instantaneous velocity were quantified for neurons transfected with expression vectors for **A)** BDNF-mRFP1 (pg. 82), **B)** BACE1-mRFP1 (pg. 83) and **C**) NeuropeptideY-mCherry (pg. 84). Error bars represent SEM.

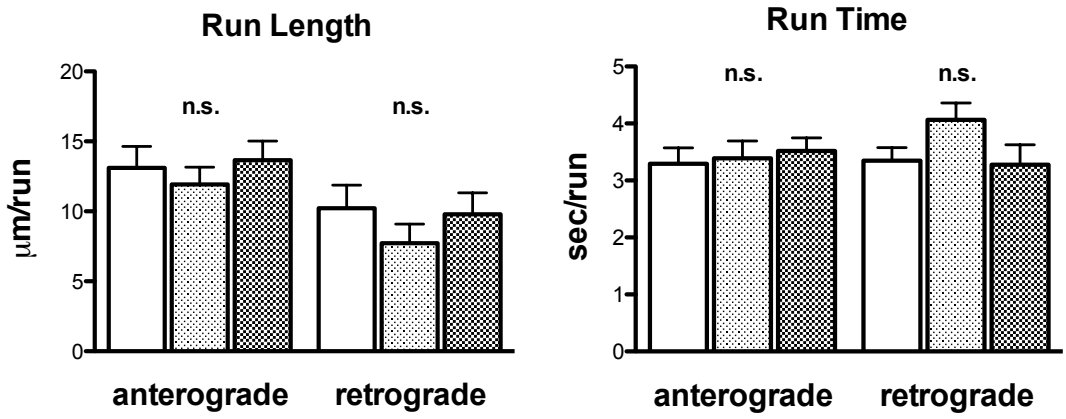
BACE1-mRFP1
Tau Knockout

B



NeuropeptideY-mCherry
Tau Knockout

C



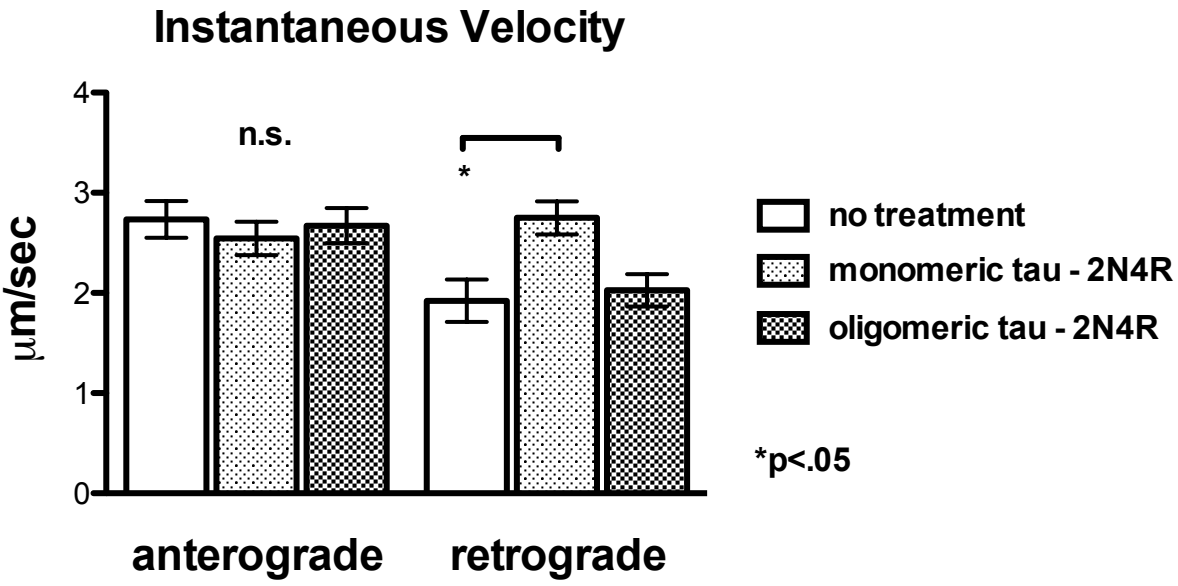


Table 3-1: Summary of endogenous tau disruption by extracellular tau oligomers

	Percent tau disruption (***p<.001)				Absolute change vs. control				Fold change vs. control			
Isoform (50nm total protein)	**no treatment**	**monomer**	**oligomer**	**fibrillar**	**no treatment**	**monomer**	**oligomer**	**fibrillar**	**no treatment**	**monomer**	**oligomer**	**fibrillar**
2N4R	20.42	18.60	**34.07*** **	16.60	0.00	-1.82	**13.65**	-3.82	1.00	0.91	**1.67**	0.81
Standard error	0.83	0.79	0.85	0.41								
2N3R	19.10	15.74	**28.47*** **	19.18	0.00	-3.35	**9.37**	0.09	1.00	0.82	**1.49**	1.00
Standard error	1.79	0.62	0.77	0.84								
1N4R	-	12.17	15.02	12.81	-	0.00	2.84	0.63	-	1.00	1.23	1.05
Standard error	-	0.76	1.00	0.87								
1N3R	-	16.59	16.89	16.44	-	0.00	0.30	-0.15	-	1.00	1.02	0.99
Standard error	-	1.64	1.20	1.80								
0N4R	-	12.54	**19.37*** **	-	-	0.00	**6.83**	-	-	1.00	**1.54**	-
Standard error	-	0.50	0.73	-								
0N3R	-	17.18	17.41	-	-	0.00	0.24	-	-	1.00	1.01	-
Standard error	-	0.80	0.80	-								
2N3R/2N4R (equimolar)	19.84	16.71	**26.91*** **	17.67	0.00	-3.13	**7.06**	-2.17	1.00	0.84	**1.36**	0.89
Standard error	0.67	0.59	1.11	0.66								
All six isorms (equimolar)	19.84	28.86	**51.64*** **	34.45	0.00	9.01	**31.79**	14.61	1.00	1.45	**2.60**	1.74
Standard error	0.67	0.73	1.25	0.95								

Table 3-2: Summary of fast axonal transport disruption by extracellular tau oligomers

2N4R tau treatment	Anterograde				Retrograde				Anterograde (Tau Knockout)				Retrograde (Tau Knockout)			
	no treatment	***monomer***	***oligomer***	**sig?^**	***no treatment***	***monomer***	***oligomer***	**sig?**	***no treatment***	***monomer***	***oligomer***	**sig?**	***no treatment***	***monomer***	***oligomer***	**sig?**
BDNF-mRFP1 (kinesin-1)																
Run Length	**11.97**	**11.08**	**27.71**	***,*	**7.53**	**8.95**	**26.69**	n.s.	**9.16**	**11.28**	**9.41**	n.s.	**7.90**	**13.15*,***	**8.02**	
+/- standard error	1.61	1.96	5.12		1.02	1.20	11.53		1.14	1.17	0.85		1.05	1.30	0.91	
Run Time	**8.69**	**8.98**	**12.64**	n.s.	**6.73**	**8.80**	**22.16**	***,***	**3.85**	**4.01**	**3.74**	n.s.	**3.68**	**5.02*,****	**3.51**	
+/- standard error	0.71	0.75	1.50		0.49	0.64	6.29		0.38	0.23	0.21		0.33	0.42	0.23	
Instantaneous Velocity	**1.27**	**1.21**	**1.98**	***,*	**1.00**	**1.07**	**1.01**	n.s.	**2.27**	**2.67**	**2.45**	n.s.	**2.05**	**2.51**	**2.25**	n.s.
+/- standard error	0.12	0.18	0.14		0.09	0.13	0.22		0.16	0.18	0.16		0.15	0.13	0.18	
BACE-1-mRFP1 (kinesin-1)																
Run Length	**10.47**	**8.25**	**18.99**	**,**	**7.03**	**9.55**	**11.25**	n.s.	**6.68**	**10.35**	**9.29**	n.s.	**9.52**	**10.64**	**13.35**	n.s.
+/- standard error	1.31	1.98	2.46		0.97	2.30	1.66		1.95	1.98	1.53		2.25	1.55	2.74	
Run Time	**5.48**	**5.22**	**7.42**	n.s.	**4.79**	**5.02**	**6.56**	n.s.	**6.58**	**6.95**	**6.55**	n.s.	**7.44**	**7.27**	**9.21**	n.s.
+/- standard error	0.45	0.43	0.60		0.33	0.58	0.70		0.62	0.69	0.65		0.91	0.75	1.31	
Instantaneous Velocity	**1.72**	**1.34**	**2.36**	*,***	**1.24**	**1.58**	**1.54**	n.s.	**0.88**	**1.35**	**1.28**	n.s.	**1.15**	**1.38**	**1.31**	n.s.
+/- standard error	0.12	0.18	0.22		0.13	0.21	0.13		0.13	0.17	0.12		0.17	0.11	0.19	
NeuropeptideY-mCherry (kinesin-3)																
Run Length	**15.50**	**13.57**	**18.68**	n.s.	**12.39**	**15.53**	**28.91**	n.s.,**	**13.11**	**11.92**	**13.65**	n.s.	**10.21**	**7.72**	**9.80**	n.s.
+/- standard error	3.75	2.69	2.58		3.08	2.22	2.96		1.53	1.25	1.37		1.65	1.37	1.53	
Run Time	**9.23**	**11.88**	**9.77**	n.s.	**9.23**	**12.11**	**12.55**	n.s.	**3.29**	**3.39**	**3.52**	n.s.	**3.35**	**4.06**	**3.28**	n.s.
+/- standard error	2.29	1.26	1.11		1.86	1.00	0.83		0.28	0.30	0.23		0.23	0.30	0.35	
Instantaneous Velocity	**1.83**	**1.41**	**1.66**	n.s.	**1.75**	**1.58**	**2.09**	n.s.,**	**2.73**	**2.54**	**2.67**	n.s.	**1.92**	**2.75*,ns**	**2.03**	
+/- standard error	0.17	0.15	0.11		0.41	0.13	0.17		0.18	0.17	0.18		0.21	0.16	0.16	

^significance relative to oligomer treatment unless noted

Chapter 4 -

Effects of extracellular tau oligomer treatment on components of the Axon Initial Segment

Chapter 4: Effects of extracellular tau oligomer treatment on components of the Axon Initial Segment

INTRODUCTION

Under normal conditions, tau has a polarized distribution in the axon, while diseased neurons exhibit extensive accumulation of tau, both soluble and inclusions, within the somatodendritic compartment [202]. This redistribution of tau is associated with a number of toxic effects, including glutamate toxicity resulting from tau interaction with the kinase, fyn, and spastin cleavage of microtubules (discussed in chapter 1-11.) Accumulation of somatodendritic tau has been shown to result from tau mutation, hyperphosphorylation and over-expression of tau [202]. Studies presented in chapter 3 demonstrate that exposure of primary neurons to extra-cellular tau oligomers also resulted in considerable accumulation of somatodendritic tau.

The axon initial segment (AIS) is a region just beyond the axon hillock that plays an important role in the maintenance of axonal polarity, separating the somatodendritic compartment from the axonal domain. The AIS is responsible for regulating the high degree of polarized distribution within neurons of various protein complexes, ion channels, and organelles, thereby acting as both a physical and physiological barrier between the axon and somatodendritic compartment [253].

This polarity is maintained by multiple proteins which the AIS comprise. Ankyrin G is a cytoskeletal scaffolding protein that is a major regulator of membrane domains [254].

Ankyrin G is restricted to the AIS in neurons and localization of sodium channels to the AIS requires their interaction with ankyrin G [255]. Localization of many other components to the AIS, such as potassium channels, neuronal cell adhesion molecule (NrCAM), neurofascin, βIV spectrin, and other AIS plasma membrane proteins is contingent on ankyrin G, which functions as a scaffold with which all other AIS interact, directly or indirectly [256-259].

The exact nature of this barrier is not yet understood, though it is known that the mobility of membrane proteins, lipids and large cytoplasmic dextrans within the AIS can be increased dramatically by disruption of the actin cytoskeleton [260-262]. Sodium channel stability is also contingent on the actin cytoskeleton, pointing to an active role for actin in maintaining polarity [263]. Actin interacts with the N-terminal domain of βIV spectrin and it is hypothesized that βIV spectrin promotes neuronal polarity by stabilizing the interaction of actin cytoskeleton with other AIS components [260].

The axon initial segment has also been shown to play a role in maintaining the polarized distribution of tau and by preventing its diffusion out of the axon into the somatodenritic compartment. Fluorescence recovery after photobleaching (FRAP) experiments show that tau diffuses readily throughout the neuron, but that once it enters the axon its re-diffusion into the soma is blocked by a retrograde barrier at the AIS. It is known that this barrier is contingent on microtubules, as treatment with nocodozole, which de-polymerizes microtubules, causes tau accumulation in the soma, as does increased phosphorylation of tau by phosphatase inhibition [264]. While it is uncertain how or if AIS microtubules interact with the actin barrier established by ankyrin G and βIV

spectrin, based on the dramatic accumulation of somatodendritic tau observed following treatment of primary neurons with 2N4R oligomeric tau (see chapter 3), I hypothesized that the extracellular oligomeric tau causes disassembly of the ankyrin G- βIV spectrin-actin complex that contributes to the diffusion barrier within the AIS.

Preliminary experiments were therefore performed to determine whether tau oligomer treatment had an effect on the distribution of ankyrin G and βIV spectrin. The data presented in this chapter, though preliminary, indicate that the distribution of ankyrin G and to a lesser extent βIV spectrin is disrupted following exposure to tau oligomers, but not tau monomer, pointing to a possible loss of AIS integrity. By quantitative image analysis, there is also an increase in the colocalization of both ankyrin G and βIV spectrin with tau, as well as MAP2 and tau, indicating that tau levels are increasing both at the AIS and in the somatodendritic compartment. These results suggest a possible cause of somatodendritic tau accumulation and are worthy of further investigation.

RESULTS

Tau disruption following extracellular tau oligomer treatment is associated with alterations in ankyrin G and βIV spectrin

In the previous study, treatment of primary neurons with 2N4R tau oligomers, but not monomers or fibrils resulted in a striking accumulation of endogenous tau in the somatodendritic compartment. To investigate whether this mislocalization of tau was associated with disruption in barriers to tau passage that exist at the AIS[264], primary neurons were exposed to tau monomer or tau oligomers. Cells were stained with antibodies to ankyrin G, the major organizer of the AIS [265], and βIV spectrin which connects ankyrin G to the actin cytoskeleton [259] in addition to tau and MAP2 (Figure 4-1). Treatment with tau oligomers again demonstrates accumulation of tau in puncta along the axon, with no change in MAP2 staining. Cells treated with tau monomer exhibit smooth, continuous βIV spectrin staining, while those treated with tau oligomers showed slight disruptions in staining along notable with varicosities in βIV spectrin distribution.

A much more profound response was seen for ankyrin G. Following monomer treatment ankyrin G exhibited continuous staining similar to βIV spectrin under the same conditions. Upon tau oligomer treatment however, ankyrin distribution is profoundly disrupted, including areas of ankyrin G accumulation that colocalize with tau puncta (Figure 4-3). These data indicate that endogenous tau mislocalization is associated with disruption in key components of the AIS.

Treatment with extracellular tau oligomers leads to increased presence of tau in the AIS and somatodendritic compartment

In addition to tau monomers and tau oligomers that were aggregated in 75 µM arachidonic acid, neurons were also exposed to tau oligomerized in 150 µM AA to analyze the effects of two different levels of tau oligomerization. Following overnight exposure to tau, cells were again analyzed with antibodies to tau, MAP2 and either βIV spectrin or ankyrin G. Immunofluorescence micrographs for βIV spectrin or ankyrin G were used to defined ROIs of 2 by 2 pixels. Fluorescence intensity values for MAP2 and Tau5 staining were measured using these same ROIs, and fluorescence ratios were obtained at each ROI for tau relative to βIV spectrin or ankyrin G, and to MAP2. These ratios are plotted as frequency distributions (Figs. 4-2 and 4-4.) Values were also obtained for βIV spectrin or ankyrin G fluorescence versus MAP2 as a control.

Neurons treated with either preparation of oligomeric tau showed a similar increase in the fluorescence ratio of tau to βIV spectrin when compared to monomeric tau, as indicated by a shift in frequency distribution to higher ratios (Fig. 4-2, A). Tau fluorescence relative to MAP2 in areas positive for βIV spectrin also increased (Fig. 4-2, B). MAP2/βIV spectrin fluorescence ratios remained unchanged following all three treatments (Fig. 4-2, C.)

A similar effect was observed in cells stained for ankyrin G (Fig. 4-4, A-C). Tau/ankyrin G fluorescence ratios exhibited an increase in maximum frequency following treatment with 75 µM AA tau oligomers and even greater shift upon 150 µM AA tau oligomer exposure (Fig 4-4, A). A similar effect was observed for tau fluorescence relative

to MAP2 in the ROIs, with frequency distributions shifted to higher values for both oligomeric tau preparations (Fig 4-4, B). MAP2 and ankyrin G ratios did exhibit a modest increase upon oligomer treatment (Fig 4-4, C).

The data collected in this chapter indicate the possibility that somatodendritic tau accumulation is related to disruptions in major components of the AIS. Localization of βIV spectrin, and most strikingly ankyrin G, is disrupted following tau oligomer treatment indicating the possibility that AIS disruption is responsible for loss of tau polarity and somatodendritic tau. It is possible, however, that these alterations are merely coincidental with tau translocation to the cell body. While this study is preliminary, further experiments are warranted to analyze the role of the AIS during tau mislocalization.

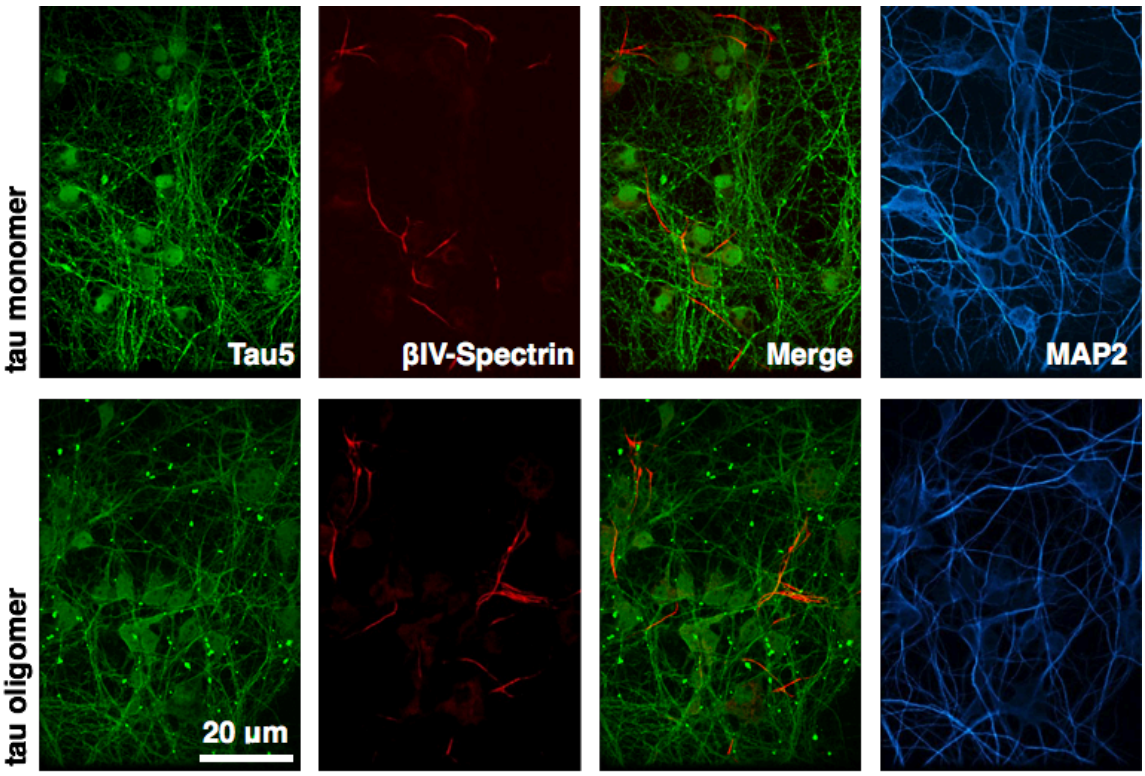


Figure 4-1: Extracellular tau oligomers disrupt βIV Spectrin distribution Tau oligomers but not monomers cause accumulation of tau accompanied with discontinuity and varicosities in βIV spectrin staining. MAP2 staining appears unperturbed.

Figure 4-2: (following page) Exposure to tau oligomers causes encroachment of endogenous tau into areas of the AIS positive for βIV Spectrin. Graphs represent frequency distributions of fluorescence intensity ratios at ROIs defined by βIV Spectrin staining. Neurons treated with oligomeric tau show an increase in the average fluorescence ratio of A) tau/βIV spectrin and B) Tau/MAP2, indicating increased colocalization of these proteins with tau. Treating cells with tau oligomerized at different AA concentrations has roughly the same effect. C) MAP2/βIV Spectrin ratios are similar following all three treatments.

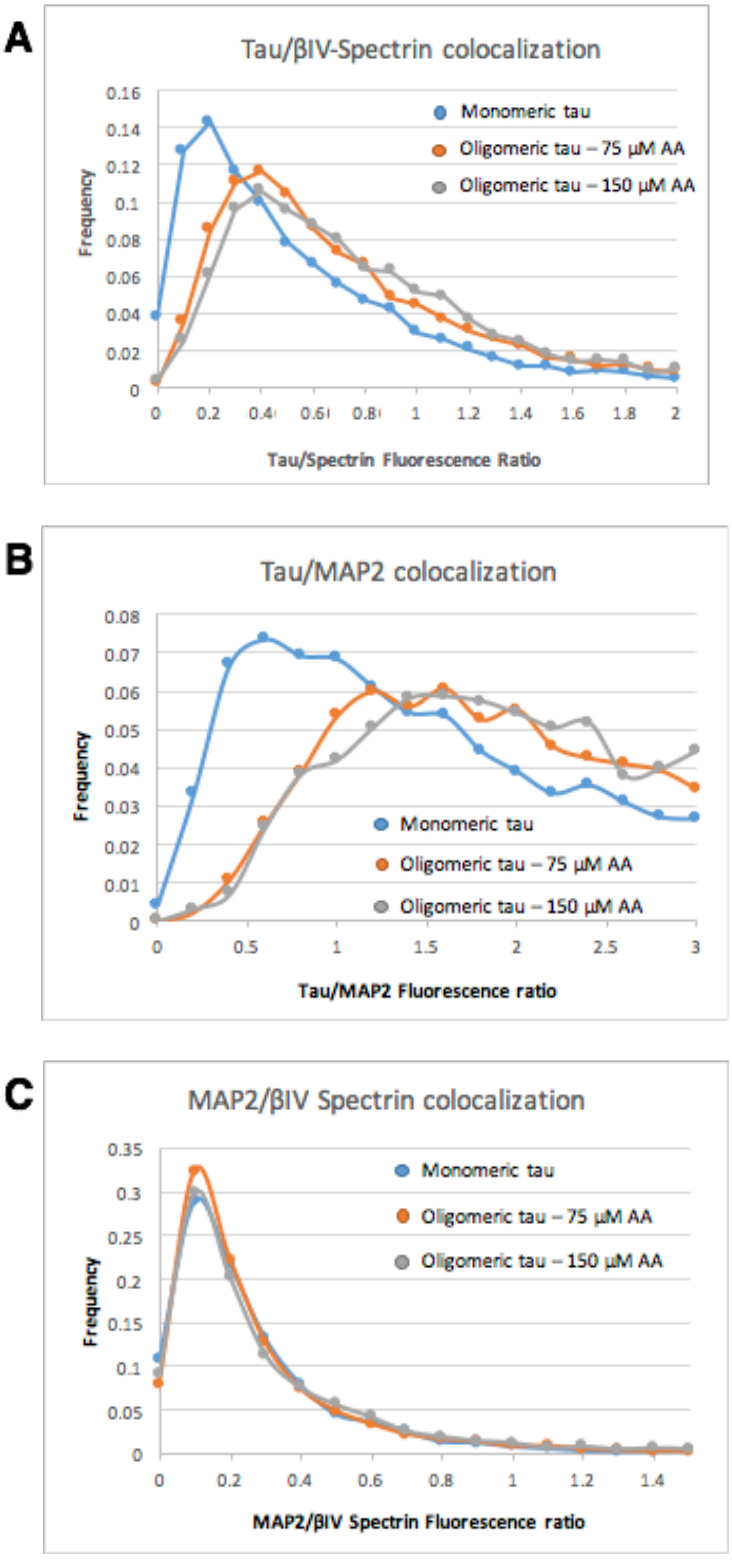

A
Tau/βIV-Spectrin colocalization
Frequency
Tau/Spectrin Fluorescence Ratio
Monomeric tau
Oligomeric tau – 75 μM AA
Oligomeric tau – 150 μM AA
B
Tau/MAP2 colocalization
Frequency
Tau/MAP2 Fluorescence ratio
Monomeric tau
Oligomeric tau – 75 μM AA
Oligomeric tau – 150 μM AA
C
MAP2/βIV Spectrin colocalization
Frequency
MAP2/βIV Spectrin Fluorescence ratio
Monomeric tau
Oligomeric tau – 75 μM AA
Oligomeric tau – 150 μM AA

Figure 4-2

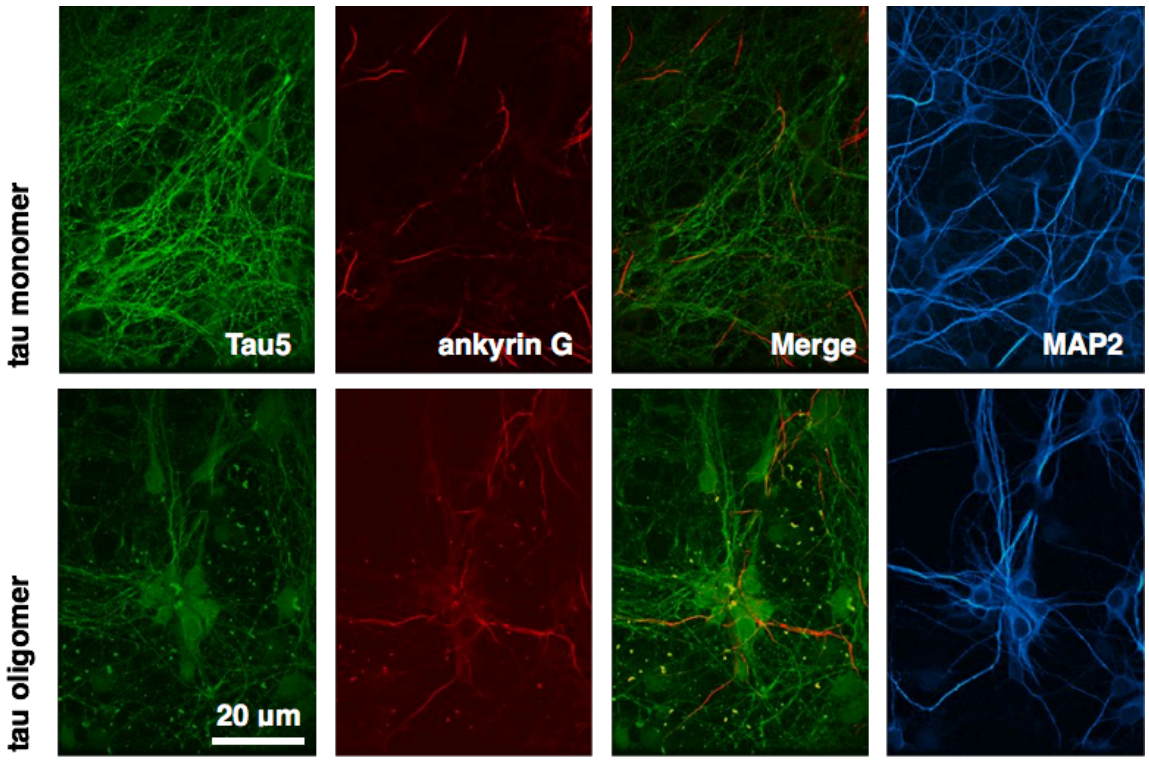


Figure 4-3: Extracellular tau oligomers extensively disrupt Ankyrin-G Tau oligomers but not monomers cause accumulation of tau accompanied with extensive disruption and accumulation of ankyrin G. MAP2 staining appears unperturbed.

Figure 4-4: (following page) Exposure to tau oligomers causes encroachment of endogenous tau into areas of the AIS positive for ankyrin G. Graphs represent frequency distributions of fluorescence intensity ratios at ROIs defined by ankyrin-G staining. Neurons treated with oligomeric tau show an increase in the average fluorescence ratio of A) tau/Ankyrin-G and B) Tau/MAP2, indicating increased colocalization of these proteins with tau. Treating cells with tau oligomerized at higher AA concentrations further increases relative tau fluorescence in the AIS. C)MAP2/Ankyrin-G ratios demonstrate only a minor change in the fluorescence ratio.

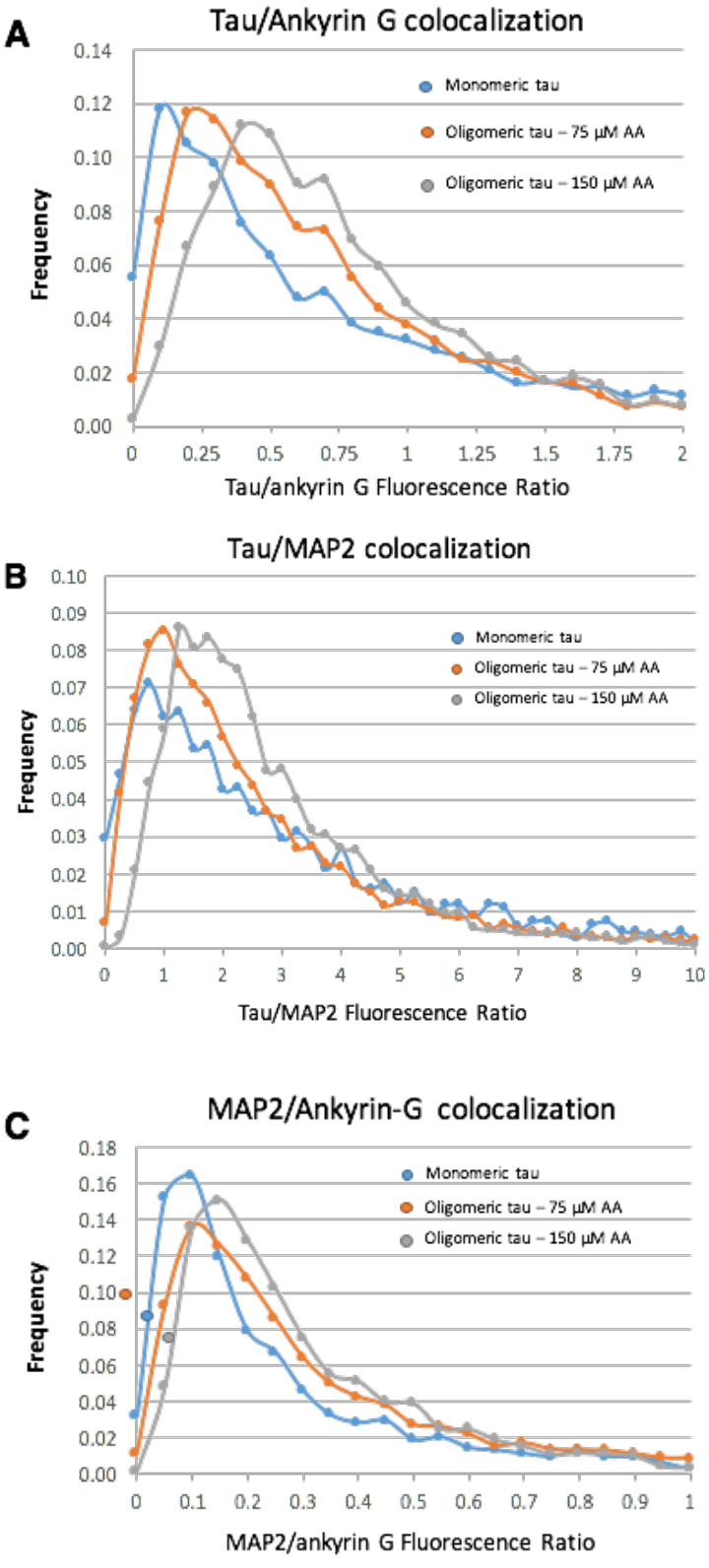

A
Tau/Ankyrin G colocalization
Frequency
Tau/ankyrin G Fluorescence Ratio
Monomeric tau
Oligomeric tau – 75 µM AA
Oligomeric tau – 150 µM AA
B
Tau/MAP2 colocalization
Frequency
Tau/MAP2 Fluorescence Ratio
Monomeric tau
Oligomeric tau – 75 µM AA
Oligomeric tau – 150 µM AA
C
MAP2/Ankyrin-G colocalization
Frequency
MAP2/ankyrin G Fluorescence Ratio
Monomeric tau
Oligomeric tau – 75 µM AA
Oligomeric tau – 150 µM AA

Figure 4-4

Chapter 5 -

Discussion

Chapter 5: Discussion

A growing body of evidence from human AD brain [217], AD model mouse brain [220,226,227], and cultured cells [184,223-225,238,243,266] has indicated that intracellular tau aggregation can be triggered by a prion-like mechanism [214,229,267] following uptake of aggregated extracellular tau. Mechanistic insight into this process has come primarily from cultured cell studies, many of which have made use of extracellular filaments made from tau fragments corresponding to the MTBRs and non-neuronal cells stably transfected to express the MTBR. These collective studies have yielded impressive gains in understanding the tau aggregation process, yet several other important issues have been largely ignored prior to the work presented here. Notable examples include the effects of aggregated extracellular tau on the endogenous full length tau in cultured neurons; the relative abilities of extracellular, full length oligomeric versus fibrillar tau to provoke intracellular tau accumulation; isoform-specific effects of the extracellular tau on intracellular tau aggregation; and perhaps most importantly, other cell biological responses of *bona fide* neurons to aggregation of their endogenous tau.

This dissertation represents efforts to shed light on all of these issues. Adapting a quantitative image analysis paradigm, this work demonstrates that intracellular accumulation of endogenous neuronal tau into apparent aggregates is induced more potently by extracellular oligomers than by extracellular filaments made from full length tau, that tau isoforms containing two N-terminal inserts are more potent than isoforms containing 0 or 1 N-terminal insert, that 4R tau likely exerts a more potent effect than 3R

tau, and that a mixture of all 6 CNS tau isoforms are even more potent (Figs 3-6, 3-10 – 3-13 and Table 3-1). Importantly, the accumulation of tau does not seem to signify fragmentation of microtubules, because with one exception, it is not accompanied by altered distribution of βIII-tubulin, a neuron-specific tubulin isoform [247] or of MAP2, a dendrite-specific microtubule-associated protein that comprises a MTBR region with high sequence identity to the comparable region of tau [246]. The lone exception concerns extracellular oligomers assembled from a mixture of all 6 CNS tau isoforms, which cause apparent disruption of both tau and MAP2 (Fig. 3-13, B), and thus may be cytotoxic. These data also demonstrate that extracellular tau oligomers can induce invasion of endogenous tau from its normal region of highest concentration, the axon, into the somatodendritic compartment, and that intracellular tau accumulation alters microtubule-based fast axonal transport. Altogether, these results imply that extracellular tau oligomers can disrupt normal neuronal homeostasis by mechanisms that depend on intracellular tau.

Since tau was first identified as a component of neurofibrillary tangles [268,269] the majority of research in the field of tauopathies has focused on these large insoluble aggregates and the hyperphosphorylated tau filaments they comprise as the major actors in the etiology of tauopathies. Tau oligomers, low-*n* soluble species of tau, have in recent years received increased focus regarding their role in these disorders. Tau oligomers have been shown to cause excitotoxicity, disrupted calcium homeostasis, decreased neurite growth, and neuronal loss [225] and have been identified at the earliest stages of disease progression in AD in both brain tissue [270] and CSF [271]

The present study extends an earlier report that extracellular tau oligomers can provoke intracellular tau aggregation [225] by demonstrating that oligomeric tau is much more potent in this regard than tau filaments. It is notable, however, that short extracellular tau filaments have been shown to be taken up by cultured neurons more efficiently than long tau filaments or monomeric tau [238]. These earlier reports, combined with the data presented here, imply that the prion-like propagation of tau pathology *in vivo* relies on small, readily diffusible forms of aggregated tau, such as oligomers and short filaments.

This is the first study demonstrating that full-length, wild type tau when applied extracellularly can lead to the redistribution and accumulation of intracellular wild-type neuronal tau. We demonstrate that extracellular oligomers, but not monomeric or fibrillar, tau induce intracellular tau accumulation. Previous studies have shown that fibrils can in fact be taken up into cells and affect the aggregation of endogenous tau [184,224,231]. While this may be the case in our assays at longer time points, it is worth noting that in these previous experiments tau fibrils were sonicated prior to treating cells[224], or truncated forms or tau were utilized to produced fibrils [184], These differences may be responsible for the discrepancy between earlier studies and the work described here.

Production of the oligomers used in the present study was adapted from a method previously developed by another lab [205]. These oligomers migrate by western blot at the same molecular weight as soluble tau species isolated from human AD brain, and an antibody raised against such oligomers preferentially stains AD brain sections [272]. The

mechanism by which extracellular oligomers affects intracellular tau in the current study is unclear, and a signaling cascade in selectively vulnerable neurons may be responsible, at least in part, for the observed tau redistribution [233]. However research from other lab groups has demonstrated uptake of low-*n* and short tau fibrils into neurons [237,238] indicating tau oligomer uptake is likely to occur under our experimental conditions.

Two curious new findings reported here concern the isoform composition of the extracellular tau oligomers. First, by testing each of the 6 isoforms individually, this study shows that intracellular tau aggregation is promoted robustly by 2N3R or 2N4R tau oligomers, but is induced barely, if at all, by 0N or 1N tau isoforms (Fig 3-6, 3-10 – 3-12). The 2 N-terminal inserts are encoded by *MAPT* exons 2 and 3, neither of which is expressed in 0N tau and only the first of which is expressed in 1N tau. Co-expression of exons 2 and 3 therefore seems to enhance the ability of extracellular tau oligomers to provoke intracellular tau accumulation. While there exists the possibility that exon 3 expression alone is required for this feature of tau, no such tau isoforms are known to occur naturally. Additionally, 2N and 0N isoforms including a fourth C-terminal MTBR, encoded by exon 10, demonstrated an increase in tau disruption when compared to like isoforms with only three MTBRs, indicating that 4R isoforms may provoke a more potent response than 3R isoforms. Secondly, oligomers assembled from an equimolar cocktail of all 6 isoforms were found to be the most potent inducers of intracellular tau aggregation (Fig 3-13 B). Although I cannot offer any obvious explanations for these results, they might relate to any of several factors. These include, for example, the possible presence of extracellular oligomers that individually comprise more than 1 tau isoform or potential

synergistic effects of multiple oligomeric species, such as those that might form from a mixture of all tau 6 isoforms. The isoform composition of the endogenous intracellular tau in the neurons we studied, which is an approximately 1:1 ratio of 0N3R:0N4R tau may also be important. In this context, it is noteworthy that intronic tau gene mutations that alter the ratio of 3R:4R tau without altering tau sequence or steady state tau levels can be fully penetrant for non-Alzheimer's tauopathies [56]. Changes in the isoform ratio have also been shown to have an extensive effect on the capacity for tau aggregation in in transgenic models of tauopathy [193] and in mice treated with extracellular tau [220]. The pathogenic potential of wild type tau can thus be dramatically affected by isoform composition, which the data presented here further indicate, but the underlying mechanisms remain mysterious.

The extent of tau disruption we observed in this study differed based on the various isoforms of tau in the oligomers, with both extra N-terminal exons and an additional MTBRs causing increased tau disruption. It is encouraging to think that the isoform effects seen in these experiments are similar to those observed with different tau "seeds", tau aggregates of varying morphologies isolated from cells that reproduce these differing morphologies upon exposure to tau-expressing cells [221,223,228]. It is known that different isoforms of tau exhibit differing propensities for aggregation [166,273,274] and given that each isoform was exposed to AA for an equal time period prior to UV exposure, it is possible that each isoform oligomerized to its own unique degree within an incubation period of constant length. The extent of intracellular tau accumulation quantified in this study correlates well with what is known about tau isoform aggregation,

namely that 4R isoforms have a lower threshold to aggregation than 3R isoforms, and that 2N tau has a lower aggregation threshold than 0N tau. 1N tau has been shown to be most aggregation prone, which makes our 1N data an outlier. One possible explanation for the discrepancy between my results and the known aggregation propensity of 1N tau is that in my experiments 1N tau formed a distinct class of oligomers with diminished capacity to drive intracellular tau aggregation.

Isoform effects such as the differential seeding of aggregates described here could play a role in the phenotypic heterogeneity observed in tauopathies. It has been hypothesized that disorders such as CBD, FTD and PSP represent a spectrum of disorders, with the clinical course and histological presentation reflecting the region of the brain were tau dysfunction originates [167]. Differential seeding of normal or mutant tau, resulting from the conformational diversity and location of the first pathogenic seeds to form in the brain, could produce a great deal of heterogeneity in both the clinical presentation and morphology of tau pathology [214,222,223,275].

AD tau has long been known to be phosphorylated at multiple sites that are rarely phosphorylated in normal tau [140,276-278], and to adopt immunologically sensitive conformational changes [248-250,279]. Interestingly, we found that extracellular tau oligomers did not alter the immunoreactivity of intracellular tau with two monoclonal antibodies, Alz50 [248,249] and MC1 [250], that detect AD tau conformations, nor with the PHF1 monoclonal, which recognizes tau phosphorylated at S396/S404 [171,251] (Fig. 3-8). While the experiments presented here focus on effects in the first 24 hours of tau oligomer exposure, it is possible that longer exposure to tau oligomers or other types of

tau aggregates would induce pathological epitopes consistent with those seen in AD and non-Alzheimer's tauopathies *in vivo*.

Because of the high levels of tau phosphorylation in pathological inclusions, kinase inhibition has been proposed as a possible therapeutic to reduce overall levels of phosphorylated tau in these patients [280]. While this may prove to be a viable therapy, the tau aggregates described in this study were not positive for a few conformational or phospho-tau epitopes common in disease. While tau may start to oligomerize and aggregate due to hyperphosphorylation in certain disease cases [78], the recombinant tau oligomers used in this study were non-phosphorylated at a key disease epitope Ser 396/404, a site phosphorylated by GSK3β [281]. Still, a more in-depth examination of the phosphorylation state of the endogenous tau, and of kinase and phosphatase activity in these cells could fill in gaps in the current literature and understanding.

One of the most striking effects of extracellular tau oligomers that we found is invasion of endogenous intraneuronal tau into the somatodendritic compartment (Fig. 3-5). This phenomenon mimics an early event in AD pathogenesis [202] and signifies breakdown of a key feature of neuronal polarity. Excess dendritic tau has been implicated in synaptotoxicity mediated by NMDA receptors [122] , and is potentiated by tau acetylation [282] Movement of tau into dendrites has also been reported by others in a small fraction of cultured neurons exposed to amyloid-β oligomers (AβOs) [283]. Others in the Bloom lab have observed this phenomenon in some of our prior studies of AβOs [140,277] although these data have not been published. The contrast between AβOs and tau oligomers for this activity is striking, though, because the vast majority of neurons

exposed to tau oligomers in the current study accumulated somatodendritic tau. When considered together, the quantitatively different effects of AβOs and tau oligomers on tau localization in the somatodendritic compartment raise the possibility that AβOs drive formation of intracellular tau oligomers that can escape the neurons and then "infect" other neurons, whose endogenous tau then enters the somatodendritic compartment. This AβO-induced process might lead to a slow buildup of extracellular tau oligomers and correspondingly slow appearance of mislocalized endogenous tau. We suspect that directly supplying extracellular tau oligomers to neurons, as was done in the current study, would dramatically increase the rate of endogenous tau mislocalization into the soma and dendrites.

Chapter 4 represents early research into possible AIS dysfunction that causes an increase in somatodenritic tau. While the data presented are preliminary, they suggest a mechanism by which some of the toxic effects could occur in neurons. Ankyrin G is the major organizer of all other subunits of the AIS [265], including ion channels involved in action potentials [257]. Loss of ankyrin G has been demonstrated to have a profound effect on all other AIS components and this disruption could itself be a major cause of neuronal dysfunction and loss of polarity [253,256,258,259]. Mice lacking spectrin also have impaired neuronal polarization [284]. Further experiments along these line could provide valuable insight into the role of the AIS in tau dysfunction.

A major benefit of using primary neurons in this study is the ability to examine the effect of this tau redistribution on cellular processes that are best studied in true neurons. Besides inducing intracellular tau missorting, extracellular tau oligomers altered several

parameters of microtubule-based fast axonal transport in a cargo-specific manner. We measured run lengths, run times and instantaneous velocities for fluorescently tagged BDNF, BACE1 and neuropeptide Y in the anterograde and retrograde directions in both wild type and tau knockout neurons (Figs. 3-15 and 3-16, Table 3-2). Because axonal microtubules are almost uniformly polarized with their plus ends facing the axon terminal [285,286], plus end-directed microtubule motors power anterograde transport, whereas minus end-directed motors are responsible for retrograde transport. More specifically, BDNF and BACE1 rely mainly on kinesin-1 as an anterograde motor [103,252], neuropeptide Y uses kinesin-3 for anterograde transport [103] and all 3 cargoes presumably depend on cytoplasmic dynein for retrograde transport.

Both BDNF and BACE1 exhibited large increases in run length and instantaneous velocity for anterograde transport after exposure of wild type neurons to oligomers, but not monomers of extracellular tau. Retrograde run times for BDNF were also dramatically increased by extracellular tau oligomers. In contrast, tau oligomers caused increases in retrograde run length and instantaneous velocity for neuropeptide Y.

We interpret these results in light of prior reports of tau acting as a virtual “speed bump” that can dislodge motile cargo from microtubules [113-116]. My study suggests that aggregation of intracellular tau causes the tau to dissociate from microtubules, thereby removing the speed bumps, and permitting more rapid and longer range motile events in a manner that is sensitive to the combination of microtubule motors associated with the cargo.

The results from our axonal transport experiments may at first seem counterintuitive, as many studies associate decreased axonal transport with diseased neurons[200]. While this may be indicative of later stages of pathogenesis, we hypothesize that the effect seen here may be indicative of an earlier stage of neuronal dysfunction, prior to loss of microtubule integrity, in which transport becomes less retrained because of the loss of microtubule bound tau. It is worth considering what the long term consequences of such a boost in fast axonal transport may have on neurons. Kinesin-1 transports many cargo types in addition to BDNF and BACE1 [103], and at least some of those cargoes may need to be delivered to sites along the length of the axonal plasma membrane. The speed bump function of tau may enable such scattered cargo delivery, and dissociation of tau from microtubules would remove these speed bumps and bias delivery to toward the axon terminal. Past studies have indeed shown that run length does increase as tau concentration decreases [115]. It is easy to imagine that such impaired axonal transport could gradually compromise neuron health as AD pathogenesis proceeds at the level of individual neurons. This could result from disrupted energy homeostasis along the axon or excitotoxic effects at the synapse. Overexpression of BDNF at the synapse, for example, has been tied to increased seizure activity [287] and increased numbers of vesicles at the synapse has been desribed in mouse models of neurodegeneration [200]. While previous studies have indicated tau has no effect on velocity, this was observed in cases where tau was over-expressed, not depleted. The trafficking velocities we observed in tau-null neurons were elevated at levels similar to those observed in oligomer treated wild-type neurons, further supporting this hypothesis.

The collective results presented here implicate tau oligomers in the spread of tau pathology from the extracellular space to the neuronal cytoplasm, pointing to a likely role for these toxic species in the spread of AD and other tauopathies. By utilizing cultured primary neurons, we were able to tie extracellular tau oligomers to two early steps in neurodegeneration: mislocalization of endogenous tau into the soma and dendrites, and dysregulation of fast axonal transport.

Soluble tau oligomers thus represent potential targets for early diagnosis and therapeutic intervention for AD and non-Alzheimer's tauopathies. Small molecules that block recruitment of native proteins to existing aggregates could prevent the further spread of prion-like inclusions. As fluid-phase endocytosis, possibly mediated by heparan sulfate proteoglycans has been proposed as a predominant uptake mechanism for misfolded proteins [231,288], blocking of the endocytic pathway may be beneficial, although off target effects will need to be taken into account. Furthermore, immunotheraphy may be valuable in the removal of prion-like species. While immunotherapy is probably incapable of affecting intracellular aggregates, the release of these species into the extracellular space provides a situation in which these therapies may be effective [289,290]. The heterogeneity of various seeds will require a great deal of optimization in these cases, as therapies effective against one conformational or linear epitope may be ineffective against others. Delivery of any of these interventions across the blood brain barrier also represents a challenge that future therapies will need to overcome [291].

Chapter 6 -

Future Directions

Chapter 6: Future Directions

The experiments described in this thesis point to a role for extracellular oligomeric tau in the pathogenesis of AD and non-Alzheimer's tauopathies. Alterations in fast axonal transport observed in oligomer treated cells, correlating with loss of axonal tau, additionally point to a mechanism of neuronal dysfunction following tau disruption. These results provide an early snapshot into the dysfunction that results from tau aggregation and mislocalization and future experiments will be able to build upon these observations.

An expanded series of time courses would be most valuable in building upon the data presented in chapter 3. 50 nM was the lowest concentration of 2N4R tau oligomers tested that produced tau aggregation at 18 hours. It would be interesting to see if any of the isoforms that produced little to no effect overnight might have a more potent effect over longer treatment intervals or at higher concentrations. The use of primary neurons does present some confounding factors in these experiments, however. Fetal tau undergoes a change from 3R tau to 4R tau during the course of development, a transition that can be seen observed even in cultured neurons. It may be difficult to parse out effects resulting from changes in intracellular isoform ratios from those due to extend oligomer exposure. Given the effects of tau isoforms that have been observed in disease [56], it may also be valuable to treat primary neurons in younger or older cultures to see if tau oligomer treatment for the same time period produces varying results. The image analysis paradigm developed for these experiments will prove useful in comparing a multitude of variables as these experiments are expanded upon.

A mouse line, hTau, expresses all six human tau isoforms utilizing a phage artificial chromosome vector driven by the tau promoter [193]. These mice were crossed to a tau knockout line, in which a targeted disruption of the tau gene was produced by insertion of GFP cDNA into tau exon 1 [128], leading to only human tau expression in these mice. Deletion of the mouse tau gene leads to inclusion formation from all six human isoforms. The isoform ratios in these mice are not completely consistent with those observed in humans, and it is unknown as of yet what these ratios are when hTau neurons are grown in primary culture. However, we have obtained these mice and it would be valuable to test the effects of extracellular oligomers against endogenous tau isoform ratios that more closely represent those observed in adult human neurons.

As discussed in the introduction, a number of post-translational modifications have been demonstrated to have an effect on the aggregation of tau [76,78]. Prion-like properties of tau have been demonstrated to be phosphorylation-independent, and while it does not appear that there is an alteration in phosphorylation associated with the tau accumulation we observe in these cases, a kinase activity analysis may be of value to look at this more carefully. This is because although AβOs have been demonstrated to activate various kinases and cause tau phosphorylation [140,292,293], it is unknown what effect extracellular tau oliogomers may have on tau aggregation beyond template mediated conformational alterations. Other less studied modifications such as N-glycosylation, O-GlcNAcylation, acetylation and isomerization, are additionally altered in AD cases (see chapter 1-8.) As detection techniques for these various modifications become more readily available it may be worth seeing if any of these modifications prime tau for

aggregation. We have antibodies that discriminate *cis* from *tra*ns forms of tau [147,294], and hope to analyze them in this system.

A number of tau oligomer specific antibodies exist, including one raised against the type of oligomers used in this study [272,295]. While these antibodies label oligomers preferentially on western blots, when they were used to stain oligomer-treated cells by immunofluorescence a high level of background in control and untreated cells made it difficult to draw any conclusion. Optimization of these antibodies for staining cells would be helpful in further analyses.

Time is a limiting factor on the axonal trafficking experiments described here, as the data analysis is very labor intensive. Still, it would be worthwhile to expand upon the trafficking dysfunction identified in chapter 3. Experiments were performed using the mitochondria and lysosome specific dyes, lysotracker and mitotracker (Invitrogen). Because these dyes stain every cell however they do not allow for individual cell imaging as there is simply too much signal to identify individual axons. This could be improved upon through the use of effective organelle-specific fluorescence vectors, much like those utilized in chapter 3. Energy homeostasis is important in the axon [296], and it would be valuable to study mitochondrial transport under this experimental paradigm, especially given that depletion of tau has been proposed as a clinical therapy.

The inhibitory effect that tau has on fast axonal transport has also been demonstrated to be more potent for 4R than 3R Tau [115] so repeating some of these experiments in hTau mice would be valuable as well. Finally, I didn't test any kinesin-2 cargos, which include plasma membrane precursors, such as N-cadherin, β-catenin, and

choline acetyl-transferase. While it is unknown if tau regulates kinesin-2, it may be worth preliminary investigation.

Several attempts with limited success were made to grown mouse primary cortical neurons in microfluidic devices, in which neurons are plated on one side of a chamber connected by nano-grooves to another media filled chamber. This results in the growth of axons down the nano-grooves and into the other well [297]. These would require optimization for use in our lab, but could prove useful in these experiments in several ways. Such microfluidic chambers have two different media environments: one for the soma, dendrites and proximal axon regions, and the other for distal axon regions and axon terminals. These could be used to identify where on the neurons tau oligomers are acting. One could also determine if any regions of the axon are more prone to tau disruption. These devices also great simplify axonal transport analysis as it isolates axons and makes it easier to determine directionality.

Perhaps the most fertile area for future study is the effects of tau oligomer treatment on structural elements of the AIS. βIV spectrin, and especially ankyrin G, play an extremely important role in the organization of the AIS, targeting among other things ion channels and the actin cytoskeleton to this region [253]. Loss of AIS functions such as the initiation of action potential and establishment neuronal polarity could lead to extensive dysregulation of neuronal physiology. We plan to further investigate the effects of tau oligomers on these and other components of the AIS in the near future.

With no disease modifying therapy currently available for AD and related neurodegenerative disorders, and with the financial burden imposed by these diseases

set to explode in the upcoming decade, valid therapeutic targets are exceedingly important. The studies presented within this thesis show tau oligomers are a potent disruptor of intracellular tau and fast axonal transport. The results point towards tau oligomers as an actor in the progression of pathology and neuronal dysfunction from cell to cell, a role that future studies may be able to expand upon even further.

Chapter 7 –

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