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Role of 4R tau in astrocyte-mediated neuronal toxicity and the progression of neurodegenerative disease

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by

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ABSTRACT OF THE DISSERTATION

Role of 4R Tau in Astrocyte-Mediated Neuronal Toxicity and the Progression of Neurodegenerative Disease

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The microtubule-binding protein tau is associated with neurodegenerative diseases, including Alzheimer's disease, progressive supranuclear palsy, and a subset of frontotemporal dementias. In these tauopathies, tau becomes hyperphosphorylated and forms intracellular neurofibrillary tau tangles, contributing to synaptic dysfunction, neuronal death, and severe astrogliosis. Tau can be classified as having a 3-repeat (3R) or 4-repeat (4R) structure, resulting from alternative splicing of exon 10 within the *MAPT* gene. While the higher deposition of 4R tau characterizes many primary tauopathies, the role of 4R tau in neurodegenerative disease pathogenesis remains unclear. To investigate the role of tau isoforms in disease, we created tau splicing antisense oligonucleotides (ASOs) that alter the ratio of 3R:4R tau. In earlier studies, ASO-induced preferential expression of 4R tau in a murine model increased both seizure severity and phosphorylated tau deposition. The absence of overt neuronal or synaptic loss to explain the observed 4R tau-mediated phenotype in mice prompted a closer examination of astrocytes, which also exhibit pathologic tau deposition in tauopathies. Human tau (hTau) expressing mice treated with a 3R to 4R tau splicing AO exhibited co-localization of 4R tau with reactive astrocytes and increased expression of pan-reactive and neurotoxic genes. In primary murine astrocytes, increased

astrocytic 4R tau levels provoked a similar morphological change, neurotoxic genetic profile, a vulnerability to oxidative damage, and a decreased ability to buffer glutamate. These results were replicated in a human iPSC model, and healthy neurons exhibited a higher firing frequency and hyper-synchrony when cultured with 4R tau-expressing human iPSC-derived astrocytes. Neurons cultured with 4R tau expressing astrocytes also exhibited higher levels of cytotoxicity. Furthermore, lowering 4R tau expression decreased levels of cytotoxicity in both models and prevented neuronal hyper-excitability and death when cultured with human astrocytes treated to reduce tau levels. These findings support a novel pathway of astrocytic 4R tau that mediates reactivity and dysfunction and suggest that astrocyte-targeted therapeutics against 4R tau may play an important role in mitigating neurodegenerative disease progression.

Epigraph

Glia – more than just brain glue
- Nicola Allen and Ben Barres 2009

The good thing about science is that it's true whether or not you believe in it.
- Neil deGrasse Tyson

Chapter 1

Introduction and Perspective

Tauopathies are a heterogeneous group of neurodegenerative diseases characterized by abnormal metabolism of misfolded tau proteins leading to intracellular accumulation and formation of neurofibrillary tangles (NFT) in the cytosol of neurons and glial cells that currently impact over 30 million people in the world. A majority of patients present with motor, cognitive, and behavioral symptoms (Josephs 2017)

Tau Biology

1.1.1 Tau function and subcellular distribution

Tau is a microtubule-associated protein that is involved in many essential cellular processes (Wolfe 2012). The most well-characterized of which is the interaction between tau and microtubules. As it is a microtubule-binding protein, it promotes the assembly and stabilization of microtubules and axonal transport (Didonna 2020). More recent evidence suggests that tau may also play a role in modulating synaptic plasticity, iron transport, actin polymerization, and maintaining DNA integrity (Caceres and Kosik 1990; X. P. Hong et al. 2010). Additionally, tau has been shown to interact with a variety of other proteins, including the protein kinase Fyn (Putra et al. 2020; D. Xia, Li, and Götz 2015; Vossel et al. 2015) and the postsynaptic density protein 95 (PSD95), suggesting that tau plays an essential role at the synapse and is involved with neuronal cell signaling (Hummel et al. 2017; Ittner et al. 2010).

Tau can localize to a variety of areas around the cell. It had previously been thought to only localize to the axon unless under developmental or pathological conditions, but this is no longer the case(Arendt, Stieler, and Holzer 2016). Evidence has shown that tau can be observed in

dendrites, the nucleus, at the synapse and can interact with the plasma membrane along with the actin cytoskeleton(K. S. Kosik and Finch 1987; Fulga et al. 2007; Brandt, Léger, and Lee 1995). More recently, groups have identified that the different isoforms of tau can also have different subcellular localization patterns(Bachmann et al. 2021). It was determined that the 0N3R tau isoform was most enriched in the axon compared to other isoforms (Bachmann et al. 2021) and that 2N isoforms localized poorly to the axon potentially because of their large size (Bachmann et al. 2021).

Tau proteins are widely conserved and expressed in many species, including *Caenorhabditis elegans (C. elegans)*, *Drosophila*, fish, rodents, goats, monkeys, and humans (Pîrşcoveanu et al. 2017). In the central nervous system, neurons express the highest levels of tau, but glial cells, including astrocytes and oligodendrocytes, express tau at lower levels (Zhang et al. 2016).

Tau is encoded by the gene *MAPT* on chromosome 17q21.31. It comprises16 exons(Arendt, Stieler, and Holzer 2016) and is divided into its functional domains: an N-terminal projection domain, a proline-rich region, or PRR, a microtubule-binding region (MTBR), and a C-terminal domain. The MTBR consists of four partially repeated sequences R1, R2, R3, and R4. The alternative splicing of exons 2,3 and 10 creates the six isoforms of tau expressed in humans (McMillan et al. 2008). Inclusion or exclusion of exons 2 and 3 creates differences at the N-terminus (0N, 1N, and 2N), with exon 3 never being expressed without exon 2, and inclusion or exclusion of exon 10 creates different numbers of the microtubule-binding domain repeats (3R and 4R) (Sarkar, Kuret, and Lee 2008). The N-terminus is involved in tau subcellular localization (J. Chen et al. 1992; Brandt, Léger, and Lee 1995) and the regulation of cargo

vesicle release (Y. Wang and Mandelkow 2016). The MTBR is located in the C-terminus of tau and is made up of four highly conserved partially repeating sequences that interact with microtubules. Doing so stabilizes them, allowing for cytoskeletal organization (Y. Wang and Mandelkow 2016; Kadavath et al. 2015). The MTBR is also flanked by the proline-rich region, which is responsible for increasing the binding affinity of tau to the microtubule (Kadavath et al. 2015). While the coding sequence is well conserved in mammals, the RNA splicing pattern has changed significantly throughout evolution (Andreadis, Brown, and Kosik 1992; Janke et al. 1999).

1.1.2 Isoform expression

Tau isoforms are thought to have specific functions as they are differentially expressed throughout development and the body. The smallest isoform of tau, 0N3R, is expressed in the developing fetal brain during neurogenesis (M. Goedert et al. 1989; Trabzuni et al. 2012) and is considered necessary for the flexibility needed in the fetal cytoskeleton (Felgner et al. 1997) in order for process formation and neurite elongation. Following the formation of synapses, 4R tau begins to be expressed; it has a higher affinity to the microtubule than 3R tau, leading to a more stable cytoskeletal framework and faster microtubule assembly (Hernández et al. 2020; Felgner et al. 1997). In a healthy adult human brain, 3R and 4R tau are expressed in approximately equal amounts (Malmanche et al. 2017). Humans continue to express the fetal form of tau even in adulthood. It has been suggested that this allows humans to have higher levels of synaptic plasticity (Bullmann et al. 2009) throughout their life. The 1:1 expression ratio of 3R:4R tau seen in humans is not conserved in other species, as adult rodents almost exclusively express 4R tau (Götz et al. 1995; H. Yoshida, Crowther, and Goeder 2002; Hernández et al. 2020). This

difference may be because the 5' splice site of exon 10 is only partially conserved between mice and humans (Grover et al. 1999; Arendt, Stieler, and Holzer 2016).

To date, there have been over 100 intronic, and exonic mutations in *MAPT* identified (Alzforum tau mutation), with over 80 that are linked to diseases such as frontotemporal dementia (FTD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and others. These mutations can either be missense mutations or splicing mutations which can alter the ratio of 3R:4R tau (D'Souza et al. 1999) or an increase in tau pathology. Most mutations that occur in *MAPT* are clustered near the MTBR of tau, with over half occurring in and around exon 10 (Michel Goedert et al. 2018). This makes the alternative splicing of exon 10 of particular interest. Tauopathies can be classified by which isoform is primarily identified in the aggregates seen in disease. 3R tauopathies such as Pick's disease exhibit increased deposition of 3R, 4R tauopathies such as PSP, CBD, argyrophilic grain disease (AGD), globular glial tauopathy (GGT), and others (Lacovich et al. 2017; Kovacs 2015) have higher deposition of 4R tau. Additionally, it is possible to have mixed isoform tauopathies such as Alzheimer's disease (Alonso et al. 2001).

1.1.3 Tau haplotypes

An ancient 900-kb inversion of the entire *MAPT* gene created the two main haplotypes seen in humans (Baker et al. 1999). H1 contains the inversion, and H2 does not. While the overall amino acid sequence is unchanged between the two, H2 has a 238bp deletion in intron 9 (Refenes et al. 2009). The single nucleotide polymorphism (SNP) rs242557 in the H1 haplotype creates the H1c sub-haplotype, which is associated with an increase in 4R tau expression (Myers et al. 2007) and is linked to an increased risk of progressive supranuclear palsy (PSP) and corticobasal

degeneration (CBD) (Rademakers, Cruts, and Van Broeckhoven 2004; Pittman et al. 2005). Alternatively, the H2 haplotype is strongly negatively associated with PSP (Vega et al. 2008). Additionally, those with the H2 haplotype exhibit increased inclusion of exon 3, suggesting that it may be protective against neurodegenerative disease (Caffrey, Joachim, and Wade-Martins 2008).

1.1.4 Post-translational modifications of tau

Alternative splicing of tau regulates isoform expression, but the regulation of tau takes place mainly through post-translational modifications (PTMs) such as phosphorylation, glycosylation, cleavage, and others (Martin, Latypova, and Terro 2011). These modifications have been suggested to play a role in the toxic gain of function of tau (Del C. Alonso, Grundke-Iqbal, and Iqbal 1996), regulating how tau aggregates as well as functions, and therefore are essential to understand how best to prevent tau pathology in disease.

Phosphorylation is the most common tau PTM and has been implicated in the regulation of tau function and disease progression. Tau has seventy-nine phosphorylation sites in its longest isoform (2N4R) (Martin, Latypova, and Terro 2011; Buée et al. 2000), and most of these are in the regions surrounding the MTBR. It has been shown that phosphorylated tau has a lowered affinity for stabilizing microtubules (Barbier et al. 2019). When specific sites in the MTBR are phosphorylated, tau does not bind to the microtubule as strongly, which then causes it to disassociate from the microtubule and be more likely to aggregate (Avila et al. 2004). This could also be due to a conformational change that occurs in phosphorylated tau (Y. Xia et al. 2020). It has also been shown that the degradation and cleavage of phosphorylated tau are altered, leading

to the buildup of tau in the cell (Arai, Guo, and McGeer 2005). Hyperphosphorylation of tau, commonly seen in disease, may induce tau pathology (Dickey et al. 2007). Hyperphosphorylated tau may also accumulate in the somato-dendritic compartment instead of axons, causing synaptic dysfunction (Tackenberg and Brandt 2009; Weissmann et al. 2009; Hoover et al. 2010).

1.1 Tau in disease

1.2.1 Classification of tauopathies

Tauopathies are a group of over twenty different neurodegenerative diseases which can be further divided into primary and secondary tauopathies. Primary tauopathies are diseases where tau is believed to be the significant contributing factor of the neurodegenerative process (Josephs 2017). Primary tauopathies are grouped under the umbrella term frontotemporal lobar degeneration (FTLD), or diseases characterized by neuronal and glial tau inclusions (Götz, Halliday, and Nisbet 2019). They include PSP, AGD, DBD, Pick's disease, GGT, aging-related tau astrogliopathy, and others (Kovacs et al. 2016; Arendt, Stieler, and Holzer 2016). Secondary tauopathies are diseases where there is tau pathology, but it is associated with other pathologies (Williams 2006), the most well-known of which is Alzheimer's disease. Other examples of secondary tauopathies include Down's syndrome, Lewy body disorders, myotonic dystrophy, chronic traumatic encephalopathy, Niemann-Pick disease type C, and others (Arendt, Stieler, and Holzer 2016). These diseases are more heterogeneous and may be caused by environmental factors and mutations (Arendt, Stieler, and Holzer 2016).

1.2.2 Tau mutations

Genetics plays a prominent role in disease progression in both primary and secondary tauopathies. Mutations in the *MAPT* gene are considered pathogenic as they may affect tau RNA splicing, impair tau function and promote tau fibrillization (V. M. Y. Lee, Goedert, and Trojanowski 2001). As mentioned earlier, most tau mutations occur around exons 9-12 or in the MTBR and impact tau protein expression and the alternative splicing of the pre-mRNA (Spillantini and Goedert 2013). Several tau mutations, including G272V, P301L, P301S, V337M, G389R, and R406W, cause a decreased affinity to the microtubule as well as a reduced ability to promote microtubule assembly leading to increased neurofibrillary tangle formation and tau positive inclusions (M. Hong et al. 1997). These mutations can also increase the phosphorylation of tau by turning it into a better substrate for protein kinases and impair axonal transport (Alonso et al. 2004; Yu, Tan, and Hardy 2014). Specific mutations can also impair the splicing of exon 10, resulting in the shift of isoform expression commonly seen in disease (Rizzu et al. 1999; Grover et al. 2002; M. Yoshida 2014; D'Souza et al. 1999). Finally, when mutations occur within the intron of exon 10, they commonly increase 4R tau expression and accumulation (Strang, Golde, and Giasson 2019).

1.2.3 Tau seeding and propagation

Evidence has emerged showing that tau pathology spreads throughout the brain along neuroanatomically connected brain regions (Dujardin et al. 2014; Calafate et al. 2015; Congdon et al. 2016). It has also been suggested that the propagation of tau can occur in a prion-like manner which involves the transfer of tau seeds from a donor cell to a recipient cell and further recruiting normal tau to further the spread of the tau seeds (Clavaguera et al. 2009; De Calignon

et al. 2012). Groups have identified that tau seeds taken from brain homogenates of human tauopathy patients, symptomatic transgenic mice, cells that have tau aggregation, or their media could induce tau pathology (Mudher et al. 2017). Additionally, it has been shown that when brain homogenates were taken from tauopathy patients and injected into the brains of mice that express non-aggregated forms of tau as well as wild-type mice, the inclusions that form are the same as those in the original tauopathy (Clavaguera et al. 2013). These data suggest that tau can spread throughout the brain in a prion-like manner through tau seeds causing further tau aggregation and disease progression.

While tau has long been considered only to be found intracellularly, new data suggest that tau can also be found in the extracellular space (Sato et al. 2018; Barthélemy et al. 2020). Both full length and truncated forms of tau have been identified in cell culture media from cells over-expressing tau, primary neurons, induced pluripotent stem cell (iPSC) derived neurons, as well as brain interstitial fluid and cerebrospinal fluid of both mice and humans (Karch, Jeng, and Goate 2013; Bright et al. 2015; Kanmert et al. 2015; Chai, Dage, and Citron 2012). This extracellular tau may be involved in furthering tau pathology throughout the brain.

1.2.4 Tau pathology

The pattern of tau deposition in various cell types throughout the brain is an additional way of characterizing tauopathies. Depending on the disease, neurons, astrocytes, and oligodendrocytes all exhibit different forms of tau pathology, allowing these diseases to be distinguished from one another.

Neuronal tau pathology includes pre-tangles which can be classified as tau positive cytoplasmic granules and can be identified by diffuse cytoplasmic staining using an anti-phosphorylated tau antibody (Arendt, Stieler, and Holzer 2016), neurofibrillary tangles, Pick bodies, spherical cytoplasmic inclusions, dystrophic neurites, threads, and grains which are tau-containing protrusions in dendrites, specific to argyrophilic grain disease (Kovacs 2015).

Tau pathology in glial cells is relatively similar to its pathology in neurons. Tau pathology in oligodendrocytes is more limited than astrocytes, with oligodendrocytes exhibiting coiled bodies or globular glial inclusions (Kovacs 2015; Arendt, Stieler, and Holzer 2016). However, astrocytic tau pathology is incredibly diverse and depends on the type of tau involved (mutated, 3R or 4R), the PTMs of tau, and the modifications to the cytoskeleton of the astrocytes. Tufted astrocytes are found in PSP, astrocytic plaques are found in CBD, thorny astrocytes are seen in AGD, globular astroglial inclusions are seen in GGT, and ramified astrocytes which are seen in Pick's disease and have thick processes and nuclei that are no longer found in the center of the cell (Komori 1999; Togo et al. 2002; Iwasaki et al. 2004; Isidro Ferrer, Santpere, and Van Leeuwen 2008; Kouri et al. 2011). The accumulation of tau may impact homeostatic astrocytic function and hinder their ability to prevent neuronal death. Astrocytes have also been shown to propagate tau pathology even in the absence of neuronal tau deposition (Maté De Gérando et al. 2021).

1.2.5 Modeling tauopathies

A variety of animal and cell models have been created in order to understand the mechanism of tau dysfunction in neurodegenerative diseases. Animals that have been used include *C. elegans*, *Drosophila melanogaster*, *Xenopus* oocytes, and mouse models. To date, there are thirty-nine rodent models with thirty-six mouse and three rat models (Alzforum 2020). As mice do not typically get tau pathology, human tau has been either knocked into the model, or particular human mutations are inserted into the genome in order to create the mouse model (Elder, Gama Sosa, and De Gasperi 2010). This also makes using mice to model human tauopathies difficult since the differences are vast, making human cell culture models more attractive.

Advances in cell culture methods have made modeling human tauopathies *in vitro* more feasible and reliable. Induced pluripotent stem cells (iPSCs) created from fibroblasts taken from tauopathy patients are powerful tools to study the molecular mechanisms underlying disease progression (Karch et al. 2019). iPSCs can be differentiated into neurons and glial cells that are impacted in disease, and these cells are then able to recapitulate disease pathology, including tau hyperphosphorylation, tau insolubility, vulnerability to specific cellular stressors, and other hallmarks of disease (Ehrlich et al. 2015; Hallmann et al. 2017; Imamura et al. 2016; Iovino et al. 2015; Jiang et al. 2018; Seo et al. 2017; Silva et al. 2016; Sposito et al. 2015; Wren et al. 2015). Due to their ability to scale up, these models provide the ability to screen potential therapeutics.

1.2.6 Tau targeted therapeutics

Tau has been shown to play a significant role in the pathology of multiple neurodegenerative diseases making both physiological and pathological tau attractive targets for therapeutics. To date, therapies for the inhibition of protein kinases, the inhibition of tau aggregation, active and passive immunotherapies, and tau silencing by antisense oligonucleotides have all been proposed (Jadhav et al. 2019). All of these have been effective at reducing tau lesions and were shown to improve cognitive and motor impairment in a variety of disease animal models. While anti-tau antibodies were thought to be the most promising and most advance potential therapeutic, the recent failure of Semorinemab in phase II clinical trials and other anti-tau antibiotics suggests that different strategies should be considered.

2 Antisense Oligonucleotides

Antisense oligonucleotides (ASOs) are single-stranded oligonucleotides of 8 to 50 bases in length that specifically bind to selected RNA sequences and regulate the expression of genes by several mechanisms depending on their chemical properties and targets, which will affect protein synthesis at posttranscriptional processing or protein translation levels.

2.1 Structure and modifications ASOs

ASOs are short single strands of either DNA or RNA made up of sugar rings connected with a phosphate backbone (Rossor, Reilly, and Sleigh 2018). Typically, oligonucleotides have weak binding affinities and are easily degraded by nucleases (DeVos and Miller 2013); however, modifying either the phosphate backbone or the sugar rings can reduce their likelihood of degradation.

One of the first and most widely used modifications in ASO design is the phosphorothioate (PS) backbone modification (DeVos and Miller 2013). The phosphate-oxygen atoms are replaced with sulfur atoms, allowing them to be a better candidate for a systemic therapeutic. The substitution of the sulfur atoms causes the ASO to be more resistant to nuclease degradation, which allows the ASO to reach its target. This modification enables the ASO to recruit RNaseH to promote cleavage of the target RNA and lower the protein levels (Cerritelli and Crouch 2009). Additionally, the PS modification allows the ASO to be taken up by cells and tissues more readily (Brown et al. 1994). If the goal is to have the ASO be incredibly stable, highly resistant to nuclease degradation, and prevent RNaseH degradation, then the thiophsophoramidate modification is ideal. Here, one of the phosphate-oxygen atoms is replaced with a nitrogen atom (Gryaznov et al. 1996). Unlike the PS modification, this is most suited for ASOs that will be used in a non-degrading way, such as for alternative splicing changes, translation inhibition, and microRNA hindrance (DeVos and Miller 2013). The final backbone modification is when the sugar-phosphate backbone is replaced with an isostere removing the negative charge from the ASO; these ASOs are referred to as morpholino ASOs (DeVos and Miller 2013). Like the thiophsophoramidate modified ASOs, they do not recruit RNaseH. They have successfully been used to inhibit gene translation inhibition or other steric blocking mechanisms, including mostly alteration of gene splicing (Summerton 1999). They have also been used with success *in vivo,* in particular with respect to spinal muscular atrophy (Osman et al. 2016).

Modifications of the sugar rings are an additional way to improve the efficacy of ASOs. These modifications are used to enhance the binding ability of the ASO to its target. While three

modifications are commonly used, the 2'-O-methyl and 2'-O-methoxyethyl (MOE) sugar modifications are the most popular (DeVos and Miller 2013) as these modifications provide increased resistance to nuclease degradation, enhanced RNA affinity, an improvement in cellular uptake as well as reduced toxicity and activation of the inflammatory response (Teplova et al. 1999). The third sugar ring modification is the locked nucleic acid modification which significantly increases the affinity of the ASO for its target but also creates a higher toxicity response causing it not to be used as often as the other sugar ring modifications (DeVos and Miller 2013). As the modification of the sugar ring leads to greater affinity of the ASO for its target and prevents RNaseH cleavage, it may not be ideal for ASOs used to reduce the expression of a specific protein. In order to maintain the strong affinity but still recruit RNaseH, a "gapmer" design can be used. Instead of modifying all of the sugar rings, an unmodified area of about ten nucleotides is flanked on both sides with fully modified nucleotides. This modification allows for increased stability and target affinity of a fully modified ASO but still will allow for target protein degradation (DeVos and Miller 2013).

2.2 ASO mechanism of action

ASOs can modulate the expression of their target in one of two main mechanisms: by recruiting RNaseH to degrade the protein or not. As mentioned earlier, the ASOs that recruit RNaseH need to have at least a portion of their sequence that is unmodified. Degrading ASOs mimic the normal DNA/RNA complex that occurs in dividing cells by binding to their target RNA in the nucleus; this binding triggers the activation of RNaseH to degrade the mRNA preventing the protein from getting translated (Rossor, Reilly, and Sleigh 2018). These ASOs have been used successfully to reduce the levels of toxic proteins associated with neurodegenerative diseases

such as amyotrophic lateral sclerosis (ALS), where SOD1 lowering ASOs have shown promise in reducing disease pathology (Schoch and Miller 2017). The ASOs that do not recruit RNaseH can be further divided into two groups: those that block protein translation by preventing the ribosome from attaching to the mRNA and those that are involved in modulating the processing of the mRNA transcript (DeVos and Miller 2013). These ASOs can bind to pre-mRNA and mask either splicing enhancers or repressor sequences (Bennett and Swayze 2010), which can cause exon skipping and the inclusion of an alternatively spliced exon. This allows the ASOs to be used as a potential therapeutic in diseases where altering the ratio of types of protein expressed can help prevent disease progression.

2.3 Pharmacokinetics and delivery of ASOs

ASOs can be delivered through a variety of methods, including intraperitoneally, subcutaneously, or intravenously. These methods allow for widespread throughout the peripheral tissue; however, because ASOs cannot cross the blood-brain barrier, they need to be delivered directly into the CSF through an intrathecal injection (Kordasiewicz et al. 2012; Smith et al. 2006). It has been identified that ASOs are taken up by all cell types in the central nervous system and through an active manner (Iversen et al. 1992). While the exact mechanism of ASO uptake is still unknown, recent data have identified that ASOs are taken up into the cell via the endocytic pathway in the liver and spleen as well as in cancer cells (Crooke et al. 2017; Linnane et al. 2019; Juliano et al. 2013; C. M. Miller et al. 2018). Further, various forms of *RAB* interact with the late endosome once the ASO has been taken up by the cell and allow the ASO to escape before being degraded by the lysosome (C. M. Miller et al. 2018). Once in the cytoplasm, ASOs can quickly move between the cytoplasmic and nuclear compartments (Lorenz et al. 2000).

ASOs have been shown to have a long-lasting impact on their targets. In a study where an RNaseH recruiting ASO was infused into the CSF, the target mRNA levels were suppressed for up to 16 weeks, and it has been suggested that non-RNaseH recruiting ASOs may have an even longer impact (Hua et al. 2010; Smith et al. 2006). All of these factors make ASOs an excellent potential therapeutic for preventing disease progression.

2.4 Therapeutic potential

As ASOs can either lower the levels of their target or alter the splicing pattern of the mRNA, they are an incredibly powerful therapeutic in potentially preventing neurodegenerative disease progression.

Many neurodegenerative diseases are caused by the toxic buildup of a single protein making the RNaseH recruiting ASOs an attractive therapy. The first protein degrading PS 2' O-methyl modified ASO to be used successfully *in vivo* targeted *SOD1* in a rat model of ALS. This ASO reduced *SOD1* mRNA levels by 50% in the brain and spinal cord of the rats, which translated into a 37% extension of life expectancy (Smith et al. 2006). These findings were promising that the ASO was advanced to phase I and subsequent phase II clinical trials (T. Miller et al. 2020). Because this ASO proved successful, other ASOs, including those that alter splicing patterns, were then tried for other neurodegenerative diseases, including spinal muscular atrophy, Huntington's disease, and tauopathies (Schoch and Miller 2017).

Spinal muscular atrophy (SMA) is caused by a deletion of the survival motor neuron (*SMN1*) gene. Unlike other species, humans express both *SMN1* and *SMN2* (Gladman et al. 2010). When

SMN1 is lost, as happens in SMA, *SMN2* can partially compensate, which makes the disease not fatal in humans (Zou et al. 2010); the degree of restoration is dependent on the number of copies of *SMN2* (Maretina et al. 2018). However, the complete rescue of function is impossible due to a C to T nucleotide variation within exon 7 of *SMN2*, which weakens the 3′ splice site and causes exon 7 skipping. The resulting protein product is unstable and degraded (Cho and Dreyfuss 2010). Therefore, the field has focused on identifying how to increase the amounts of functional *SMN* protein to rescue disease pathology. In 2016, the ASO nusinersen was approved for the treatment of SMA (I. Chen 2019). Nusinersen functions by binding to its target mRNA and increasing the inclusion of exon 7, thereby increasing the levels of functional *SMN* protein (Schoch and Miller 2017). After administration of nusinersen, children with SMA can better control head movement, roll, sit, crawl, and stand and walk. These children also survived longer than those that received the placebo therapy.

ALS is characterized by the progressive loss of motor neurons and muscle wasting. It is typically diagnosed between the ages of 40-70, and patients usually die from the disease within 2-5 years of diagnosis. Gene mutations cause about 10% of ALS cases. Of those, 15-20% are caused by mutations in the Cu/Zn superoxide dismutase 1 gene (SOD1) (Ghasemi and Brown 2018), with an additional 40% thought to be caused by a hexanucleotide repeat expansion within the chromosome 9 open reading frame 72 (C9orf72). Because SOD1 and C9orf72 cause more than half of all familial ALS, they are attractive therapeutic targets. As mentioned earlier, the SOD1 degrading ASO has successfully passed phase I and II clinical trials (T. Miller et al. 2020). All patients who received the ASO exhibited decreased SOD1 levels compared to those who received the placebo. As few adverse effects were reported, the trial was moved onto phase III,

which is scheduled to complete in July of 2021 ("Tofersen | ALZFORUM" n.d.). An ASO that targets the hexanucleotide repeats in C9orf72 and recruits RNaseH to degrade them was explored (Ly and Miller 2018; Schoch and Miller 2017). Studies reported that following administration of the C9orf72 ASO that bound downstream of the expansion. There was a significant reduction in C90rf72 mRNA levels while the ASO that bound within or immediately upstream of the intronic expansion did not significantly alter C9orf72 mRNA levels (Ly and Miller 2018). However, both ASOs significantly reduced RNA foci, increased survival from glutamate excitotoxicity, and reduced aberrant gene expression patterns both *in vivo* and *in vitro*. The phase I clinical trial for BIIB078, a C9orf72 ASO, is currently ongoing.

Huntington's disease (HD) is an adult-onset, neurodegenerative condition characterized by a locomotor and cognitive decline, with patients being diagnosed around the ages of 30-40 due to worsening symptoms. HD is an autosomal dominantly inherited disease caused by increased *CAG* repeats in the gene *huntingtin* (*Htt*) (Heinz, Nabariya, and Krauss 2021). While medium spiny neurons of the striatum are affected most strongly, it does appear that other neuronal populations may be vulnerable (Šonský et al. 2021). The phase III clinical trial of the ASO tominersen was stopped for not meeting end goals. This ASO had previously shown promise in phase I and II trials at lowering *Htt* levels (Kwon 2021). There are still, however other ASOs still in development that may be better suited as a therapeutic.

The most common neurodegenerative disease is Alzheimer's disease, which affects over 10 million people worldwide ("Dementia" n.d.). Alzheimer's disease is caused by the toxic buildup of two proteins: amyloid-beta and tau (Mary 2012). There are currently no effective therapies

that can prevent neurogenerative disease progression. As ASOs have proven to be an effective therapeutic in difficult to treat diseases, they are now being tested for Alzheimer's disease and other dementias like tauopathies. Tau lowering ASOs showed incredible promise in reversing disease pathology and preventing neuronal loss in mice and non-human primates (DeVos et al. 2017) and are now in phase I clinical trials.

ASO therapies may also be a better alternative to CRISPR-based technologies. The use of ASOs as therapeutics for difficult-to-treat diseases shows incredible potential. ASOs are easily altered and administered, long-lived, incredibly specific to their target, and show little to no toxicity. There is no need for exogenous gene-editing machinery that can generate permanent genetic changes with unknown consequences.

3 Astrocytes

Astrocytes are a star-shaped glial cell that plays a role in development, homeostasis, injury, and repair of the central nervous system (Hart and Karimi-Abdolrezaee 2021). They regulate blood flow, maintain the blood-brain barrier, glucose and other ion metabolism and storage, synapse formation and function, and axon myelination (Isidre Ferrer et al. 2014). In disease, astrocytes can take on a reactive phenotype which can either be harmful or beneficial depending on the environment (Sofroniew 2009). While previously thought to be a supporting cell of the brain, new evidence has emerged showcasing the importance of better understanding astrocytes and their role in disease progression.

3.1 Astrocyte Biology

3.1.1 Development

Astrocytes originate from a heterogeneous population of cells known as radial glial cells (RGCs) (Hart and Karimi-Abdolrezaee 2021). RGCs are derived from neuroepithelial cells found between the ventricular and pial surfaces of the developing CNS. Their radial fibers are used as scaffolding for migrating cells (Barry and McDermott 2005; Poluch and Juliano 2007; Rakic 1972). Initially, RGCs are differentiated into neurons in a process known as neurogenesis. After the bulk of neurogenesis has occurred, gliogenesis begins (Sloan and Barres 2014). Various secreted factors, including the IL-6 family of cytokines bone morphogenic protein (BMP), Notch signaling, and their downstream effector proteins, activate astrogenesis (Sloan and Barres 2014). In mice, gliogenesis begins at E17.5 and continues through P7 (Bond et al. 2020), whereas in humans, it starts around 18 weeks post-conception and continues through the first two weeks of life (Semple et al. 2013).

3.1.2 Astrocyte morphology

Astrocytes are highly morphologically, physiologically, genetically, functionally, and metabolically diverse. This is thought to be partially driven by the progenitors they originate from (Zhang et al. 2014) and the region in which they are found. Astrocytes found in humans are much larger, with more complex processes that can contact hundreds of more synapses than those found in rodents (Oberheim et al. 2009). Astrocytes found in the brain's white matter are referred to as fibrous astrocytes, and those found in the grey matter are known as protoplasmic astrocytes (WL 1893). Protoplasmic astrocytes have extremely ramified processes that allow them to interact with neurons at multiple synapses (Bushong et al. 2002; Oberheim, Goldman,

and Nedergaard 2012), where they are involved in neuromodulation. Fibrous astrocytes are smaller than protoplasmic astrocytes, have fewer processes, and contain many glial filaments in the cytoplasm (Vasile, Dossi, and Rouach 2017). They are found along white matter tracks in the brain and can only contact neurons at Ranvier's nodes, where they can provide homeostatic support in the region (Lundgaard et al. 2015).

3.1.3 Genetics

In addition to morphological differences, there are also differences in genetic expression in astrocytes that contribute to their differences in functionality. While *GFAP* has long been considered a classical astrocytic marker, it is now known that it is a reactive astrocyte marker, and in the healthy CNS, not all astrocytes express it (Pekny et al. 2016; Walz and Lang 1998; Eng, Ghirnikar, and Lee 2000). Other genes that can be used to identify astrocytes include glutamine synthetase (GS), which stains astrocytes in the entorhinal cortex (Anlauf and Derouiche 2013), and *Aldh1L1* which while expressed more highly in healthy astrocytes than *GFAP*, labels primarily cortical astrocytes (Waller et al. 2016).

3.1.4 Homeostatic functions of astrocytes

Astrocytes are involved in many functions that ensure the CNS can function properly, including promoting neuronal maturation, synapse formation and function, regulating blood flow, and maintaining the homeostatic environment (D. D. Wang and Bordey 2008) by providing metabolic support and taking up excess ions at the synapse. Astrocytes have been identified as a significant source of extracellular matrix (ECM) proteins and adhesion molecules in the CNS, which are responsible for either promoting or inhibiting neurite outgrowth (D. D. Wang and

Bordey 2008). Secretion of thrombospondins by astrocytes is critical for the formation and function of synapses (Barres 2008; Christopherson et al. 2005). They have also been shown to secrete factors that can induce C1q expression at the synapse in order for microglia to prune them (Stevens et al. 2007). Astrocytic processes can make contact and interact with blood vessels which can regulate blood flow within the CNS. They have also been shown to secrete factors that can cause blood vessels to constrict or dilate (Iadecola and Nedergaard 2007; Gordon, Mulligan, and MacVicar 2007). Through their contact with blood vessels, astrocytes can also regulate blood flow to particular areas based on synaptic activity (Schummers, Yu, and Sur 2008).

3.1.5 Astrocytes at the synapse

As astrocyte processes can surround synapses, they play a crucial role in maintaining synaptic homeostasis and function. Astrocytes can control the pH of the synapse through proton shuttling, using the Na^+/H+ exchanger, bicarbonate transporters, monocarboxylic acid transporters, and the vacuolar-type proton ATPase (Obara, Szeliga, and Albrecht 2008). Astrocytes express high levels of various transporters used to take up neurotransmitters such as glutamate, GABA, and glycine from the synaptic cleft (Sattler and Rothstein 2006; Schummers, Yu, and Sur 2008). Once these are taken up, they get converted into their precursors and recycled back to the synapse for reconversion into active transmitters. It has also been suggested that astrocytic networks linked by gap junctions can take up small molecules such as glutamate and potassium that otherwise could cause neuronal toxicity (Seifert, Schilling, and Steinhäuser 2006) through glutamate transporters and Kir4.1 channels, respectively. Unlike neurons, astrocytes cannot propagate action potentials, but this does not mean that they are physiologically silent. Astrocytes can regulate their intracellular calcium levels. These oscillations are essential in

astrocyte-astrocyte and astrocyte-neuron communication (Cornell-Bell et al. 1990; Bazargani and Attwell 2016).

Additionally, increases in intracellular calcium levels can lead to the release of synaptically active molecules, including glutamate, purines (ATP and adenosine), GABA, and D-serine (Ota, Zanetti, and Hallock 2013) which can subsequently alter neuronal excitability. This has given rise to the tripartite synapse hypothesis, which states that astrocytes play direct and interactive roles with neurons during synaptic activity in a manner essential for information processing by neural circuits (Chung, Allen, and Eroglu 2015; Um 2017; Haydon and Nedergaard 2015).

3.2 Astrocytes in disease

3.2.1 Astrocyte activation

Astrocytes can become reactive in response to changes in the surrounding environment such as insult or injury in the CNS and occur as a series of changes in gene expression and cellular changes. Reactive astrocytes can secrete extracellular molecules, such as neurotrophic factors (BDNF, VEGF, and bFGF), inflammatory factors (including IL-1β, TNF-α, and nitric oxide), and cytotoxins (such as Lcn2). Depending on which factors are being secreted, astrocytes can play either a neuroprotective or neurotoxic role (such as provoking inflammation or increasing damages) in the CNS (Giordano, Kavanagh, and Costa 2009; Kajihara et al. 2001). Healthy astrocytes do not all express *GFAP*, maintain individual domains, and are not particularly proliferative (Sofroniew and Vinters 2010); this changes upon astrocyte activation. In mild to moderate activation, almost all astrocytes become *GFAP* positive, some cellular hypertrophy is

seen, but the astrocytes maintain their individual domains and are not particularly proliferative. If the stimulus causing astrocyte activation is removed, the astrocytes can return to their healthy state. With severe astrogliosis, there is more upregulation of *GFAP* expression, along with a disruption of individual astrocyte domains , causing a long-lasting reorganization of tissue architecture, and the astrocytes become much more proliferative (Sofroniew and Vinters 2010).

Additionally, there are changes in molecular gene expression, functional activity, and severe cellular hypertrophy. Infections, as well as neurodegenerative disorders, commonly cause this. Finally, reactive astrocytes can form a glial scar (Sofroniew and Vinters 2010) that can persist for an extended period of time and act as barriers to axon regeneration and inflammatory cells, infectious agents, and non-CNS cells to protect healthy tissue from nearby sites of severe damage.

3.2.2 Mechanism of dysfunction in disease

In disease, astrocytes have been shown to lose their ability to provide homeostatic support and exhibit toxic gain of function. It has been shown that astrocytes may lose their ability to take up excitatory neurotransmitters from the synapse causing excitotoxicity (Mahmoud et al. 2019). Reactive astrocytes may also begin to produce higher levels of cytokines, reactive oxygen species (ROS), and other toxic molecules (Palpagama et al. 2019). They have also been shown to release glutamate, which can also cause excitotoxicity and propagate seizures (Vargas-Sánchez et al. 2018). Reactive astrocytes may also contribute to the degradation of the blood-brain barrier, which then allows for neurotoxic molecules to enter the CNS (Kondo et al. 2015). These

findings show that when astrocytes cannot function properly, it creates an environment that can contribute to disease progression.

3.2.3 Astrocytes in Epilepsy

Epilepsy is a disorder that is characterized by the occurrence of unpredictable and periodic seizures. These are caused by abnormal and excessive neuronal activity in the cortex (Fisher et al. 2005). While the cause of most cases is still unknown, epilepsy may be caused by brain injury, stroke, brain tumors, infections of the brain (Goldberg and Coulter 2013). Some known genetic mutations can lead to cases of epilepsy (Pandolfo 2011).

As astrocytes play such an essential role in regulating synaptic function, it stands to reason that the astrocytes themselves can cause neuronal hyper-excitability. In the healthy CNS, astrocytes express ion channels, transmitter receptors, and transporters used to recycle and remove neurotransmitters that could otherwise cause hyper-excitability (Siracusa, Fusco, and Cuzzocrea 2019). However, in the brains of epilepsy patients, it has been identified that there is a decrease in glutamine synthetase, which causes a slowing in glutamate-glutamine cycling leading to the buildup of glutamate at the synapse (Steinhauser et al. 2012). There is also an increase in depolarization at the synapse in epilepsy patients, which leads to a rise in hyper-excitability (Steinhauser et al. 2012). These findings suggest that dysfunctional astrocytes play a role in epilepsy.

3.2.4 Astrocytes in Parkinson's disease

Parkinson's disease is a neurodegenerative disorder that is characterized by the loss of dopaminergic neurons in the pars compacta of the substantia nigra (SN) and intraneuronal aggregates of α-synuclein (called Lewy bodies and Lewy neurites) in specific regions of the brain stem, spinal cord, and cortex (Balestrino and Schapira 2020). It has been suggested that glial cells are involved in neuronal loss initiation and the neurodegeneration that follows. Early in the disease, protoplasmic astrocytes have been shown to take up α-synuclein, which has been shown to cause astrocytic dysfunction (B. D. Lee et al. 2010). When α-synuclein accumulates in astrocytes, they begin to produce higher levels of proinflammatory chemokines and cytokines as well as neuroinflammatory mediators. Additionally, the early accumulation of α-synuclein causes a disruption in glutamate transport, leading to neuronal death (Gu et al. 2010).

3.2.5 Astrocytes in ALS

As described earlier, ALS is a progressive, fatal neurodegenerative disease involving the brain and spinal cord's upper and lower motor neurons. It has been shown that in ALS, glutamate uptake by astrocytes is significantly reduced, which leads to an increase in glutamate levels at the synapse, which can cause motor neuron death due to excitotoxicity (Rothstein, Martin, and Kuncl 1992; Rothstein et al. 1995; Howland et al. 2002; H. Guo et al. 2003). Additionally, astrocytes in ALS exhibit dysfunctional mitochondria, which leads to increased production and release of ROS, along with increased superoxide radical formation, and other defects associated with nitro-oxidative damage (Cassina et al. 2008). It has also been shown that Kir4.1 levels are significantly decreased in astrocytes in ALS, which prevents astrocytes from maintaining water and potassium homeostasis in CNS and further contributing to neuronal death and disease progression (Zhong et al. 2008).

3.2.6 Astrocytes in AD

AD is the most common cause of dementia and is pathologically defined by extracellular amyloid-beta plaques and intracellular neurofibrillary tau tangles. Reactive astrocytes have been identified near amyloid-beta deposits in post-mortem AD patient brains as well as in animal models (Nagele et al. 2003; Olabarria et al. 2010; Simpson et al. 2010). It has been shown that astrocytes are able to internalize amyloid-beta in order to prevent further damage. However, this amyloid-beta then impacts astrocytic function (Kuchibhotla et al. 2009). Astrocytes that have been exposed to amyloid-beta have higher ROS production, leading to higher levels of oxidative stress which then leads to alterations in calcium signaling (Phatnani and Maniatis 2015). It has also been shown that there is reduced connectivity between astrocytes in AD leading to higher levels of glutamate and ATP being released, causing neuronal toxicity (Phatnani and Maniatis 2015).

3.3 Astrocytes in tauopathies

3.3.1 Astrocytic tau expression and diseases with glial tau deposition

While tau is most highly expressed by neurons, glial cells do express tau mRNA and protein, although at much lower levels (Zhang et al. 2016). Some tauopathies, however, can have predominantly glial tau pathology (Kahlson and Colodner 2015). As described earlier, tau deposition in astrocytes creates a variety of astrocytic phenotypes, including tufted astrocytes, astrocytic plaques, globular glial inclusions, ramified astrocytes, thorny astrocytes, and granular/fuzzy astrocytes (Kovacs et al. 2016). Interestingly, it appears that 4R tau may

preferentially be expressed or be deposited in astrocytes in tauopathies (Reid et al. 2020). Even in 3R tauopathies such as Pick's disease, there is 4R tau deposition in astrocytes (Hogg et al. 2003; Kahlson and Colodner 2015).

Many tauopathies also exhibit reactive gliosis in affected brain regions, with activated astrocytes being found in areas of severe neurodegeneration (Bierer et al. 1995; Togo et al. 2002; Togo and Dickson 2002). It also seems that tau deposition within astrocytes may cause them to become reactive. PSP, CBD, AGD, GGT, Pick's disease, aging-related tau astrogliopathy (ARTAG), AD, primary age-related tauopathy (PART), and chronic traumatic encephalopathy (CTE) all present with tau deposition (Kahlson and Colodner 2015; Reid et al. 2020), showcasing the importance of understanding how tau deposition impacts astrocytic function and the progression of the disease.

3.3.2 How tau impacts astrocytic function

There is accumulating evidence suggesting that tau accumulation in astrocytes leads to a decreased ability to perform homeostatic functions. When normal tau was expressed in astrocytes in aged mice, there was a disruption to the BBB, which suggests that the presence of tau in astrocytes leads to them not being able to maintain the BBB (Bartels et al. 2009; Zlokovic 2008). Additionally, astrocytes that overexpressed either normal tau or the P301L mutation had fewer glutamate transporters which led to a decrease in glutamate transport capacity (Dabir et al. 2006). This can lead to calcium-dependent excitotoxicity, which has been linked to a number of neurodegenerative disorders (Maragakis and Rothstein 2001). Further, when 3R or 4R tau were overexpressed in hippocampal astrocytes alone, it caused a change in mitochondrial distribution

in the cells from the distal processes to the soma and led to changes in their redox state and decreased motility (Richetin et al. 2020). Richetin et al. also found that 3R tau overexpression led to changes in mitochondrial shape and function in the distal astrocytic processes. It was also found that overexpression of 3R tau in astrocytes led to impairment of the neural network and impaired the synchronous activity as well as spatial memory in mice. These data strongly implicate that tau deposition or expression in astrocytes impacts their ability to perform homeostatic functions which can lead to disease progression.

Summary of dissertation

The work described in the subsequent chapters of this dissertation aims to show the importance of understanding the role that tau expression and deposition in astrocytes plays in the progression of tauopathies. Chapter 2 explores the impact of tau expression in a primary astrocyte model. I was able to identify that astrocytes that have increased levels of 4R tau take on a neurotoxic phenotype and lose the ability to provide homeostatic functions in order to protect neurons. Chapter 3 expands upon the work done in primary cells and examines the impact of 4R tau expression in a human cell model using astrocytes derived from IVS 10+16 *MAPT* mutation carriers and their isogenic controls. I found that the human astrocytes exhibited a similar increased expression of neurotoxic genes and reduced ability to provide homeostatic support to neurons. Additionally, I identified that neurons co-cultured with 4R tau expressing astrocytes exhibited hyper-excitability and were more susceptible to death. This was reduced when tau levels were lowered in the astrocytes alone, suggesting that tau in astrocytes directly influences

neuronal excitability and survival. Chapter 4 describes preliminary data where pre-trans-splicing molecules were used to alter tau isoforms in primary astrocytes. This work can be expanded in the future to change tau isoforms in a cell-specific manner to identify how altering the levels of 4R tau in astrocytes alone *in vivo* will impact disease progression. The final chapter summarizes the findings and the impact of the experiments. It describes potential future directions that can lead to a greater understanding of how 4R tau impacts astrocytic function in disease and why astrocytes should be considered as candidates for possible therapeutics.

Chapter 2

Astrocytic 4R tau expression drives astrocyte reactivity and dysfunction *in vivo* and *in vitro*

Adapted from a manuscript that is under revision at JCI insight:

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Summary

The protein tau and its isoforms are associated with several neurodegenerative diseases, many of which are characterized by greater deposition of the 4R tau isoform; however, the role of 4R tau in disease pathogenesis remains unclear. We created antisense oligonucleotides (ASOs) that alter the ratio of 3R:4R tau to investigate the role of specific tau isoforms in disease. Preferential expression of 4R tau in human tau (hTau)-expressing mice increased seizure severity and phosphorylated tau deposition without neuronal or synaptic loss. However, we observed strong co-localization of 4R tau within reactive astrocytes and increased expression of pan-reactive and neurotoxic genes following 3R to 4R tau splicing ASO treatment in hTau mice. Increasing 4R tau levels in primary astrocytes provoked a similar response, including a neurotoxic genetic profile and diminished homeostatic function. These findings support a novel pathway by which astrocytic 4R tau mediates reactivity and dysfunction and suggest that astrocyte-targeted therapeutics against 4R tau may mitigate neurodegenerative disease progression.

Introduction

Tauopathies are a class of neurodegenerative diseases characterized by the pathogenic aggregation of hyperphosphorylated tau protein in neurofibrillary tangles (Y. Wang and Mandelkow 2016). Tau, encoded by the *MAPT* gene, plays a role in many important cellular functions, including intracellular signaling, synapse formation and function, and axonal transport regulation (Allen et al. 2018; T. Guo, Noble, and Hanger 2017; Ballatore et al. 2012). Tau hyperphosphorylation and aggregation can disrupt essential cellular functions that are critical for maintaining homeostasis in the central nervous system (Piacentini et al. 2017) and can lead to neurodegenerative disease (Y. Wang and Mandelkow 2016; Michel Goedert, Eisenberg, and Crowther 2017; M. Liu et al. 2020).

Tau can be classified as 3-repeat (3R) or 4-repeat (4R), corresponding to the number of microtubule-binding repeat domains that result from alternative splicing of exon 10 of *MAPT* (Sealey et al. 2017; Buée et al. 2000; Pîrşcoveanu et al. 2017). In adults, the ratio of 3R to 4R tau is approximately 1:1; however, certain disease mutations in the *MAPT* gene can cause a change in the ratio of the isoforms toward greater expression of either 3R or 4R tau (Strang, Golde, and Giasson 2019; Kovacs et al. 2018; Reid et al. 2020). Many primary genetic *MAPT* mutations are found in and around exon 10, resulting in tau mis-splicing, biased 4R tau production, or mutant tau protein expression (Y. Wang and Mandelkow 2016; F. Liu and Gong 2008; Qian and Liu 2014). Thus, an altered ratio of tau isoforms, particularly affecting the 4R tau isoform, is sufficient to drive tau pathology. In a mouse model expressing all six isoforms of

human tau (hTau), including both 3R and 4R tau (Andorfer et al. 2003), an antisense oligonucleotide (ASO) strategy to increase 4R tau was sufficient to induce tau phosphorylation and aggregation and increased seizure severity (Schoch et al. 2016). These results further confirmed that 4R tau toxicity might be involved in tau pathology and disease. While there were substantial effectsin neuronal function as evidenced by behavioral changes, there were minimal neuronal changes in terms of pathology or number. This discrepancy prompted us to consider non-neuronal cells as a potential contributor to 4R toxicity. We focused first on astrocytes because of the well-described pathological changes within astrocytes in human disease (Jain, Wadhwa, and Jadhav 2015; Ikeda et al. 2018; Okamoto et al. 2019; Garwood et al. 2017).

To examine an astrocyte-specific role for 4R tau in disease, we used tau isoform switching ASOs, previously characterized in our lab (Andorfer et al. 2003), to alter the ratio of tau isoform expression in hTau-expressing murine astrocytes. Our findings demonstrate that, despite the low level of tau expression in astrocytes, 4R tau interferes with normal astrocyte functions and causes the astrocytes to take on a neurotoxic phenotype and may contribute to neurodegeneration.

Materials and Methods

Mass spectrometry: Immunoprecipitation and mass spectrometry were performed as previously described with some modifications (Sato et al. 2018; Barthélemy et al. 2019). Briefly, cultured astrocytes and neurons were collected in PBS, pelleted, and frozen until analyses. Cell pellets were lysed with detergent and caotropic reagents (final 1% NP-40, 5mM guanidine) and subjected to immunoprecipitation with Tau1 and HJ8.5 tau antibodies. Tau was trypsinized, desalted with Toptip columns, and analyzed by a high-performance mass spectrometer, Orbitrap Eclipse (Thermoscientific). Internal standard N15 labeled 2N4R recombinant tau, and AQUA peptides were spiked into the samples for normalization.

Animals: Age- and sex-matched male and female human tau-expressing (hTau) (Andorfer et al. 2003), mouse tau knockout (mTau-/-) (Tucker, Meyer, and Barde 2001; Schoch et al. 2016)(17, 58), and C57BL/6 mice were aged to 12 months prior to experimental treatment. All husbandry and surgical procedures were approved by the Washington University Institutional Animal Care and Use Committee in accordance with federal standards.

Antisense oligonucleotides (ASOs): All ASOs were designed with a phosphorothioate backbone and either uniform or "gapmer" 2'-O-methoxyethyl sugar ring modifications to achieve tau splicing or knockdown, respectively (DeVos et al. 2013; DeVos and Miller 2013). The fully modified design prevents target degradation, while the gapmer design, consisting of 10 central, unmodified nucleotides flanked by five modified nucleotides, enables RNaseH-mediated target degradation. An ASO control similar in structure and modifications but without target specificity was included in experimental treatments to account for potential toxicity or off-target effects. All

ASOs were synthesized by Ionis Pharmaceuticals (Carlsbad, CA) and generously provided for use. Sequences are as follows: tau splicing control ASO (5'-TCATTTGCTTCTACAGG-3'), 3R to 4R tau splicing ASO (5'-GGCGCATGGGACGTGTGA-3'), 4R to 3R tau splicing ASO (5'-GGACGTGTGAAGGTACTC-3'), For, in vitro experiments, ASOs were diluted to 10μM in astrocyte media and replaced every other day for 12 days. hTau astrocytes treated with a control ASO exhibited similar levels of select mRNA gene expression as compared to those that were treated with saline-supplemented media; therefore, a control ASO was used for all subsequent treatments. For in vivo experiments, ASOs were continuously administered to the right lateral ventricle via osmotic pump (ALZET, Cupertino, CA) as described below.

Intraventricular delivery of ASOs: Osmotic pumps were prepared as previously described (Schoch et al. 2016; DeVos et al. 2017). Mice were anesthetized using a constant flow of 2-3% inhalant isoflurane and placed onto a stereotaxic head frame (Kopf, Tujunga, CA). While anesthetized, the scalp was cut, and a pump was placed into a subcutaneous pocket above the scapula. A metal catheter attached to the pump via flexible tubing was positioned to -1.1mm M/L, -0.5mm A/P, -2.5mm D/V from bregma and secured to the skull to prevent movement. The incision was then sutured, and mice were placed on a 37°C warming pad until ambulatory. Following the surgical procedure, all mice were individually housed.

Primary astrocyte cultures: Cortices from hTau, mTau-/- littermates and C57BL/6 mouse pups (P2-P4) were isolated as previously described (Schildge et al. 2013) and cultured until confluency on 100μg/mL poly-D-lysine (Corning CB-40210)-coated plates in primary astrocyte media (DMEM, 10% heat-inactivated fetal bovine serum (FBS), 1X penicillin-streptomycin) at

37°C, 5% CO2. The mixed glial cultures were purified by shaking at 270 rpm for 6 hours at 37°C to achieve a pure primary astrocyte culture.

Primary neuron isolation: Cortices from E15.5 C57 mouse pups were isolated as previously described (Hilgenberg and Smith 2007). Briefly, the cortices were isolated and the meninges removed; the cortices were then digested in .05% trypsin for 15 minutes. A single cells suspension was made, and the cells were counted. 150,000 neurons/mL were plated on top of hTau astrocytes. The following day, 1uM of Ara-C was added to prevent glial contamination.

Neuron astrocyte co-cultures: Cortices from hTau, mTau-/- littermates and C57BL/6 mouse pups (P2-P4) were isolated as described above and cultured until confluency on 100µg/mL poly-D-lysine (Corning CB-40210)-coated plates in primary astrocyte media (DMEM, 10% heat-inactivated fetal bovine serum (FBS), 1X penicillin-streptomycin) at 37□C, 5% CO2. The mixed glial cultures were purified by shaking at 270 rpm for 6 hours at 37□C to achieve a pure primary astrocyte culture. The purified astrocytes were treated with ASOs for 12 days prior to co-culture with primary neurons. These cells were cultured in primary neuronal media (Neurobasal, B27, N2, 1X glutamax, and 1X penicillin-streptomycin). The co-cultures were grown for 14 days to allow for the formation of mature synapses. On day 14, the media was supplemented with saline or 100µM H_2O_2 for 24 hours prior to fixation and immunocytochemical analysis.

Immunoblotting: Cells were lysed in cold RIPA lysis buffer supplemented with complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Basel Switzerland). Protein lysate

concentrations were measured using the PierceTM BCA Protein Assay Kit (ThermoFisher Waltham, MA), and 20µg of protein was separated using a 4-20% polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred onto a PVDF membrane. The membrane was probed for total tau (Tau 5, 1:1000) and GAPDH (1:1000), Eaat2 (1:1000), Stat3 (1:1000), Vinculin (1:1000) overnight and then incubated with secondary HRP antibodies (both at 1:5000). The membrane was imaged using a SYNGENE reader.

Immunofluorescence staining: Free-floating 50uM thick tissue sections were washed in 1X Tris-buffered saline (TBS) before blocking in 5% normal horse serum (NHS) diluted in 0.1% Triton-X-100. The sections were incubated in primary antibody (3R 1:1000, Millipore-Sigma Cat #05-803; 4R 1:1000, Cosmo Bio Cat # CAC-TIP-4RT-P01; GFAP 1:1000, Millipore-Sigma Cat #AB5541 at 4C overnight. The next day the sections were incubated in the blocking buffer prior to incubation with fluorescently tagged secondary antibodies (anti-mouse AlexaFluor 594, anti-rabbit AlexaFluor 647, anti-chicken AlexaFluor 488 all at 1:500). Sections were then mounted and sealed with Fluoromount media (Southern Biotech, Birmingham AL). The Nikon A1Rsi confocal microscope was used for acquiring images.

Immunocytochemical staining: Primary astrocytes were fixed using 4% paraformaldehyde (Santa Cruz Biotechnology Cat # sc-281692, Dallas, TX) and permeabilized using 0.1% Triton-X-100 in PBS. The cells were then incubated in blocking buffer (0.5% BSA diluted in PBS). The cells were incubated in primary antibody (3R 1:1000, Millipore-Sigma Cat #05-803; 4R 1:1000, Cosmo Bio Cat # CAC-TIP-4RT-P01; GFAP 1:1000, Millipore-Sigma Cat #AB5541, MAP2 1:1000 Abcam Cat #5392) at 4C overnight. The next day, cells were incubated with

fluorescently tagged secondary antibodies (anti-mouse AlexaFluor 594, anti-rabbit AlexaFluor 647, anti-chicken AlexaFluor 488 all at 1:500). Coverslips were mounted and sealed with Fluoromount media (Southern Biotech, Birmingham, AL). The Nikon A1Rsi confocal microscope was used for acquiring images.

Quantitative real-time PCR: RNA was isolated from mouse brain tissue using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Primary astrocytes were lysed using 500µL of QIAzol Lysis Reagent (Qiagen) and placed into Eppendorf tubes. Chloroform (100µL) was added to each sample, shaken vigorously for 5 seconds, and let to sit for 3 minutes at room temperature. The samples were centrifuged for 15 minutes at 12,000 RCF at 4□C. The aqueous layer was removed and combined with 1.5 times volume of 100% ethanol and together added to the RNeasy column for RNA purification according to the manufacturer's protocol. cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA) and analyzed on the QuantStudio 12K Flex Real-Time PCR System using either the Power SYBR™ Green PCR Master Mix (ThermoFisher) the PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies, Coralville, IA). Expression levels were calculated by the ΔΔCt method, normalized to GAPDH and a biological reference sample. Primer sequences or identifiers (purchased from Integrated DNA Technologies) are detailed in Supplementary Table 1.

Glutamate uptake assay: Glutamate uptake was assessed using the Glutamate Assay Kit (abcam, Cambridge, United Kingdom) per the manufacturer's instructions. Briefly, primary murine astrocyte cultures and iAstrocytes were plated onto PDL-coated 24-well plates and

treated with ASOs as described above. Cells were incubated in 100 µM glutamate in Live Cell Imaging Solution (ThermoFisher) for 2 hours at 37°C. Following the incubation, 50µL of the glutamate-containing media was combined with 50µL of the reaction buffer in a 96-well plate and incubated for 30 minutes at room temperature with shaking while protected from light. Sample absorbance was measured at 450nm on a plate reader, and a standard curve of known glutamate concentrations was used to quantify the concentration of glutamate within each sample. Samples were run in triplicate and averaged.

Cytotoxicity assay: Lactate dehydrogenase (LDH) release was quantified using the Pierce LDH Cytotoxicity Assay Kit (ThermoFisher) following the manufacturer's instructions. Briefly, 24 hours prior to the assay, astrocyte media was supplemented with 0 or 100µM of hydrogen peroxide and added to the cells. The next day, the media was collected, and LDH activity was measured at 490nm and 680nm on a plate reader. The 680nm value was subtracted from the 490nm value to obtain the level of LDH release. Samples were run in triplicate and averaged.

Statistical analysis: All data were graphed as mean + SEM and analyzed using GraphPad Prism 9 statistical software (GraphPad Software, La Jolla, CA). Quantifiable mRNA data for both 3R to 4R and 4R to 3R splicing experiments in hTau mice and hTau primary astrocytes were evaluated by two-way ANOVA with Tukey's multiple comparisons post hoc analysis. mRNA data in the C57 WT and mTau$^{-/-}$ mice and astrocytes was analyzed using one-way ANOVA with multiple corrections. Glutamate uptake by primary astrocytes treated with both 3R to 4R and 4R to 3R splicing was evaluated by two-way ANOVA with Tukey's multiple comparisons post hoc analysis. Levels of LDH release pre and post stress in 3R to 4R and 4R to 3R splicing treated

primary astrocytes was analyzed by two-way ANOVA with Tukey's multiple comparisons post hoc analysis. Percent of the viewing field covered by *MAP2* staining was analyzed using an ordinary one-way ANOVA with multiple corrections. A value of $p<0.05$ was deemed significant for all analyses.

Results

4R tau is expressed by reactive astrocytes *in vivo*

As we previously had not identified overt changes to neuronal pathology or number, we were interested to see if a difference in tau isoform localization throughout the brain could explain the seizure phenotype associated with increased 4R tau levels. We stained hTau brain tissue from mice that were treated with the control ASO for 3R tau (**Figure 2.1A**), 4R tau (**Figure 2.1B**), and GFAP to identify astrocytes (**Figure 2.1C**). We saw that both 3R and 4R tau appeared to be largely neuronal, with some 4R tau co-localizing to astrocytes (**Figure 2.1D**). In contrast, hTau tissue from mice treated with the 3R to 4R tau splicing ASO exhibited a decrease in 3R tau staining (**Figure 2.1E**) and a change in 4R tau localization **(Figure 2.1F**), coincident with astrocyte morphology (**Figure 2.1G**) which had not been seen in the hTau mice treated with the control ASO (**Figure 2.1H**). After identifying a change in astrocyte morphology, we performed qRT-PCR analysis for pan-reactive, neurotoxic, and neuroprotective genes from whole mouse brain lysates from hTau, mTau-/-, and wildtype (C57BL/6) mice treated with either control or 3R to 4R tau splicing ASO. hTau mice treated with the 3R to 4R tau splicing ASO exhibited a significant upregulation in several reactive and neurotoxic genes (Liddelow et al. 2017), including *Vim* (pan-reactive), *Serpina3n* (pan-reactive), *C3* (neurotoxic), and *Serping1* (neurotoxic) compared to the mice treated with the control ASO (**Figure 2.1I**). No change in neuroprotective genes was identified in the hTau mice treated with the 3R to 4R tau splicing ASO as compared to those treated with the control ASO (**Figure 2.1I**). These changes were not seen in either the mTau-/- mice or the C57BL/6 mice treated with the same ASOs (Supplemental Figure 2.1A and B), confirming that the mRNA changes after 3R to 4R ASO treatment are

specific to altering the ratio of 4R tau and not an inflammatory, non-specific effect of ASOs. In contrast, we found that when hTau mice were treated with a 4R to 3R tau splicing ASO, genes that had been previously upregulated (*Vim, Serpina3n, C3*) were reduced to baseline (**Figure 2.1I**). These findings suggest that the presence of 4R tau in astrocytes leads them to take on a reactive, neurotoxic phenotype, but it was unclear whether this was due to astrocytic uptake or expression of tau. Despite collective data in mice (Richetin et al. 2020; Zhang et al. 2014) and humans (Kovacs et al. 2017, 2016; Zhang et al. 2016) that support tau expression in astrocytes, the level of neuronal tau far exceeds astrocyte tau expression. To confirm that astrocytic tau protein is detectable, we performed mass spectrometry on primary hTau astrocytes, mTau-/- astrocytes, and wild-type neurons obtained from mice to analyze endogenous tau protein expression. We identified that tau protein is detectable in hTau mouse astrocytes, albeit at levels approximately 100-fold less than in neurons (**Figure 2.1J**). We were also able to detect total tau levels in primary hTau astrocytes via western blot (Supplementary Figure 2.1C). These results are consistent with previous studies that have measured relative tau RNA expression levels (Zhang et al. 2014, 2016) and confirm the presence of endogenous tau in astrocytes.

Manipulation of tau splicing towards more 4R tau promotes a neurotoxic astrocyte phenotype

Despite observing a neurotoxic astrocytic signature in hTau mice following ASO-mediated tau splicing to increase 4R tau, the non-selective nature of ASO uptake prevented us from isolating cell-type-specific changes. Therefore, we tested the effect of tau splicing manipulation in an in vitro astrocyte model. Primary hTau-expressing astrocytes from mice were treated with either a control ASO or a 3R to 4R tau splicing ASO to identify if altering the ratio of tau isoforms in

astrocytes alone would lead to similar morphologic and mRNA changes as seen in vivo. We identified a similar morphological change in the hTau astrocytes treated with the 3R to 4R tau splicing ASO as previously seen in vivo, with brighter GFAP staining and the processes retracting towards the soma (**Figure 2.2A-H**). hTau astrocytes treated with the 3R to 4R tau splicing ASO exhibited a significant increase in genes associated with a pan-reactive and neurotoxic genetic profile and again no change in genes associated with a neuroprotective phenotype as compared to astrocytes treated with the control ASO (**Figure 2.2I**). As expected, mTau-/- and C57BL/6 astrocytes treated with either the control ASO or the 3R to 4R tau splicing ASO showed no significant changes in any of the genes profiled (Supplemental Figure 2.2A and B). Additionally, when hTau astrocytes were treated with the 4R to 3R tau splicing ASO, they exhibited no change in mRNA gene expression as compared to those treated with the control ASO (**Figure 2.2I**). Together with our in vivo findings, these in vitro genetic profiles suggest astrocytes exhibiting increased 4R tau may bias toward a reactive or neurotoxic phenotype.

4R tau expression in astrocytes hinders the homeostatic function

Given the changes in astrocytic gene signatures, we tested if astrocyte functionality was altered following an increase in the 4R:3R tau ratio. As astrocytes are responsible for taking up excess glutamate at the synapse in order to prevent excitotoxicity, we tested whether an increased amount of 4R tau in astrocytes could lead to a decrease in glutamate uptake ability. We found that hTau astrocytes treated with the 3R to 4R tau splicing ASO exhibited a significantly reduced ability to take up glutamate from the cellular media (**Figure 2.2J**) as compared to the hTau

astrocytes treated with the control ASO. mTau-/- astrocytes treated with either the control or the 3R to 4R tau splicing ASO exhibited no change in glutamate uptake ability (Supplemental Figure 2.3B). We then treated the hTau astrocytes with a 4R to 3R tau splicing ASO and identified that when 4R tau levels were lowered in hTau astrocytes, the amount of glutamate that remained in the media was equivalent to those that were treated with the control ASO (**Figure 2.2J**).

In many neurodegenerative diseases, the loss of astrocytic ability to protect neurons from oxidative damage leads to the progression of disease (Hallmann et al. 2017). Therefore, we examined whether altering the ratio of tau isoforms altered the vulnerability of astrocytes to oxidative stress. Following exposure to hydrogen peroxide, a higher level of lactate dehydrogenase (LDH) release was detected in both hTau and mTau-/- astrocytes regardless of treatment, indicative of cell death (**Figure 2.2K** and Supplemental Figure 2.3B). However, hTau astrocytes treated with the 3R to 4R tau splicing ASO exhibited significantly higher levels of LDH release compared to hTau astrocytes treated with the control ASO (**Figure 2.2K**). When the hTau astrocytes were treated with the 4R to 3R tau splicing ASO, they appeared to be less vulnerable to oxidative stress as the levels of LDH release were lower than those that were treated with a control ASO or the 3R to 4R tau splicing ASO (**Figure 2.2K**). Taken together, these data suggest that increased 4R tau levels in astrocytes are sufficient to cause functional deficiencies and that by lowering the levels of 4R tau, we may be able to prevent the loss of homeostatic function.

Neurons cultured with 4R tau expressing astrocytes are more susceptible to death

We next wanted to identify if primary neurons that were co-cultured with 4R tau expressing astrocytes exhibited higher levels of cell death, given that 4R astrocytes were more vulnerable to oxidative stress. We co-cultured WT primary neurons with hTau astrocytes that were treated with either a control ASO, the 3R to 4R tau splicing ASO, or the 4R to 3R tau splicing ASO in order to identify if 4R tau expression in astrocytes impacted neuronal survival. Neurons that were co-cultured with hTau astrocytes treated with either the control ASO, 3R to 4R tau splicing ASO or the 4R to 3R tau splicing ASO showed no decrease in the percent of MAP2 staining (**Figure 2.3A- C, G**). These data suggest that at when the system is not exposed to outside stressors, there is no change in neuronal survival.

Having identified that primary neurons were more likely to die when cultured with 4R tau expressing astrocytes at baseline, we wanted to examine if this would occur when the system was stressed, as commonly occurs in neurodegenerative disease. Here, co-cultured the cells as before, but prior to fixation, we added 100μM of H_2O_2 for 24 hours. While we saw an expected decrease in MAP2 staining in all conditions (**Figure 2.3D-F, H**), there was no change in the amount of MAP2 staining when the astrocytes were treated with either the control ASO or the 4R to 3R tau splicing ASO (**Figure 2.3D, F, H**). However, when neurons were co-cultured with 4R tau expressing astrocytes and exposed to additional stress, there was significantly less MAP2 staining than in the other two conditions (**Figure 2.3E, H**). These data suggest that the increased vulnerability to oxidative stress in 4R tau expressing astrocytes leads to an increase in neuronal death and may be responsible for neurodegenerative disease progression.

Dysfunctional 4R tau expressing astrocytes may be activated by the STAT3 pathway

Having seen that 4R tau expression in astrocytes leads to disruption of their homeostatic function, and that following exposure to oxidative stress, neurons that are co-cultured with 4R tau expressing astrocytes are more prone to death; we wanted to identify a potential pathway that led to astrocyte activation. We found that hTau astrocytes had significantly decreased protein expression levels of Eaat2 than those treated with either the control ASO or the 4R to 3R tau splicing ASO (**Figure 2.4A and B**). The decreased levels of Eaat2 support the decreased ability of the astrocytes to recycle glutamate from the media.

It has been shown that decreased levels of Eaat2 may be due to activation of the Stat3 pathway, therefore, we wanted to identify if this was the case in the 4R tau expressing hTau astrocytes. We found that increased levels of 4R tau led to a slight but not significant increase in Stat3 levels as compared to those treated with the control ASO and the 4R to 3R tau splicing ASO (**Figure 2.4C and D**). These findings provide a potential mechanism of astrocyte toxicity following increased expression of 4R tau.

Discussion

The source and pathogenic mechanisms of tau in neurodegenerative diseases are primarily attributed to neurons despite concurrent tau accumulation in glial cells. Here, we demonstrate that even low levels of tau, specifically 4R tau, in astrocytes are sufficient to disrupt normal astrocytic function and may contribute to neuronal dysfunction in disease. Primary astrocytes with increased 4R tau exhibited reactive gene expression, reduced ability to take up glutamate, and increased sensitivity to oxidative stress, which were all mitigated by splicing-mediated 4R tau reduction. This suggests that increased expression of 4R tau by astrocytes causes a reactive astrocytic phenotype and is detrimental to their ability to perform homeostatic functions.

Tau pathology is most commonly associated with neurons, but astrocytes also exhibit pathologic tau deposition in many primary tauopathies (Reid et al. 2020), even in the absence of detectable neuronal tau pathology (Higuchi et al. 2002; He et al. 2020; Narasimhan et al. 2020). Tau deposition within astrocytes often serves as a defining feature for several primary tauopathies, including progressive supranuclear palsy, corticobasal degeneration, and argyrophilic grain disease, aiding in their diagnoses (Reid et al. 2020; Rösler et al. 2019). Selective deposition of tau isoforms within neurons traditionally confers the 3R or 4R tauopathy classification. However, astrocytes tend to exhibit higher 4R tau deposition, even in 3R tauopathies such as Pick's disease (Hogg et al. 2003; Josephs 2017), suggesting that a component of astrocytic biology may predispose them to express or deposit more 4R tau. Aging-related tau astrogliopathy (ARTAG) has been used to classify diseases where tau deposition occurs in astrocytes throughout the brain (Kovacs et al. 2016), and many primary tauopathies exhibit ARTAG-related astrocyte morphologies (Kovacs et al. 2018). While there is tau deposition in astrocytes in

primary tauopathies, the source of astrocytic tau – whether endogenous to astrocytes or taken up from neighboring neurons or oligodendrocytes (Narasimhan et al. 2020)– is unclear. We show that in astrocytes isolated from hTau mice, altered endogenous tau isoform levels promote reactive, dysfunctional astrocytes, which may predispose tau to deposit (Schneider et al. 1999; Alonso et al. 2001; DuBoff, Götz, and Feany 2012). Prior studies in our lab have shown that an ASO-mediated increase in 4R tau in hTau mice leads to tau phosphorylation and aggregation (Schoch et al. 2016) although we did not attribute the tau pathology to neurons versus astrocytes. Our in vitro studies here have allowed us to investigate the role of tau expression in astrocytes separate from neurons; however, we cannot exclude a neuronal contribution for pathological tau in astrocytes, as it has been well documented that astrocytes may take up various forms of extracellular tau and contribute to tau spread (He et al. 2020; Narasimhan et al. 2020; Perea et al. 2019; Holmes et al. 2014; Leyns and Holtzman 2017). Once tau is internalized, it causes a disruption to normal astrocytic functions including calcium signaling and gliotransmitter release (Kahlson and Colodner 2015) as well as impaired glutamate clearance (Maragakis and Rothstein 2001; Dabir et al. 2006). Future studies will be needed to probe cell type-specific and overlapping mechanisms of tau pathology in neurodegenerative disease.

Previously published studies have relied on tau overexpression to assess tau isoforms in astrocytes (Richetin et al. 2020; Forman et al. 2005). 4R tau-expressing astrocytes isolated from P301S mice were less able to support neurons in vitro (Sidoryk-Węgrzynowicz and Struzyńska 2019). Additionally, in a fruit fly model of tauopathy, 4R tau expression led to greater neurodegeneration and impairment in learning in memory (Sealey et al. 2017). In a separate study, 3R tau overexpression in astrocytes was sufficient to cause memory deficits and neuronal

dysfunction in mice (Richetin et al. 2020). These studies are difficult to interpret in the context of human disease as overexpression of normal tau is linked to the accumulation of filamentous actin, formation of tau tangles, and neurodegeneration (Y. Wang and Mandelkow 2016; Alonso et al. 2001; Stoothoff and Johnson 2005; Grundke-Iqbal and Iqbal 1999; Iqbal et al. 2010). Here, we chose to alter the ratios of endogenously expressed physiological levels of astrocytic tau. The tau splicing ASOs used do not alter total tau levels but instead altered the ratio of 3R tau to 4R tau (Schoch et al. 2016) by interfering with the alternative splicing of exon 10. Our data suggest that 4R tau expression in astrocytes promotes a reactive astrocytic phenotype and leads to the loss of their ability to protect neurons, placing tau in astrocytes as a potential contributor to tau-related neurodegenerative disease mechanisms.

While the role that astrocytes play in neurodegenerative disease progression is still not well understood, astrocytes are responsible for maintaining neuronal health and homeostasis under physiological settings (Yonghee Kim, Park, and Choi 2019). Therefore, when astrocyte function is disrupted, as occurs in Alzheimer's disease, neurons may be less protected from toxic proteins, stress, or inflammation. The loss of glutamate buffering ability in reactive astrocytes may lead to Alzheimer's disease progression (Gómez-Gonzalo et al. 2017; K. Li et al. 2019; Acosta, Anderson, and Anderson 2017). In disease, astrocytes may also begin to secrete pro-inflammatory cytokines that can contribute to disease progression (Liddelow et al. 2017) and may exhibit upregulation of neurotoxic genes. Astrocytes in disease also exhibit increased sensitivity to oxidative damage (Hallmann et al. 2017) and impaired glutamate buffering ability at the synapse (Acosta, Anderson, and Anderson 2017; Phatnani and Maniatis 2015). Therefore, dysfunctional astrocytes, possibly resulting from tau accumulation, may contribute to disease

progression.

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Figures:

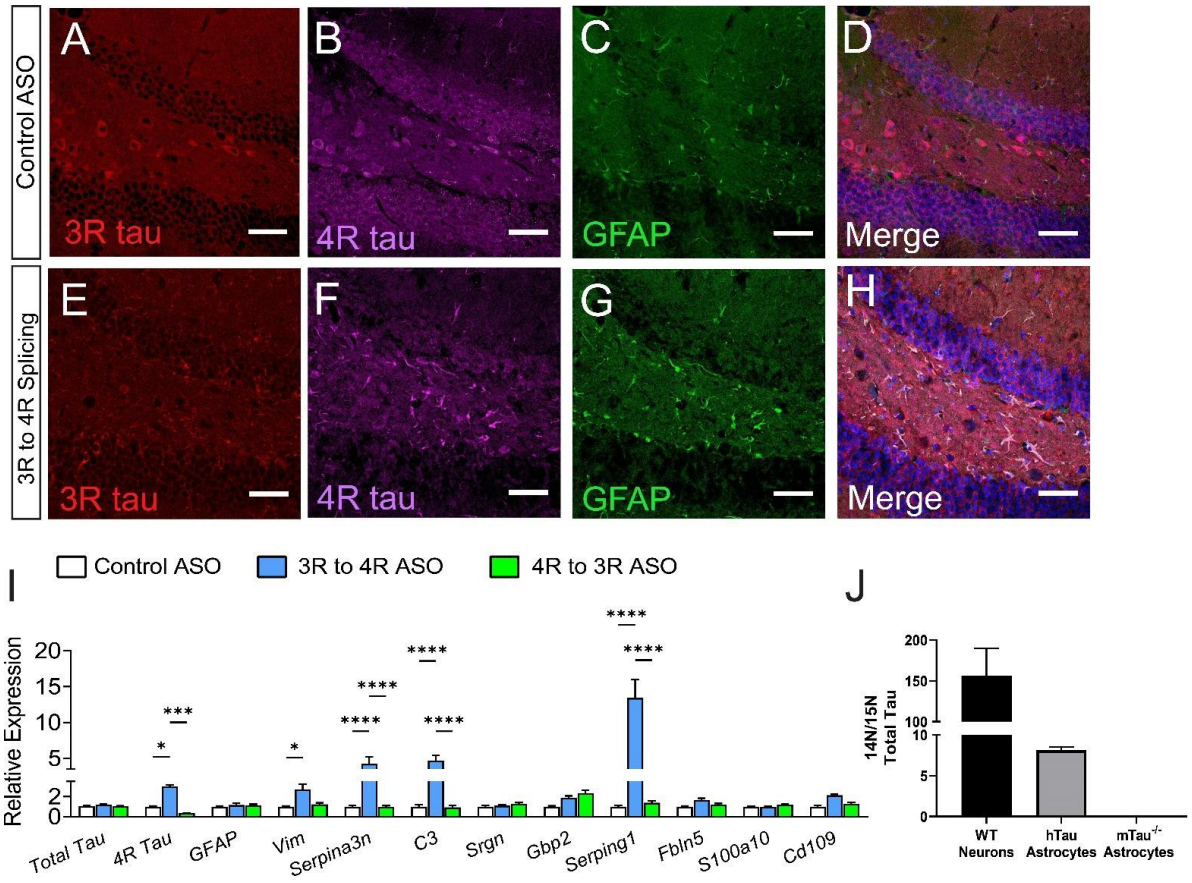


Figure 2.1: 4R tau expression in astrocytes leads to a reactive phenotype *in vivo*.

A and E) 3R tau,**B and F)** 4R tau, **C and G)** GFAP, **D, and H**) merged representative images of the dentate gyrus of thehippocampus, contralateral to ASO injection in hTau mice treated with control ASO or 3R to 4R tau splicing ASO. Scale bar = 50µm. **I**) Select gene expression in hTau mouse brain lysates after control, 3R to 4R tau splicing, or 4R to 3R tau splicing ASO treatment measured by qRT-PCR. Data are normalized to *GAPDH* relative to control ASO levels and shown as mean + SEM; two-way ANOVA withTukey's multiple comparisons; n=3-5 mice, *p<0.05, ***p<0.001, ****p<.0001. **J)** Total tau protein wasmeasured in primary C57BL/6 neuron cell lysates, hTau astrocyte cell lysates, and mTau-/- astrocyte cell lysates by mass spectrometry. n=3 wells/group. Mean + SEM.

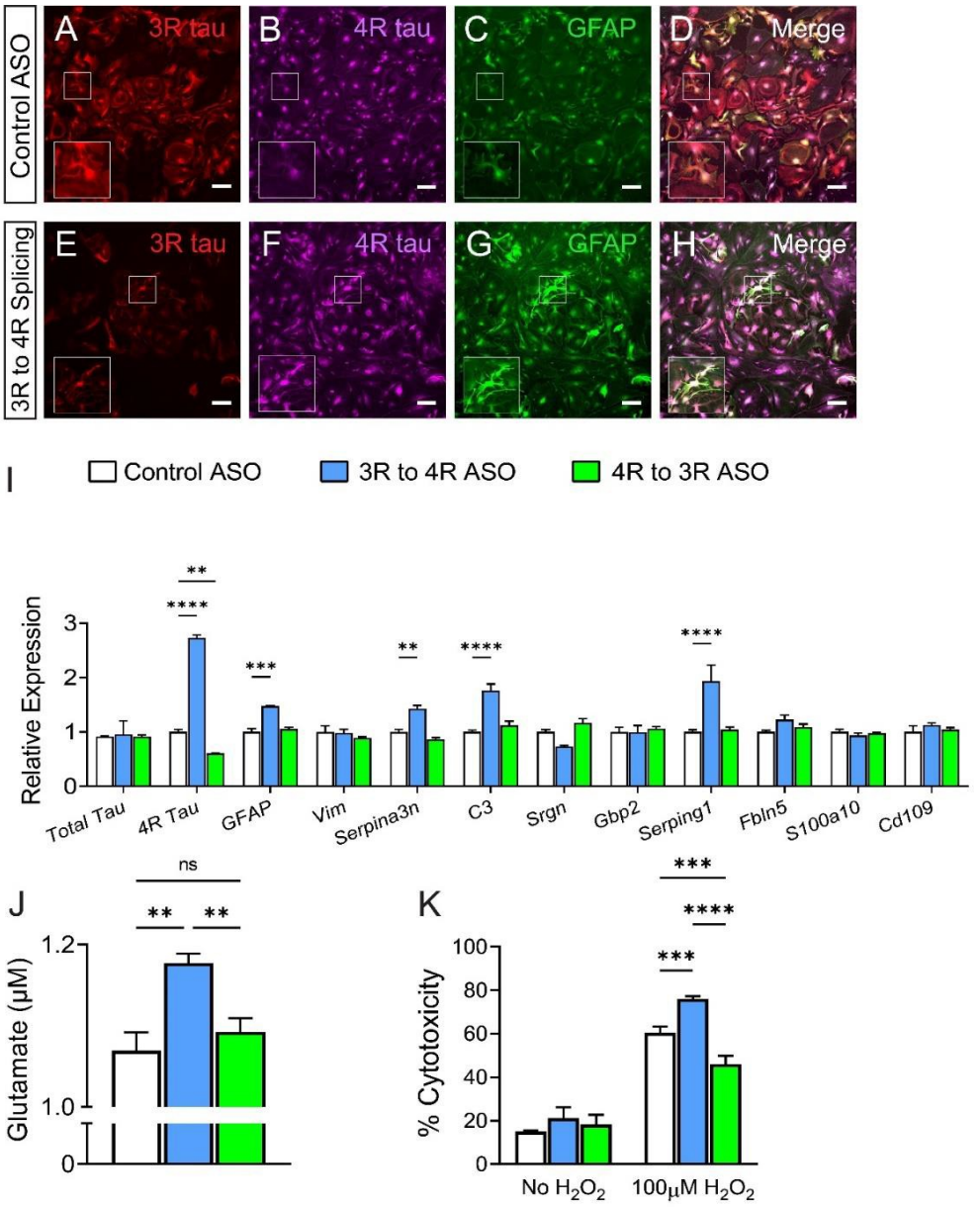


Figure 2.2: Increased 4R tau in primary hTau astrocytes induces a neurotoxic genetic signature and leads to dysfunction.

A and E) 3R tau, **B and F)** 4R tau, **C and G)** GFAP, **D and H**) merge immunofluorescence images from cultured hTau astrocytes treated with control ASO or 3R to 4R tau splicing ASO. Scale bar = 200μM. **I**) Select gene expression in cultured primary hTau astrocytes after control, 3R to 4R tau splicing, or 4R to 3R tau splicing ASO treatment measured by qRT-PCR. Data are normalized to *GAPDH* relative to control ASO levels and shown as mean + SEM; two-way ANOVAwith Tukey's multiple comparisons; n=3-5 wells, *p<0.05, **p<0.01, ***p<0.001, ****p<.0001. **J**) Glutamate measured in cellular media after control, 3R to 4R tau splicing, or 4R to 3R tau splicing ASOtreatment in hTau astrocytes. Data are mean + SEM; n=6 wells/treatment; two-way ANOVA with Tukey's multiple comparisons; **p<0.01, ns = not significant. **K**) Cytotoxicity (measured by LDH release) in control, 3R to 4R tau splicing or 4R

to 3R splicing ASO treated hTau astrocytes at baselineor following 100μM H_2O_2 treatment. Data are mean + SEM; n=6 wells/treatment; two-way ANOVA withTukey's multiple comparisons; ***p<0.001, ****p<0.0001.

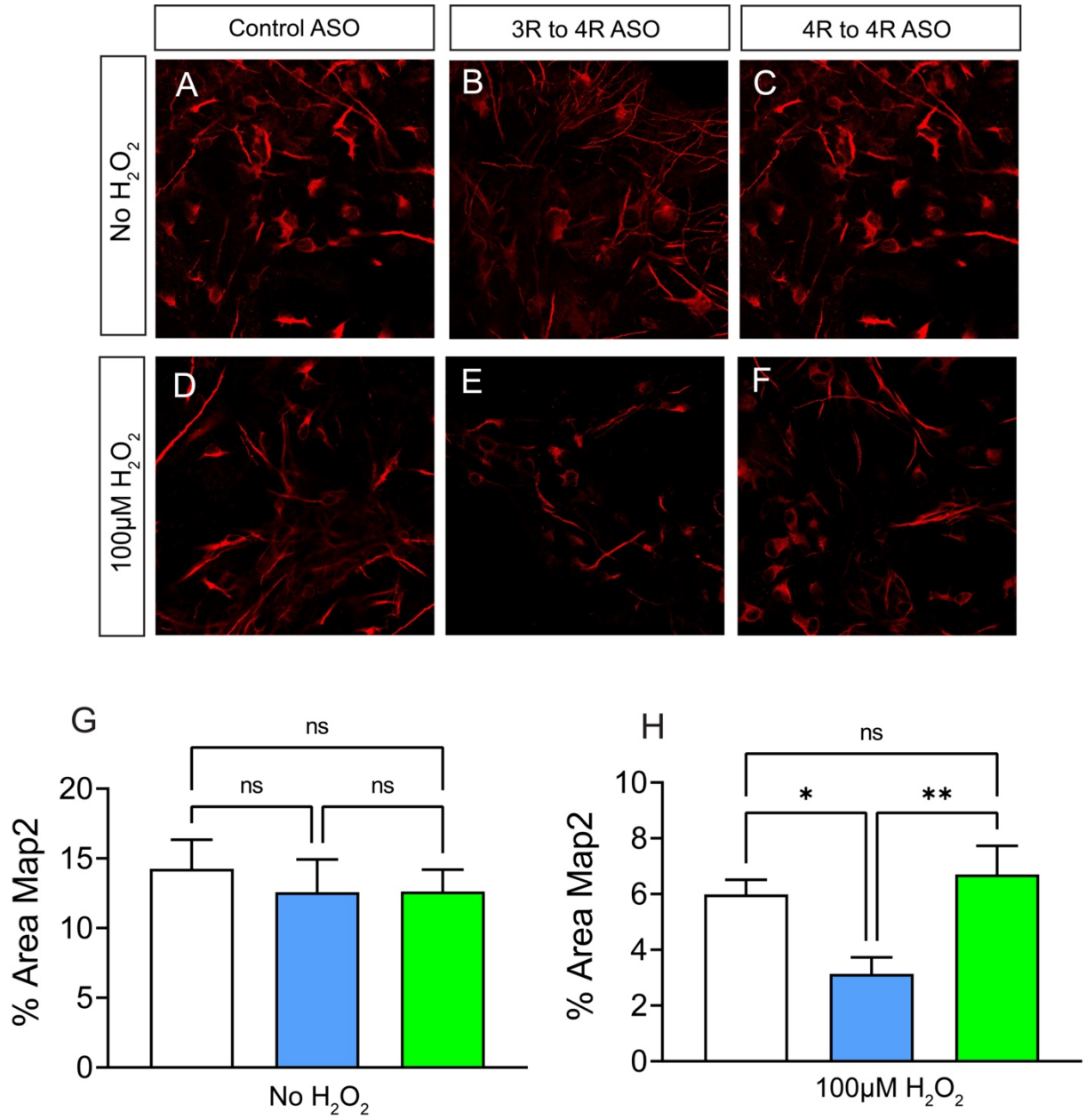


Figure 2.3: Neurons cultured with 4R tau expressing astrocytes are more prone to death following oxidative stress.

Representative images of *MAP2* staining in primary cortical neurons that were co-cultured with **A)** hTau astrocytes treated with a control ASO and not stressed with 100µM of H_2O_2, **B)** hTau astrocytes treated with a 3R to 4R tau splicing ASO and not stressed with 100µM of H_2O_2, **C)** hTau astrocytes treated with a 4R to 3R tau splicing ASO and not stressed with 100µM of H_2O_2, **D)** hTau astrocytes treated with a control ASO and stressed with 100µM of H_2O_2 **E)** hTau astrocytes treated with a 3R to 4R tau splicing ASO and stressed with 100µM of H_2O_2, **F)** hTau astrocytes treated with a 4R to 3R tau splicing ASO and stressed with 100µM of H_2O_2, **G)**

quantification of % *MAP2* area covered in co-cultures that were not exposed to H_2O_2. **H)** quantification of % *MAP2* area covered in co-cultures that were exposed to H_2O_2. Data shown as mean + SEM, n= 6 wells per treatment and 10 images per well; *p<.05, **p<.01, ns = not significant by one-way ANOVA with multiple corrections.

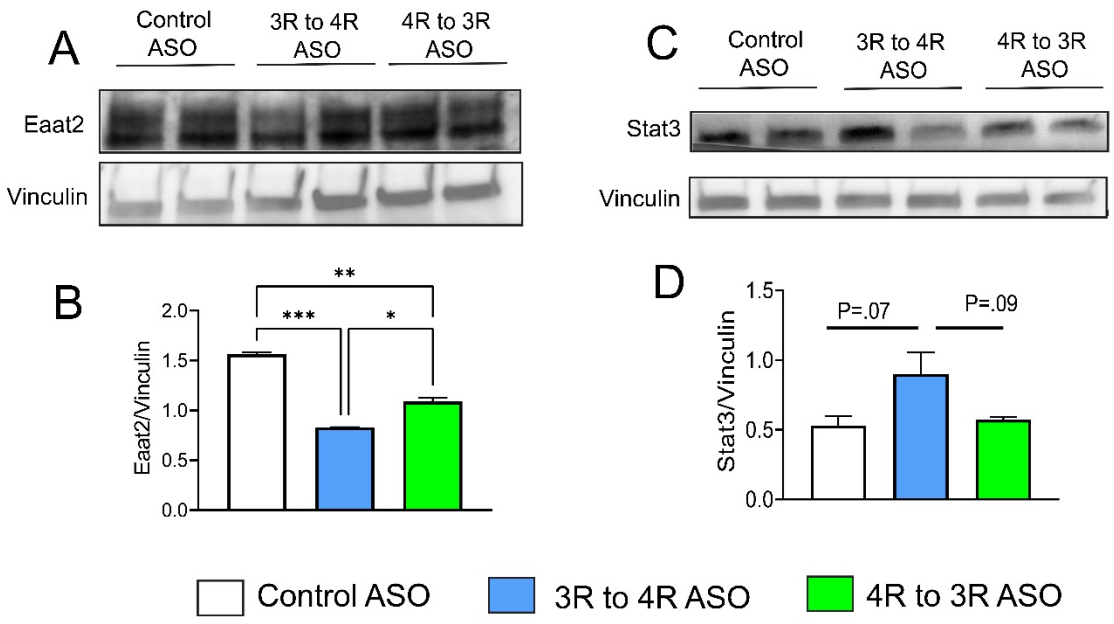
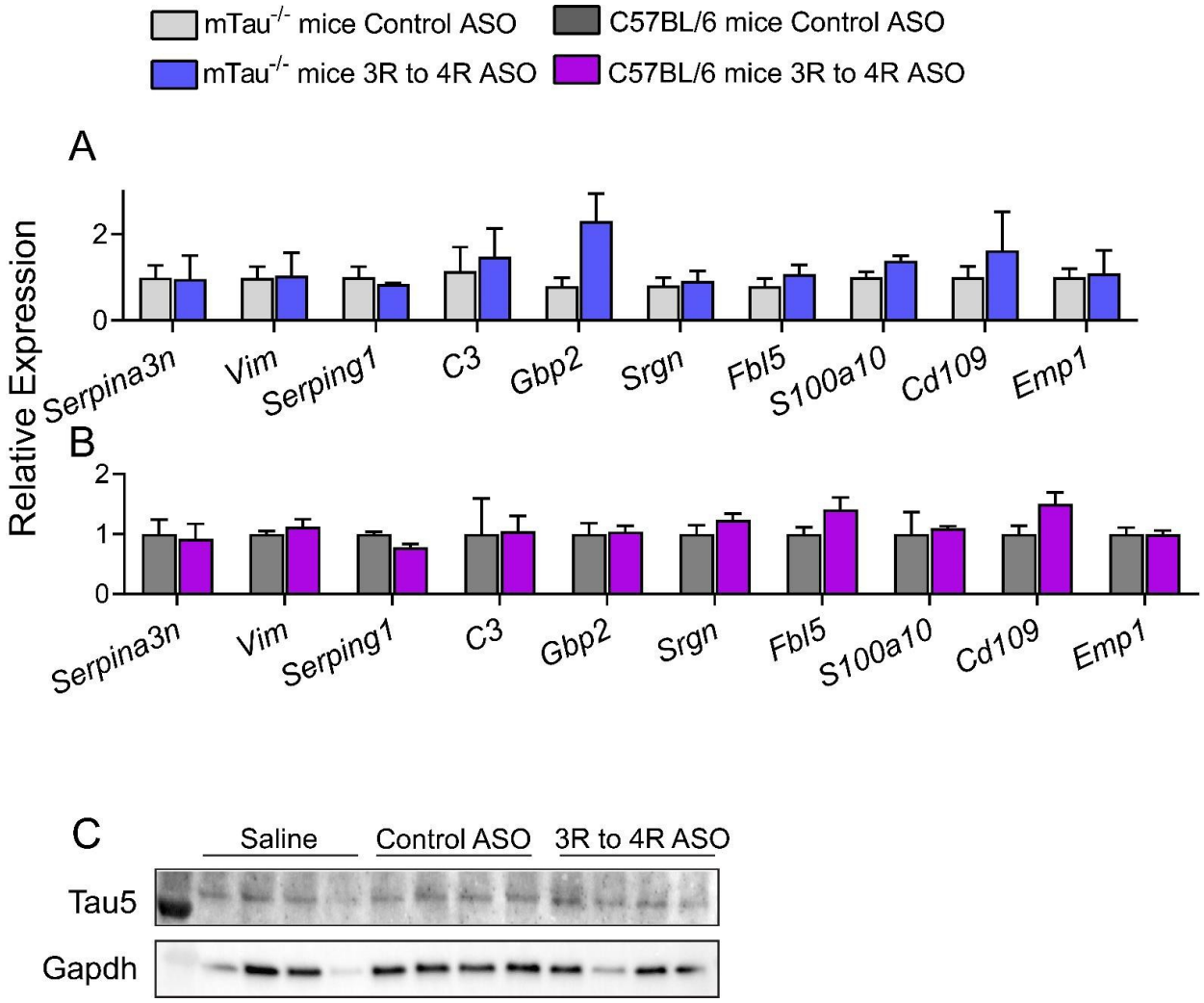


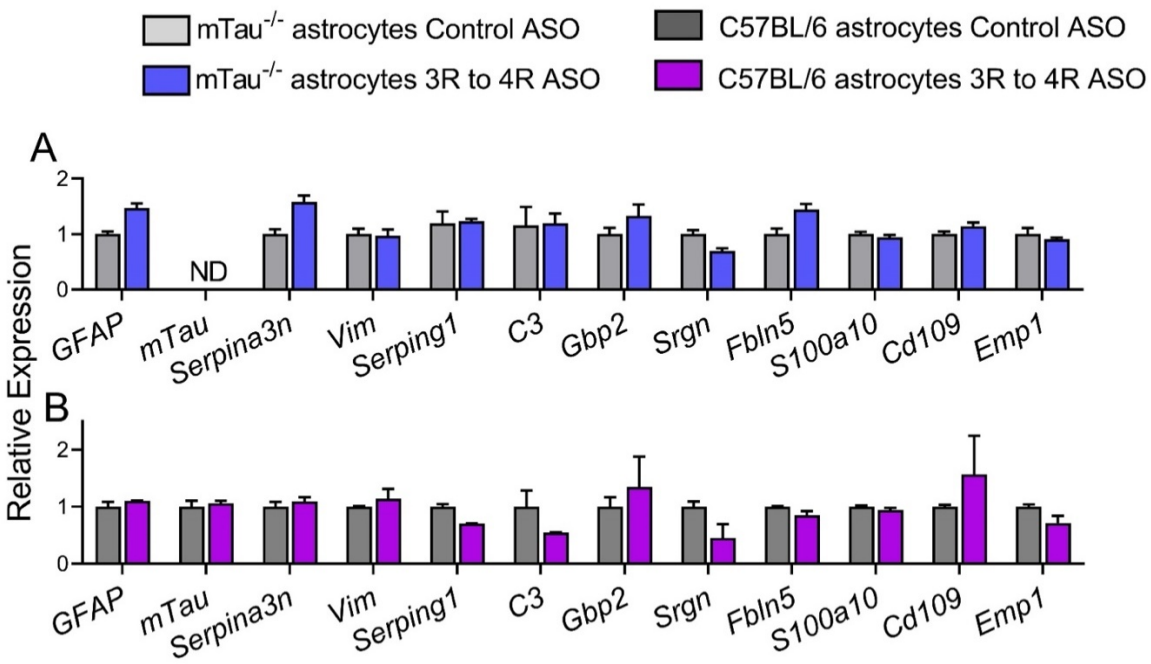
Figure 2.4: The Stat3 pathway may be involved in 4R tau astrocyte dysfunction.

A) Western blots of hTau astrocytes treated with the control ASO, 3R to 4R tau splicing ASO or the 4R to 3R tau splicing ASO and probed for Eaat2 and vinculin. **B**) Quantification of Eaat2 levels. **C**) Western blots of hTau astrocytes treated with the control ASO, 3R to 4R tau splicing ASO or the 4R to 3R tau splicing ASO and probed for Stat3 and vinculin. **D)** Quantification of Stat3 levels. Data are shown as mean + SEM, n=6 per treatment; *p<.05, **p<.01, ***p<.001, ns = not significant by one way ANOVA with multiple corrections.



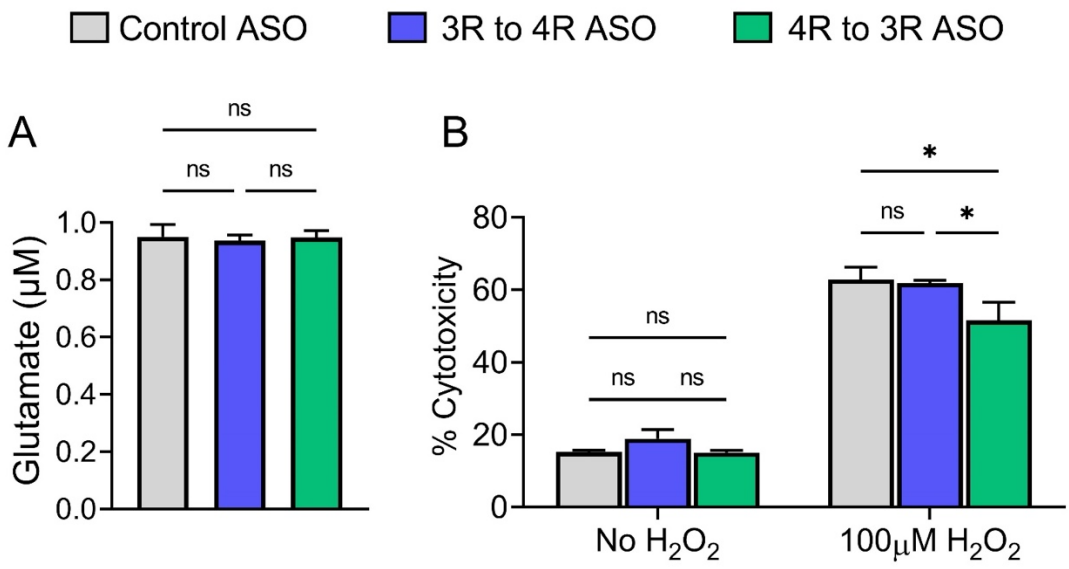
Supplemental Figure 2.1S: ASO-mediated increase in 4R tau does not alter select mRNA levels in control mice.

Astrocyte-associated gene markers in tissue from **A**) mTau$^{-/-}$ mice and **B**) C57 mice were measured by qRT-PCR, normalized to *GAPDH*, and expressed relative to control ASO levels. Data are mean + SEM. n=3-5/treatment; one way ANOVA with multiple comparisons. **C**) Western blot for total tau of primary hTau astrocytes treated with saline, the Control ASO, or 3R to 4R tau splicing ASO for 12 days.



Supplemental Figure 2.2S: ASO-mediated increase in 4R tau does not alter select mRNA levels in primary astrocyte cultures.

Astrocyte-associated gene markers from **A**) mTau$^{-/-}$ astrocytes and **B**) C57 astrocytes were measured by qRT-PCR, normalized to *GAPDH*, and expressed relative to controlASO levels. Data are mean + SEM; n=3-5/treatment; one way ANOVA with multiple comparisons. ND = not determined (cycle threshold values greater than 35).



Supplemental Figure 2.3S: **Glutamate uptake and cytotoxicity in mTau-/- astrocytes following ASO treatment.**

A) Glutamate concentration measured in cellular media after control, 3R to 4R tausplicing, or 4R to 3R tau splicing ASO treatment in mTau$^{-/-}$ astrocytes. Data are mean + SEM; n=6 wells/treatment; one-way ANOVA with Tukey's multiple comparisons; ns = not significant. **B**) Cytotoxicity (measured by LDH release) in control, 3R to 4R tau splicing, or 4R to 3R splicing ASO treated mTau$^{-/-}$ astrocytes at baseline and following 100µM H2O2 treatment. Data are mean + SEM;n=6 wells/treatment; two-way ANOVA with Tukey's multiple comparisons; *p<0.05, ns = not significant.

Gene Name	Species	Reagents	Sequence (5' to 3') or Assay ID
Serpina3n	M	Sybr	F: CAGATCCCAGCCATCAAGAG
			R: CTGGCAGCTGGCTGGTTT
S100a10	M	Taqman	Mm.PT.58.6571055 NM_009112
Cd109	M	Taqman	Mm.PT.58.6710335 NM_153098
Emp1	M	Taqman	Mm.PT.58.5886962 NM_010128
4R Tau	H	Sybr	F: GACTGGACGTTGCTAAGATC
			R: CATGCCAGACCTGAAGAATG
4R Tau Probe	H	Taqman	56-FAM/CCACTGAGAACCTGAAGCACCAGC/3IABkFQ
Total Tau	H	Sybr	F: AGAAGCAGGCATTGGAGAC
			R:TCTTCGTTTTACCATCAGCC
Total Tau Probe	H	Taqman	56-FAM/ACGGGACTGGAAGCGATGACAAAA/3IABkFQ
Gapdh	M	Sybr	F: TGCCCCCATGTTGTGATG
			R: TGTGGTCATGAGCCCTTCC
Gapdh Probe	M	Taqman	56- FAM/AATGCATCCTGCACCACCAACTGCTT/3IABkFQ
GFAP	M	Sybr	F: ACCGCATCACCATTCCTGTAC
			R: TGGCCTTCTGACACGGATTT
GFAP Probe	M	Taqman	56-FAM/TCCAGATCCGAGAAACCAGCCT/3IABkFQ
Fbln5	M	Taqman	Mm.PT.58.29865771 NM_011812
Serping1	M	Taqman	Mm.PT.58.30811631 NM_009776
Srgn	M	Taqman	Mm.PT.58.41483771 NM_011157
C3	M	Taqman	Mm.PT.58.17325540 NM_009778
Timp1	M	Sybr	F: AAGGTGGTCTCGTTGATTCG
			R: ATCTGGCATCCTCTTGTTGC
Vim	M	Sybr	F: TCCACTTTCCGTTCAAGGTC
			R: AGAGAGAGGAAGCCGAAAGC
mTau F	M	Sybr	F: GAACCACCAAAATCCGGAGA
			R: CTCTTACTAGCTGATGGTGAC
mTau Probe	M	Taqman	56-FAM/CCAAGAAGGTGGCAGTGGTCC/3IABkFQ

Supplemental Table 2.1S: Sequences of primers used.

Names and sequences of all primers and reagents used for qRT-PCR analysis. F, forward; R, reverse; M, Mouse; H, Human.

Chapter 3

4R tau expression in human astrocytes leads to dysfunction and neuronal death

Adapted from a manuscript that is under revision at JCI insight:

Lubov A. Ezerskiy, Kathleen M. Schoch, Chihiro Sato, Mariana Beltcheva, Frank Rigo, Ryan Martynowicz, Celeste M. Karch, Randall J. Bateman, Timothy M. Miller. *Astrocytic 4R tau expression drives astrocyte reactivity and dysfunction.*

Summary

In many neurodegenerative diseases, tau becomes abnormally hyperphosphorylated and insoluble, leading to its aggregation. Several primary tauopathies exhibit higher deposition of 4R tau; however, the mechanism of 4R tau toxicity remains unclear. Following tau splicing manipulation toward higher 4R tau levels in mice, seizure severity and phosphorylated tau deposition were increased, yet no neuronal abnormalities were observed. Astrocytes, which commonly exhibit pathologic tau deposition despite exhibiting lower levels of tau, were then considered. We created astrocytes from IVS 10+16 *MAPT* mutation carriers and their isogenic controls to study the impact of increased levels of 4R tau on human astrocyte function. We identified that 4R tau expressing iPSC derived astrocytes expressed higher levels of neurotoxic and pan-reactive genes, had a decreased ability to buffer glutamate and were more susceptible to oxidative stress. Additionally, healthy neurons cultured with 4R tau-expressing human iPSC-derived astrocytes exhibited a higher firing frequency and hyper-synchrony and death, which could be prevented by lowering tau expression. These results show that 4R expression in astrocytes is sufficient to cause neuronal hyper-activity and death potentially leading to the progression of neurodegenerative disease.

Introduction

The deposition of the protein tau is a hallmark pathology of a group of neurodegenerative diseases known as tauopathies (Kopach et al. 2021). These diseases include Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD)—such as corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease, globular glial tauopathy (GGT), aging-related tau astrogliopathy (ARTAG), and others (Pîrşcoveanu et al. 2017; Tudorică et al. 2017; Iqbal et al. 2010). Mutations within the gene MAPT have been shown to cause tau hyperphosphorylation, making it more prone to aggregate into the neurofibrillary tangles seen in disease (Forrest, Kril, and Halliday 2019; Nisbet et al. 2015; Iovino et al. 2015).

There are six isoforms of tau expressed in the adult human brain. They are created through the alternative splicing of exons 2, 3, and 10 found in MAPT (M. Goedert and Jakes 1990; Connell et al. 2005; McMillan et al. 2008) and are expressed at different times throughout development (C. Liu and Götz 2013; Arendt, Stieler, and Holzer 2016; Bachmann et al. 2021). 3R and 4R tau are created through the exclusion or inclusion of exon 10 respectively (M. Goedert et al. 1989), with 3R tau being expressed in the fetal brain and both isoforms being expressed at an approximate 1:1 ratio in the adult brain (Kenneth S. Kosik et al. 1989). The majority of disease-causing MAPT mutations are found in and around exon 10 and lead to the inclusion of exon 10, creating higher levels of 4R tau (Dawson et al. 2007).

Induced pluripotent stem cell (iPSC) models are a powerful tool that can be used to study how particular mutations impact disease progression in a human cell model that gives a more

representative picture of what happens in human disease (D'Aiuto et al. 2014; Karch et al. 2019). Cells derived from patients that have the IVS 10+16 MAPT mutation are unlike other iPSC derived cells because they express 4R tau directly after differentiation (Verheyen et al. 2018), making them an ideal line to identify the impact of 4R tau without needing to overexpress 4R tau or use small molecules to alter the ratios of the isoforms.

We previously (Chapter 2) identified that 4R tau expression in primary astrocytes derived from human tau (hTau) expressing mice causes them to take on a neurotoxic phenotype and a loss of homeostatic function. Here, we examined the impact that 4R tau expression had on human astrocytes. 4R tau expressing human astrocytes exhibited similar increases in neurotoxic gene expression along with a decrease in homeostatic function as compared to their isogenic control. These were rescued when the levels of tau were lowered with a tau lowering ASO. We also found that iPSC-derived neurons that were co-cultured with 4R tau expressing astrocytes exhibited hyper-excitability and were more susceptible to death. This, too, was reversed when tau levels were reduced in the astrocytes. These data suggest that 4R tau expression in human astrocytes is toxic to neurons and can contribute to disease progression.

Materials and Methods

Antisense oligonucleotides (ASOs): All ASOs were designed with a phosphorothioate backbone and either uniform or "gapmer" 2'-O-methoxyethyl sugar ring modifications to achieve tau splicing or knockdown, respectively(DeVos et al. 2013; DeVos and Miller 2013). The fully modified design prevents target degradation, while the gapmer design, consisting of 10 central, unmodified nucleotides flanked by five modified nucleotides, enables RNaseH-mediated target degradation. An ASO control similar in structure and modifications but without target specificity was included in experimental treatments to account for potential toxicity or off-target effects. All ASOs were synthesized by Ionis Pharmaceuticals (Carlsbad, CA) and generously provided for use. Sequences are as follows: tau splicing control ASO (5'-TCATTTGCTTCTACAGG-3'), 4R to 3R tau splicing ASO (5'- GGACGTGTGAAGGTACTC-3'), tau knockdown control ASO (5'-GCTTTTACTGACCATGCGAG-3'), and tau knockdown ASO (5'–CCTTCCCTGAAGGTTCCTCC-3'). For in vitro experiments, ASOs were diluted to 10μM in astrocyte media and replaced every other day for 12 days.

iAstrocyte differentiation and culture: iPSCs (GIH-36 C2 (ISV10+16/WT) and GIH-36 C2 IVS10+16 1D01 (WT/WT)) (Karch et al. 2019) were provided for use by Dr. Celeste Karch at Washington University in St. Louis. The cells were cultured in neural induction media for 2 weeks to promote a neural lineage. The neural progenitor cells were then cultured for 30 days in astrocyte media as per established protocol (TCW et al. 2017) in the Human Cells, Tissues, and Organoid Core at Washington University in St. Louis. iAstrocytes were used for experiments after 60 days in culture.

Co-culture of iAstrocytes and iPSC-derived neurons: iAstrocytes were cultured as described above and treated with ASOs for 12 days prior to co-culture with iPSC-derived mixed cortical neurons (BrainXell Cat #BX-0500). The neurons were plated at a 5:1 ratio to iAstrocytes in seeding media supplemented with the astrocyte supplement (BrainXell Cat #BX-2600). The following day, a half media change was done using the Day 1 media. On day 4, an additional half change of media was done, and this media was supplemented with Day 4 supplement (BrainXell). From day 7 onwards, a half media change was done every 3-4 days. Media composition is listed in supplementary table 3.3. After 12 days of culture, the cells were treated with media supplemented with either saline or 100µM H_2O_2 for 24 hours prior to fixation and immunocytochemical analysis.

Immunocytochemical staining: Co-cultures were fixed using 4% paraformaldehyde (Santa Cruz Biotechnology Cat # sc-281692, Dallas, TX) and permeabilized using 0.1% Triton-X-100 in PBS. The cells were then incubated in blocking buffer (0.5% BSA diluted in PBS). The cells were incubated in primary antibody (MAP2 1:1000, Abcam Cat #ab5392; GFAP 1:1000, Millipore-Sigma Cat #AB5541; Total Tau 1:1000 Cat #A0024) at 4C overnight. The next day, cells were incubated with fluorescently tagged secondary antibodies (anti-mouse AlexaFluor 594, anti-rabbit AlexaFluor 647, anti-chicken AlexaFluor 488 all at 1:500). Coverslips were mounted and sealed with Fluoromount media (Southern Biotech, Birmingham, AL). The Nikon A1Rsi confocal microscope was used for acquiring images.

Quantitative real-time PCR: iAstrocytes were lysed using 500µL of QIAzol Lysis Reagent (Qiagen) and placed into Eppendorf tubes. Chloroform (100µL) was added to each sample,

shaken vigorously for 5 seconds, and let to sit for 3 minutes at room temperature. The samples were centrifuged for 15 minutes at 12,000 RCF at 4°C. The aqueous layer was removed and combined with 1.5 times volume of 100% ethanol and together added to the RNeasycolumn for RNA purification according to the manufacturer's protocol. cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA) and analyzed on theQuantStudio 12K Flex Real-Time PCR System using either the Power SYBR™ Green PCR Master Mix(ThermoFisher) the PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies, Coralville, IA). Expression levels were calculated by the ΔΔCt method, normalized to GAPDH and a biological reference sample. Primer sequences or identifiers (purchased from Integrated DNA Technologies) are detailed in Supplementary Table 1.

Glutamate uptake assay: Glutamate uptake was assessed using the Glutamate Assay Kit (abcam, Cambridge, United Kingdom) per the manufacturer's instructions. Briefly, primary murine astrocyte culturesand iAstrocytes were plated onto PDL-coated 24-well plates and treated with ASOs as described above.Cells were incubated in 100 μM glutamate in Live Cell Imaging Solution (ThermoFisher) for 2 hours at37□C. Following the incubation, 50μL of glutamate-containing media was combined with 50μL of the reaction buffer in a 96-well plate and incubated for 30 minutes at room temperature with shaking while protected from light. Sample absorbance was measured at 450nm on a plate reader, and a standard curve of known glutamate concentrations was used to quantify the concentration of glutamatewithin each sample. Samples were run in triplicate and averaged.

Cytotoxicity assay: Lactate dehydrogenase (LDH) release was quantified using the Pierce LDH

Cytotoxicity Assay Kit (ThermoFisher) following the manufacturer's instructions. Briefly, 24 hours prior to the assay, astrocyte media was supplemented with 0 or 100µM of hydrogen peroxide and added to the cells. The next day, the media was collected, and LDH activity was measured at 490nm and 680nm on a plate reader. The 680nm value was subtracted from the 490nm value to obtain the level of LDH release. Samples were run in triplicate and averaged.

Microelectrode assay (MEA): CytoView MEA Plates (Axion Biosystems, Atlanta, GA) were coated with 100µg/mL PDL in borate buffer (100mM Boric acid from Sigma Aldrich, St. Louis, MO and 75mM NaCl from Sigma Aldrich), washed in molecular grade sterile water, and dried prior to cell seeding. Mixed human cortical neurons (BrainXell, Madison, WI) were seeded with iAstrocytes at a 5:1 ratio in seeding media. On day 4, the seeding media was supplemented with the Day 4 supplement from BrainXell. On day 7, the media was replaced with Supplement C Treatment medium for 2 hours and then changed to maintenance medium. Media compositions are listed in Supplementary Table 2. Half medium changes were performed every 3 days until the end of the experiment. On DIV 21, the electrical activity of the cultures was measured on the Axion Biosystems Maestro 768D MEA system (Axion Biosystems). The cultures were equilibrated on the machine for 15 minutes at 37□C and 5% CO2. Recordings were taken for 20 minutes and repeated 3 times for each plate at a sampling rate of 12.5 kHz. Spikes were detected using a threshold set to 6 times the estimated standard deviation of the noise. Single-channel bursts were detected with a minimum of 5 spikes and a maximum of 100 ms inter-spike intervals. Network bursts were detected with a minimum of 50 spikes, maximum of 100 ms inter-spike intervals, and a minimum of 35% participating electrodes. Analysis of individual spikes and bursts was done using the neural metrics tool from Axion Biosystems.

Statistical analysis: All data were graphed as mean + SEM and analyzed using GraphPad Prism 9 statistical software (GraphPad Software, La Jolla, CA). Quantifiable mRNA in iPSC derived iAstrocytes treated with saline was evaluated by one-wayANOVA with Tukey's multiple comparisons post hoc analysis. mRNA from iPSC derived astrocytes treated with either the control ASO or the total tau knockdown ASO was evaluated by two-way ANOVA with Tukey's multiple comparisons. Glutamate uptake by iPSC derived astrocytes treated with saline was evaluated by students t-test and glutamate uptake by iPSC derived astrocytes treated with the control ASO or the total tau knockdown ASO was evaluated by one-way ANOVA with Tukey's multiple comparisons post hoc analysis. Levels of LDH release in iPSC derived astrocytes treated with saline, the control ASO or the total tau knockdown ASO was analyzed by two-way ANOVA with Tukey's multiple comparisons post hoc analysis. Percent of the viewing field covered by *MAP2* staining was analyzed using an ordinary one-way ANOVA with multiple corrections. Analysis of tau knockdown in iPSC cortical neurons that were co-cultured with iPSC derived astrocytes that were treated with tau knockdown ASO prior to co-culture was quantified using an ordinary one-way ANOVA with multiple comparisons. Effect of the previously developed 4R to 3R tau splicing ASO in iPSC derived astrocytes was analyzed using a two-way ANOVA with multiple comparisons. Mean frequency, number of spikes, spikes per burst, the burst duration and the number of bursts in iPSC cortical neuron and iPSC derived astrocyte MEA co-cultures treated with saline was analyzed using students t-test. Mean frequency, number of spikes, spikes per burst, the burst duration and the number of bursts in iPSC cortical neuron and iPSC derived astrocyte MEA co-cultures where the astrocytes were pre-treated with either the

control ASO or the total tau knockdown ASO were analyzed by one-way ANOVA with multiple corrections. A value of $p<0.05$ was deemed significant for all analyses.

Results

4R tau-expressing human astrocytes exhibit a neurotoxic phenotype and a disruption to homeostatic function

We used iPSC-derived astrocytes (iAstrocytes) from fibroblasts collected from patients with an IVS 10+16 MAPT mutation to investigate 4R tau-mediated effects independent of ASO interventions. These cells express 4R tau immediately following differentiation, making them an excellent model to study how increased 4R tau alone impacts cellular function (30). We confirmed increased 4R tau expression in IVS 10+16 iAstrocytes, which was not evident in the corrected isogenic iAstrocytes controls (**Figure 3.1A**). In addition, we identified that, at baseline, IVS 10+16 astrocytes expressed higher levels of pan-reactive and neurotoxic genes compared to isogenic control iAstrocytes (**Figure 3.1A**). The genes that were upregulated were similar to those identified in both hTau mice and primary hTau-expressing astrocytes treated with the 3R to 4R tau splicing ASO, providing further support for increased 4R tau leading to a neurotoxic astrocytic phenotype (Liddelow et al. 2017). When tested for functional responses, iAstrocytes derived from IVS 10+16 mutation carriers were significantly less able to take up glutamate from the media compared to their control astrocytes (**Figure 3.1B**). When we examined the iAstrocytes' ability to respond to oxidative stress, we found that IVS 10+16 astrocytes were more vulnerable at baseline, which was exacerbated upon hydrogen peroxide stress (**Figure 3.1C**). These results provide a human correlate for the neurotoxicity and loss of homeostatic function in astrocytes with increased 4R tau and suggest that tau in astrocytes may be an important player in neurodegenerative disease progression.

Human astrocytes expressing 4R tau increase neuronal excitability

We observed that 4R tau-expressing astrocytes were less able to uptake glutamate from the media, which we hypothesize may be conducive to neuronal hyper-excitability. To test this, we cultured mixed cortical iPSC-derived neurons with IVS 10+16 mutation carrier iAstrocytes or isogenic control iAstrocytes and measured spontaneous neuronal activity (**Figure 3.2A-B**). Neurons co-cultured with IVS 10+16 astrocytes exhibited a significantly higher firing rate (**Figure 3.2C**), more signals being sent (**Figure 3.2D**), a higher number of signals per burst (**Figure 3.2E**), longer burst duration (**Figure 3.2F**), and more bursts (**Figure 3.2G**) than those cultured with non-mutation iAstrocytes. The higher number of bursts in neurons co-cultured with 4R tau-expressing iAstrocytes implies that the neurons are firing in hyper-synchrony, while the increase in spike number and firing rate suggests that the neurons are firing more frequently when cultured with 4R tau-expressing iAstrocytes. These data suggest that when astrocytes express higher levels of 4R tau, neurons near those astrocytes demonstrate hyper-excitability and may potentiate neurotoxicity.

Lowering levels of total tau in 4R tau expressing iAstrocytes rescues human astrocyte function

Having identified that increased 4R tau expression is detrimental to homeostatic human astrocyte function, we hypothesized that lowering the amount of total tau expressed in iAstrocytes alone would also improve function. While specifically lowering 4R tau in these astrocytes would be ideal, the previously developed 4R to 3R tau splicing ASO was not effective in IVS 10+16 astrocytes, likely due to the additional secondary structure created by the

mutation that prevented ASO binding (Supplementary Figure 3.1S). Therefore, we used a total tau mRNA lowering approach (DeVos et al. 2017). We treated IVS 10+16 mutation iAstrocytes with a tau mRNA lowering ASO and measured gene expression, glutamate uptake ability, and response to oxidative stress. We found that following a reduction of total tau levels, select pan-reactive, and neurotoxic genes were significantly downregulated (**Figure 3.3A**), suggesting that the reactive phenotype was rescued with tau knockdown. We also identified that IVS 10+16 iAstrocytes with lowered total tau were better able to take up glutamate from the media (**Figure 3.3B**) as compared to those that were treated with the control ASO. Lowering total tau levels also allowed the iAstrocytes to resist hydrogen peroxide-induced oxidative stress, as exhibited by the decrease in cytotoxicity (**Figure 3.3C**). These data strongly suggest that, by lowering tau levels in astrocytes alone, we are able to prevent astrocyte reactivity and rescue their ability to perform homeostatic functions.

Lowering levels of total tau in 4R tau expressing iAstrocytes rescues neuronal excitability

We then investigated if lowering total tau would prevent astrocytic 4R tau-induced neuronal hyper-excitability. We pre-treated the iAstrocytes with either control or total tau lowering ASO prior to co-culturing with iPSC-derived neurons in which to measure spontaneous neuronal activity and found no change in tau levels in the neurons (Supplementary figure 3.2S) (**Figure 3.4A- D**). We found that neurons co-cultured with the IVS 10+16 iAstrocytes treated with total tau lowering ASO prevented increases in neuronal firing rate, number of signals being sent, number of bursts, signals per burst, and burst duration compared to control ASO-treated IVS 10+16 iAstrocytes (**Figure 3.4E-I**). We detected no change in neuronal firing when the isogenic iAstrocytes were treated with the total tau lowering ASO as compared to the isogenic

iAstrocytes treated with the control ASO (**Figure 3.4E-I**). These results suggest that the presence of 4R tau in astrocytes is sufficient to induce neuronal hyper-excitability, which can be rescued following tau lowering.

iPSC derived neurons co-cultured with 4R tau expressing astrocytes are more susceptible to death

Given that we identified a significant increase in neuronal hyper-excitability when neurons were co-cultured with 4R tau expressing astrocytes, we wanted to identify if this would translate to a higher amount of neuronal death. We again pre-treated iAstrocytes derived from an IVS 10+16 patient or their isogenic control with a control ASO or the tau lowering ASO prior to co-culturing them with iPSC derived cortical neurons. We found no reduction in the percent of MAP2 staining in neurons that were co-cultured with the isogenic control iAstrocytes that were either treated with the control ASO or the tau KD ASO at baseline (**Figure 3.5A, B, E**). We saw a significant decrease in the percent of MAP2 staining in neurons that were co-cultured with the IVS 10+16 iAstrocytes treated with the control ASO (**Figure 3.5C, E**). This decrease was reversed when neurons were co-cultured with the IVS 10+16 iAstrocytes were treated with the total tau lowering ASO (**Figure 3.5D, E**), suggesting that by lowering tau in the 4R tau expressing astrocytes, we are able to increase neuronal survival.

As we had previously seen that astrocytes that express higher levels of tau are more vulnerable to oxidative stress, we wanted to see if this vulnerability would lead to an increase in neuronal death. We stressed the co-cultures by adding 100μM of hydrogen peroxide for 24 hours. We found that, as expected, there was a decrease in the percent of MAP2 area coverage in the

cultures (**Figure 3.5F-J**). Interestingly, while the neurons co-cultured with the isogenic control astrocytes that were pre-treated with the control ASO showed a decrease in MAP2 area coverage (**Figure 3.5F, J**), those that were co-cultured with the isogenic control astrocytes had a significantly higher amount of MAP2 staining (**Figure 3.5G, J)**. The neurons that were co-cultured with the IVS 10+16 iAstrocytes treated with the control ASO had the lowest percent area covered by MAP2 staining (**Figure 3.5H, J**). This decrease was reversed when the iAstrocytes were pre-treated with the tau lowering ASO (**Figure 3.5I, J**). These results show that 4R tau expressing astrocytes are not only more vulnerable to oxidative stress but are actively neurotoxic and that lowering the levels of tau in these astrocytes is sufficient to reduce neuronal death.

Discussion

To date, the majority of studies on tauopathies have focused on the impact of neuronal tau on disease progression, as it is most highly expressed in neurons (Fleeman and Proctor 2021). Here we studied how human astrocytes derived from IVS 10+16 MAPT mutation carriers and their isogenic controls are impacted by 4R tau expression. These cells independently express elevated 4R tau (Verheyen et al. 2018). Additionally, by using iPSC-derived cells, we were able to more accurately model what happens in human disease (Ebert, Liang, and Wu 2012; Rowe and Daley 2019). We found that 4R tau expression led to a neurotoxic genetic profile and a decreased ability to provide homeostatic support. This was reversed with tau reduction. Further, neurons cultured with IVS 10+16 MAPT astrocytes exhibited hyper-excitability and were more prone to death, suggesting 4R tau in astrocytes may promote aberrant neuronal responses, which could also be mitigated by tau reduction.

Though astrocytes express tau at much lower levels than neurons (Zhang et al. 2016), astrocytic tau pathology is a common defining feature of many primary tauopathies, including PSP, CBD, ARTAG, and others (Kovacs 2020). It has been shown that tau deposition in astrocytes can cause loss of homeostatic function and overexpression of tau in astrocytes alone was sufficient to cause disease progression (Forman et al. 2005). Data have emerged showing that astrocytes are able to propagate tau pathology even in the absence of neuronal tau (Narasimhan et al. 2020). Additionally, overexpression of either 3R or 4R tau in astrocytes led to learning and memory deficits in mice (Richetin et al. 2020). While these experiments provided support for the role that astrocytic tau plays in disease progression, they relied on tau overexpression, which has been

shown to cause toxicity and increases in levels of phosphorylated tau along with learning and memory deficits (Bakhoum and Jackson 2011; Clark et al. 1998; Hutton et al. 1998; Poorkaj et al. 1998).

To avoid potential toxicity of tau overexpression and to see if our results in a primary cell model translated to humans, we created astrocytes from iPSCs derived from IVS 10+16 mutation patients and their isogenic controls. The phenotype of 4R tau expressing iAstrocytes mirrored what was seen in the primary hTau astrocytes, with an increase in neurotoxic gene expression and a higher vulnerability to oxidative stress (Chapter 2). Further, we identified that human 4R tau-expressing astrocytes displayed a loss in glutamate uptake ability and, when cultured with cortical neurons, induced neuronal hyper-excitability and hyper-synchrony, and death. These *in vitro* findings may explain the elevated seizure phenotype identified *in vivo* with increased 4R tau (Schoch et al. 2016). Our data suggest that the presence of 4R tau in astrocytes leads to astrocyte dysfunction that is consistent with neurodegenerative diseases (Bezzina et al. 2015; Ranasinghe et al. 2020).

Collective data, including from our lab, supports the therapeutic benefit of tau lowering strategies in tauopathy (DeVos et al. 2017, 2018). Given that many primary tauopathies have mutations in and around exon 10 (Strang, Golde, and Giasson 2019; F. Liu and Gong 2008), which encodes 4R tau, tau splicing strategies that lower 4R tau may also be viable therapeutically. We investigated a MAPT mutation (IVS 10+16), which causes an increased expression of 4R tau in humans. Due to the location of the mutation at the binding site of our 4R to 3R tau splicing ASO, we were unable to manipulate 4R tau levels specifically. However,

lowering total tau levels using a total tau knockdown ASO was able to reduce expression of reactive astrocytic genes, increase astrocytic ability to take up excess glutamate, and prevent cell death after oxidative stress. Tau expression in neurons is a clear target for tau-lowering strategies, but new data support astrocyte tau in disease progression, despite its low level of expression. We show, for the first time, that 4R-tau mediated dysfunction in astrocytes can be reversed by lowering the levels of total tau in iAstrocytes. Additionally, when tau was lowered in astrocytes alone, it led to a decrease in neuronal hyper-excitability, suggesting that cell-specific tau lowering in astrocytes may be a viable therapeutic strategy. Together, these data strongly support that tau-lowering ASOs that are currently in a clinical trial for Alzheimer's disease may be able to rescue neuronal and astrocyte functions in neurodegenerative disease.

Figures:

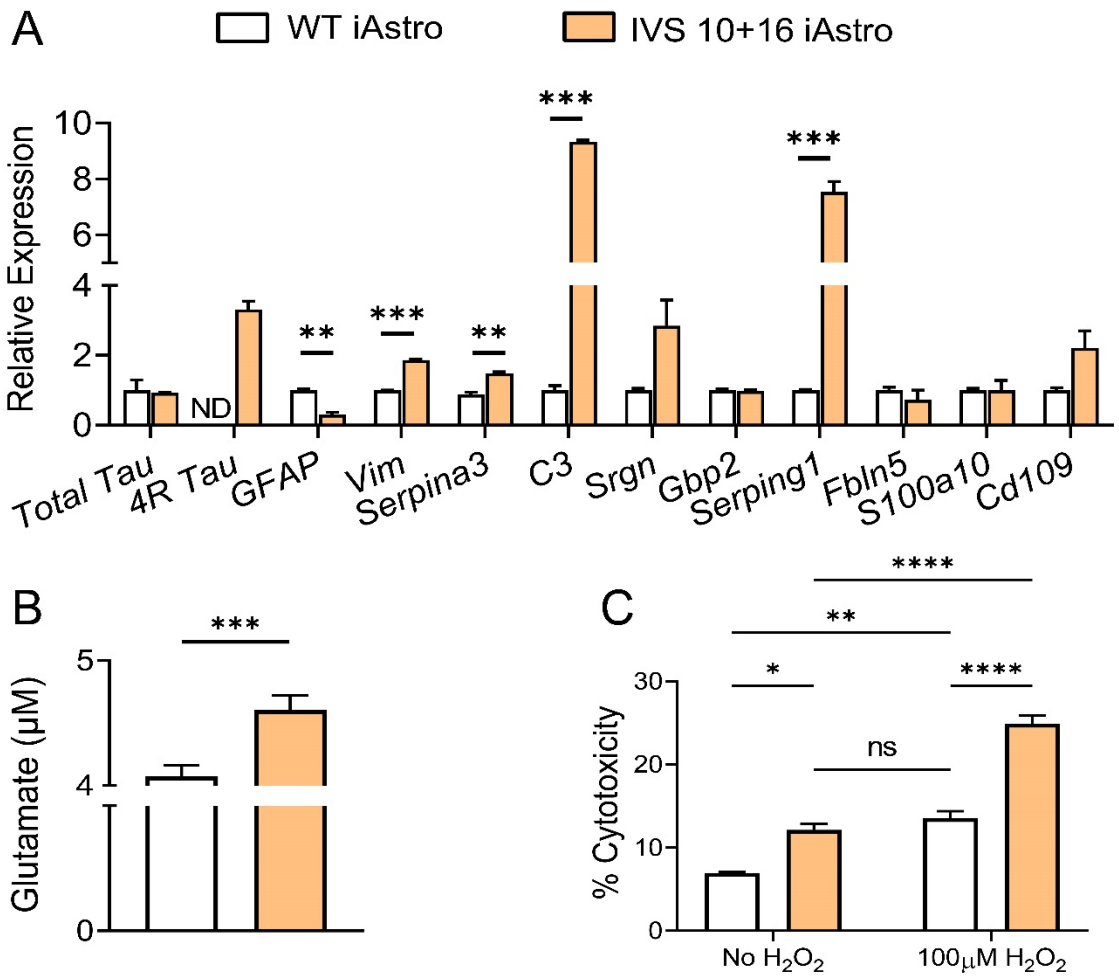


Figure 3.1: iAstrocytes exhibit a neurotoxic phenotype and disruption to homeostatic function.

A)Levels of select genes in wild-type (WT) and IVS 10+16 *MAPT* mutation iAstrocytes measured by qRT- PCR. Data are normalized to *GAPDH* relative to isogenic levels and shown as mean + SEM; one-wayANOVA with Tukey's multiple comparisons; n=3 wells; **p<0.01, ***p<0.001, ND = not determined. **B**)Glutamate concentration measured in cellular media in iAstrocytes. Data are mean + SEM; n=3 wells/group; unpaired t-test; ***p<0.001. **C**) Cytotoxicity (measured by LDH release) in iAstrocyte cultures at baseline and following 100μM H2O2 treatment. Data are mean + SEM; n=3 wells/treatment; two-way ANOVA with Tukey's multiple comparisons; *p<0.05, **p<0.01, ****p<0.0001, ns = notsignificant.

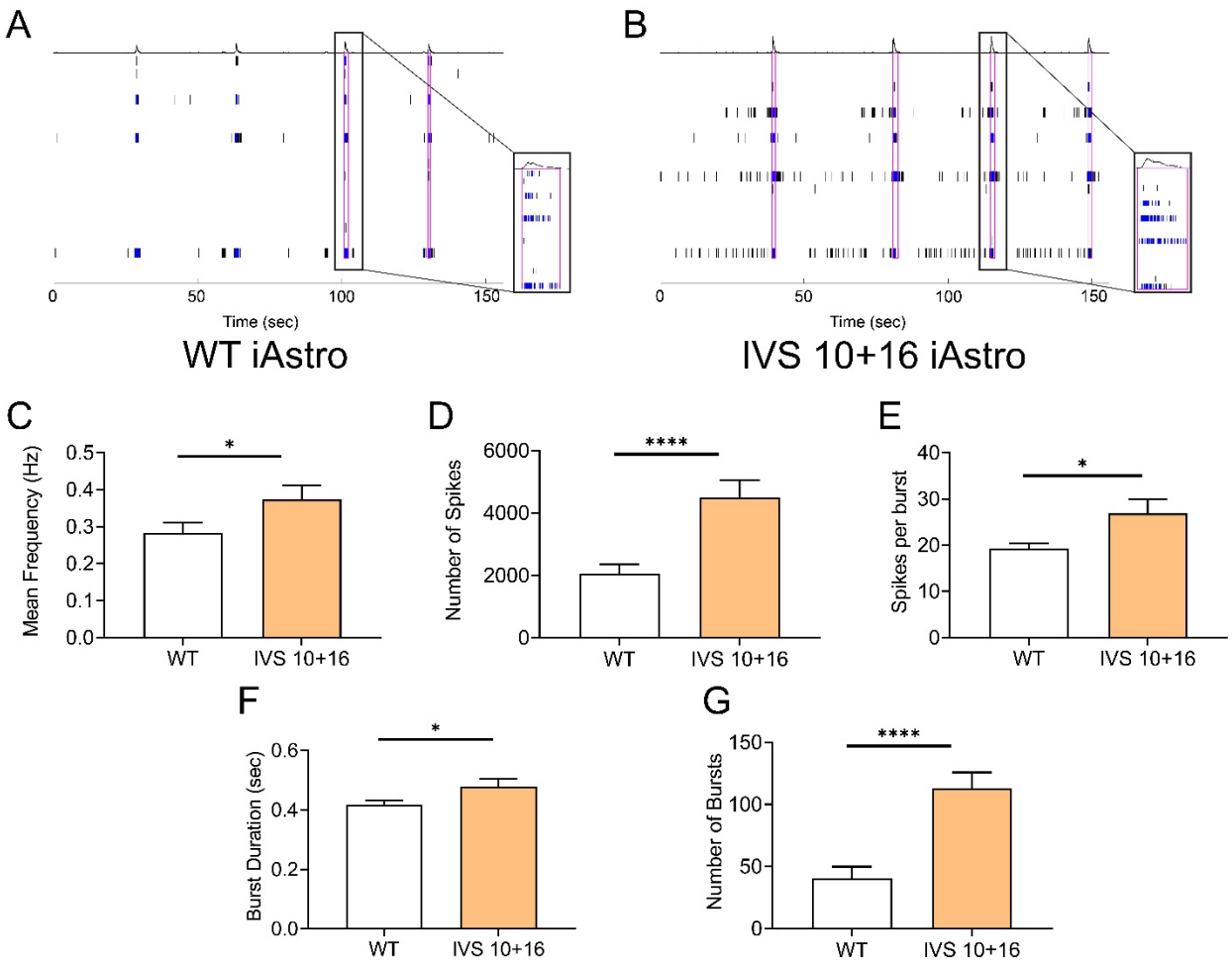


Figure 3.2: Homeostatic control of neuronal excitability is reduced in 4R tau-expressing iAstrocytes.

Representative Rastor plots of burst rates from neurons co-cultured with (**A**) wild-type (WT) or (**B**) IVS 10+16 *MAPT* mutation iAstrocytes. The blue tick marks represent spikes that were partof a single electrode firing, while black tick marks represent multi-electrode firings. The magenta outlinesindicate network bursts. **C**) Mean frequency, (**D**) number of spikes, (**E**) number of spikes per burst, (**F**)burst duration, and (**G**) number of bursts were measured from neurons co-cultured with Non-Mut or IVS10+16 iAstrocytes. Data are mean + SEM; n=45 wells/group from three recordings; *p<.05, ****p<.0001by unpaired t-test., ns = not significant.

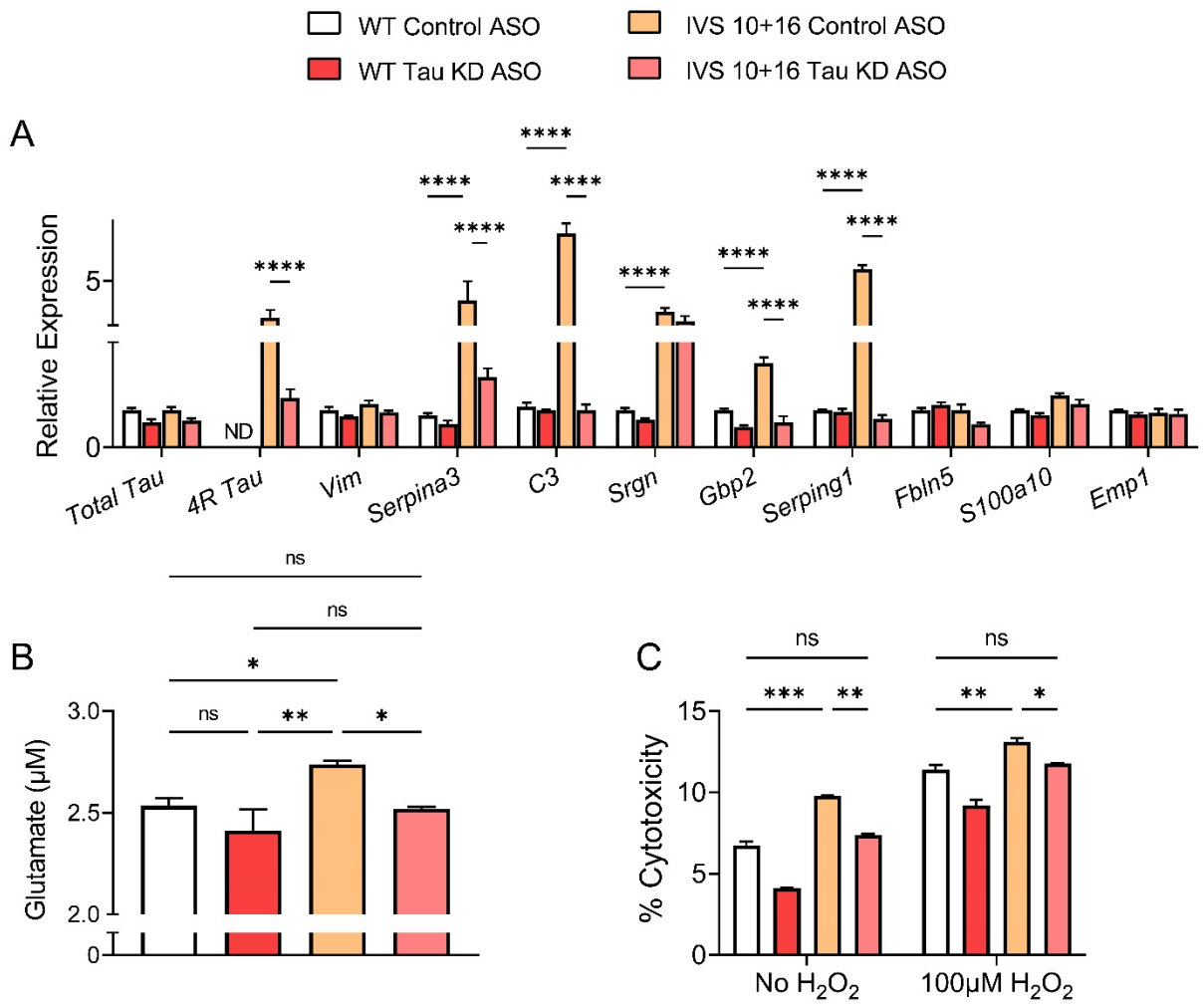


Figure 3.3: Lowering levels of total tau in iAstrocytes rescues neurotoxic phenotype and function.

A) Levels select genes in wildtype (WT) or IVS 10+16 *MAPT* mutation iAstrocytes treated with controlASO or tau knockdown (KD) ASO measured by qRT-PCR. Data are normalized to *GAPDH* relative to Non-Mut levels and shown as mean + SEM; n=3 wells/treatment; two-way ANOVA with Tukey's multiple comparisons; ****p<0.0001, ND = not determined (cycle threshold values greater than 35). B) Glutamate concentration measured in cellular media in iAstrocytes treated with control ASO or tau KDASO. Data are mean + SEM; n=6 wells/treatment; one-way ANOVA; *p<0.05, **p<0.01, ns = not significant. C) Cytotoxicity (measured by LDH release) in iAstrocyte cultures treated with a control ASOor tau KD ASO at baseline and following 100μM H2O2 treatment. Data are mean + SEM; n=3 wells/treatment; two-way ANOVA with Tukey's multiple comparisons; *p<0.05, **p<0.01, ***p<0.001, ns = not significant.

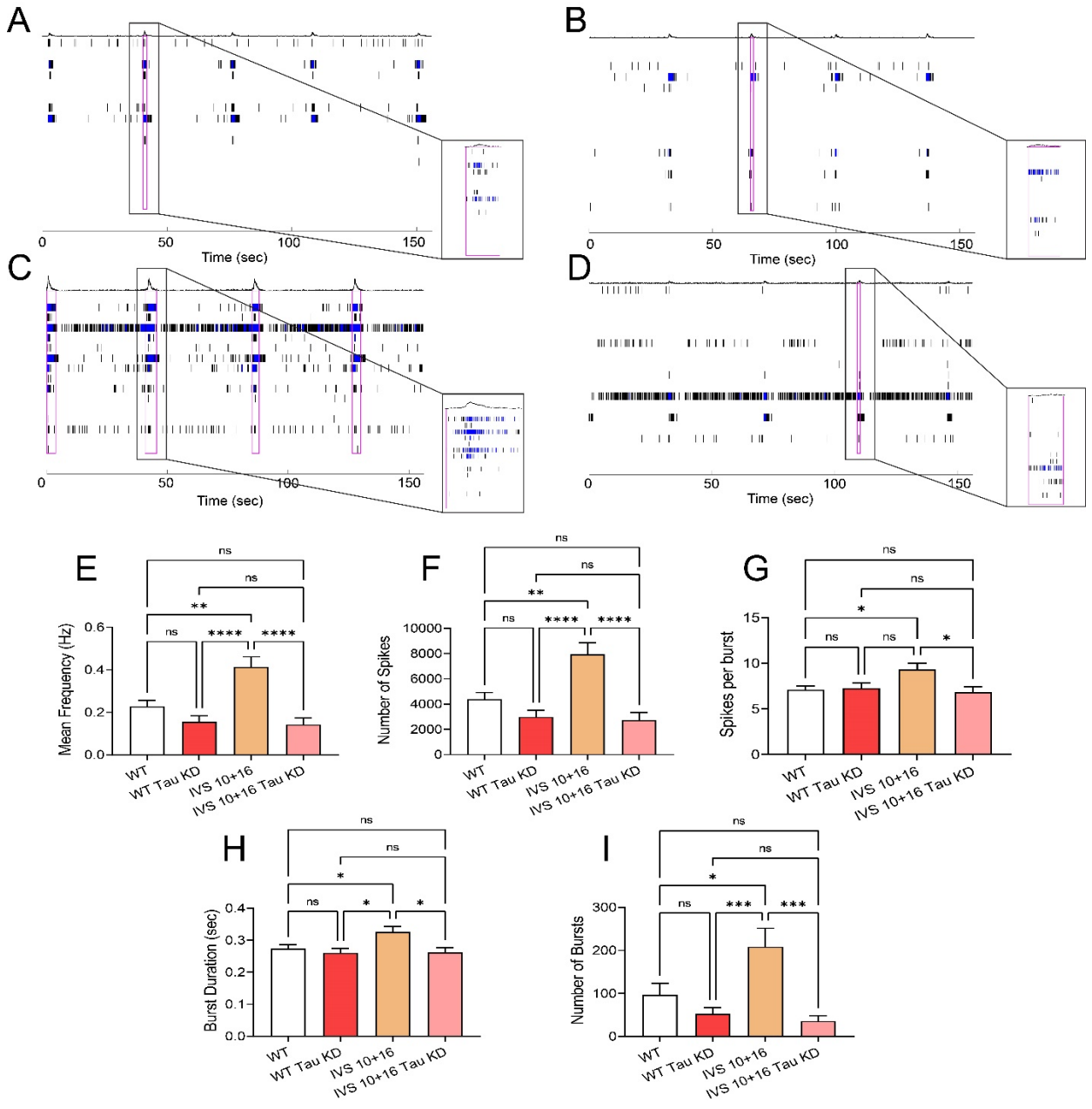


Figure 3.4: Homeostatic control of neuronal excitability is rescued following lowering of tau levels in 4R tau expressing iAstrocytes.

Representative Rastor plots of burst rates from neurons co-cultured with **A)** wild type (WT) **B**) WT tau knockdown ASO treated **C**) IVS 10+16 *MAPT* **D**) IVS 10+16 *MAPT* tau knockdown ASO treated iAstrocytes. The blue tick marks represent spikes that werepart of a single electrode firing, while black tick marks represent multi-electrode firings. The magenta outlines indicate network bursts. **E)** Mean frequency, **F)** number of spikes, **G)** number of spikes per burst, **H)** burst duration, and **I)** the number of bursts were measured from neurons co-cultured with Non-Mut, Non-Mut tau knockdown ASO treated, IVS 10+16 *MAPT* or IVS 10+16 *MAPT* tau

knockdown ASO treated iAstrocytes**.** Data are mean + SEM; n=30-45 wells/group from three recordings**;** *p<.05, **p<.01, ***p<.001, ****p<.0001, ns = not significant by one-way ANOVA with multiple corrections.

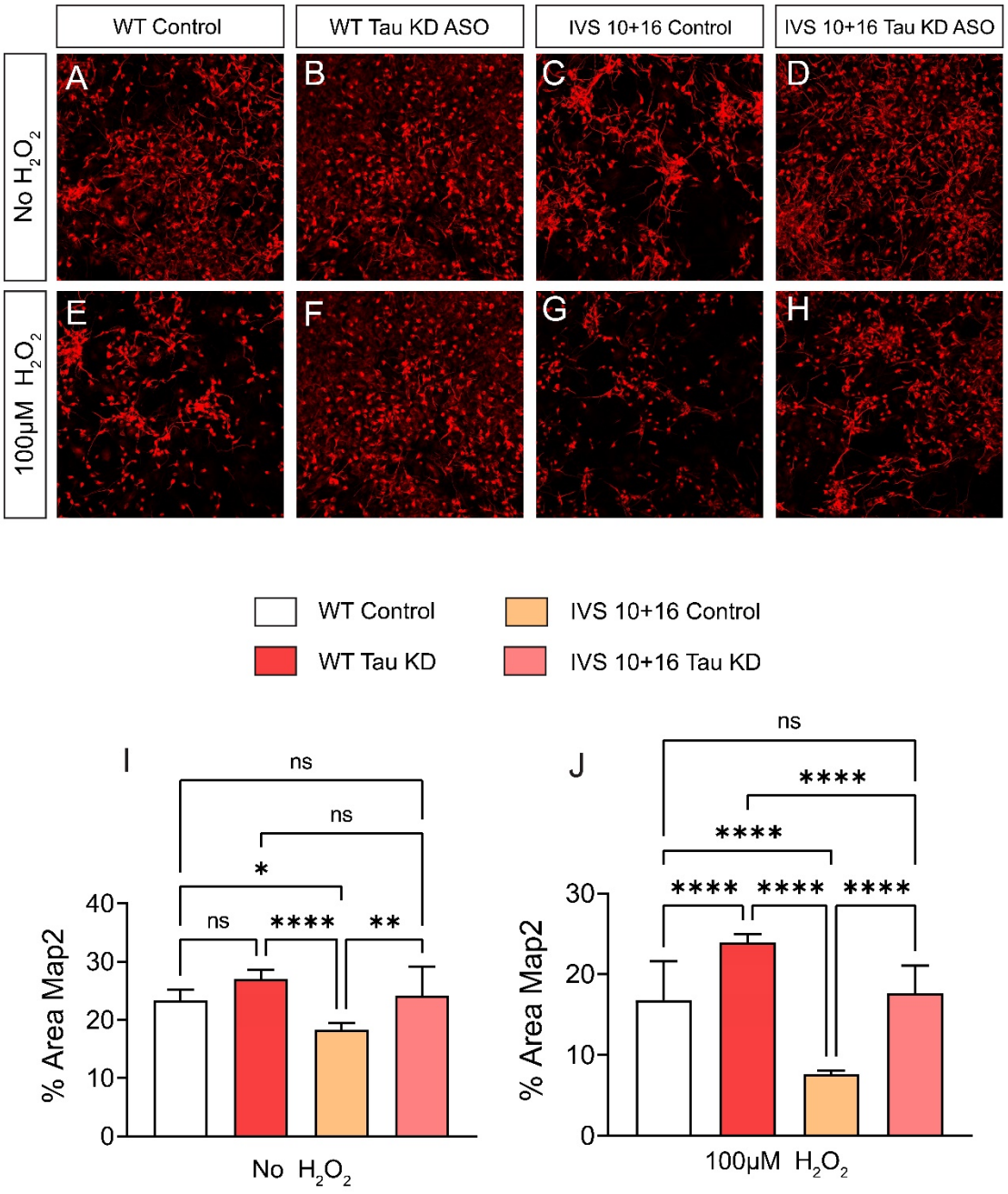
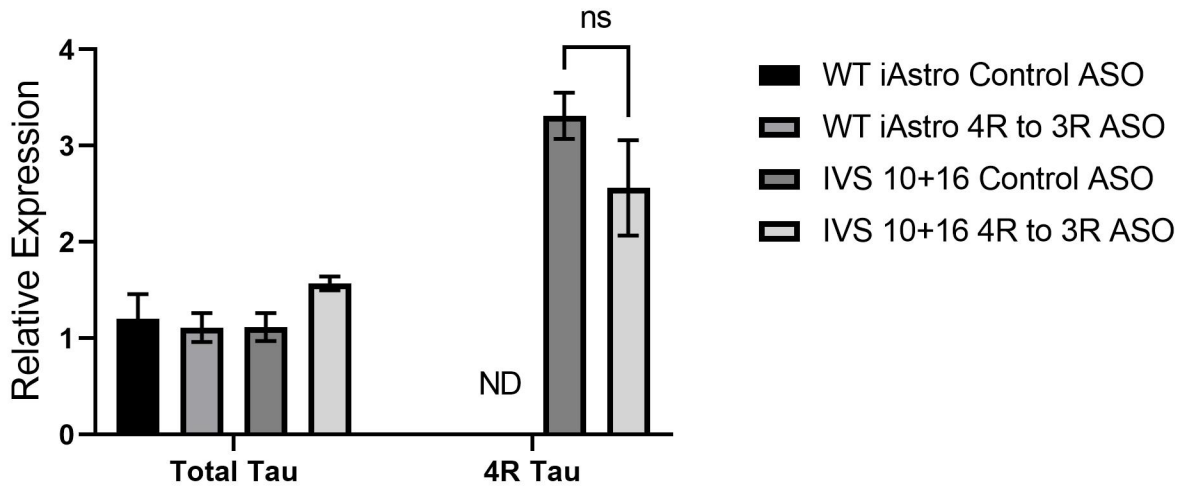


Figure 3.5: Lowering levels of tau in astrocytes improves neuronal survival.

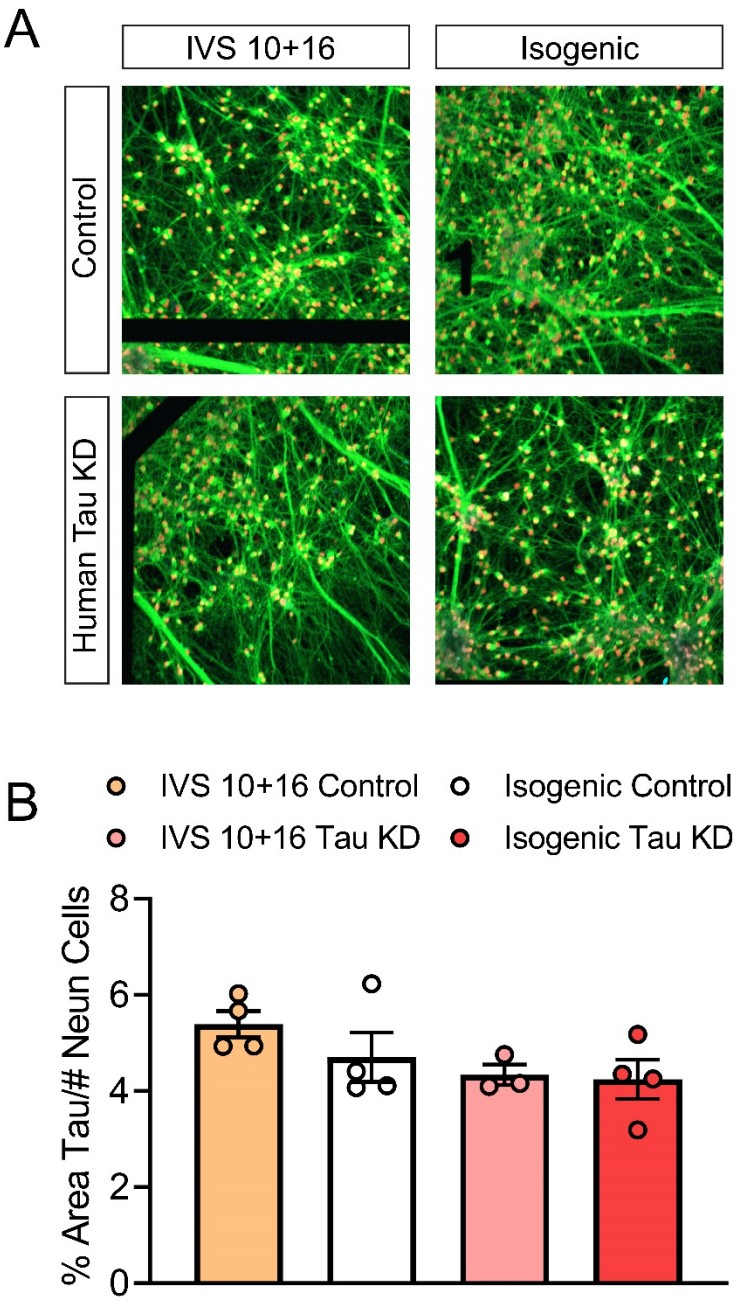
Representative images of *MAP2* staining in iPSC cortical neurons that were co-cultured with **A,)** WT iAstrocytes treated with a control ASO and not stressed with 100µM of H_2O_2, **B)** WT iAstrocytes treated with the tau knockdown ASO and not stressed with 100µM of H_2O_2, **C)** IVS 10+16 iAstrocytes treated with the control ASO and not stressed with 100µM of H_2O_2, **D)** IVS 10+16 iAstrocytes treated with the tau knockdown ASO and not stressed with 100µM of H_2O_2. **E)** WT iAstrocytes treated with a control ASO and stressed with 100µM of H_2O_2, **F)** WT iAstrocytes treated with the tau knockdown ASO and stressed with 100µM of H_2O_2, **G)** IVS

10+16 iAstrocytes treated with the control ASO and stressed with 100μM of H_2O_2, **H)** IVS 10+16 iAstrocytes treated with the tau knockdown ASO and stressed with 100μM of H_2O_2. **I)** quantification of % *MAP2* area covered in co-cultures that were not exposed to H_2O_2. **J)** quantification of % *MAP2* area covered in co-cultures that were exposed to H_2O_2. Data shown as mean + SEM, n= 8 wells per treatment and 10 images per well; *p<.05, **p<.01, ***p<.001, ****p<.0001, ns = not significant by one-way ANOVA with multiple corrections.



Supplement figure 3.1S: Previously developed 4R to 3R tau splicing ASO does not alter levels of 4R tau.

qRT-PCR data showing analysis of WT and IVS 10+16 iAstrocytes treated with either a control ASO or a 4R to 3R tau splicing ASO. Data shown are mean + SEM; n= 3 wells per treatment. ND=not determined, CT>35, ns = not significant by two-way ANOVA with multiple corrections.



Supplement Figure 3.2S: iPSC cortical neurons do not show decreased levels of tau when cultured with iAstrocytes pre-treated with tau lowering ASO.

A) representative images of tau staining in iPSC cortical neuron and iAstrocyte co-cultures. The top row represents either isogenic (WT) iAstrocytes or IVS 10+16 iAstrocytes that were treated with the control ASO prior to co-culture with neurons for 21 days. The bottom row represents either isogenic (WT) iAstrocytes or IVS 10+16 iAstrocytes that were treated with the total tau lowering ASO prior to co-culture with neurons for 21 days. **B)** quantification of the percent area covered by *MAP2* staining divided by the total number of NUEN positive cells to quantify if

there is a decrease in neuronal tau expression. Data are mean + SEM, n= 35-48 wells per group, no significant change detected by one way ANOVA with multiple corrections.

Gene Name	Species	Reagents	Sequence (5' to 3') or Assay ID
4R Tau	H	Sybr	F: GACTGGACGTTGCTAAGATC
			R: CATGCCAGACCTGAAGAATG
4R Tau Probe	H	Taqman	56-FAM/CCACTGAGAACCTGAAGCACCAGC/3IABkFQ
Total Tau	H	Sybr	F: AGAAGCAGGCATTGGAGAC
			R:TCTTCGTTTTACCATCAGCC
Total Tau Probe	H	Taqman	56-FAM/ACGGGACTGGAAGCGATGACAAAA/3IABkFQ
Gapdh	H	Taqman	Hs.PT.39a.22214836 NM_002046(1)
Gapdh	H	Sybr	F: AGGGCTGCTTTTAACTCTGGT
			R: CCCCACTTGATTTTGGAGGGA
Vim	H	Sybr	F: TGGACCAGCTAACCAACGAC
			R: GCCAGAGACGCATTGTCAAC
Serpina3	H	Sybr	F: CCTGAAGGCCCCTGATAAGAA
			R: GCTGGACTGATTGAGGGTGC
C3	H	Taqman	Hs.PT.56a.2840009 NM_000064(1)
Srgn	H	Sybr	F: AGGTTATCCTACGCGGAGAG
			R: GTCTTTGGAAAAAGGTCAGTCCT
Gbp2	H	Sybr	F: CTATCTGCAATTACGCAGCCT
			R: TGTTCTGGCTTCTTGGGATGA
Serping1	H	Sybr	F: CTGGCTGGGGATAGAGCCT
			R: GAGATAACTGTTGTTGCGACCT
Fbln5	H	Sybr	F: CTCACTGTTACCATTCTGGCTC
			R: GACTGGCGATCCAGGTCAAAG
S100a10	H	Sybr	F: GGCTACTTAACAAAGGAGGACC
			R: GAGGCCCGCAATTAGGGAAA
Emp1	H	Sybr	F: GTGCTGGCTGTGCATTCTTG
			R: CCGTGGTGATACTGCGTTCC
GFAP	H	Sybr	F: GTCCCCCACCTAGTTTGCAG
			R: TAGTCGTTGGCTTCGTGCTT

Supplemental Table 3.1S: Sequences of primers used.

Names and sequences of all primers and reagents used for qRT-PCR analysis. F, forward; R, reverse; H, Human.

Seeding Media	Stock Concentration	Final concentration
DMEM/F12 (Life Technologies #11330-032)	1X	0.5X
Neurobasal (Life Technologies #21103-049)	1X	0.5X
B27 (Life Technologies #17504-044)	50X	1X
N2 (Life Technologies #17502-048)	100X	1X
Glutamax (Life Technologies #35050-061)	200mM	0.5mM
BDNF (Peprotech #450-02)	10µg/mL	10ng/mL
GDNF (Peprotech #450-10)	10µg/mL	10ng/mL
TGF-β1 (Peprotech #100-21C)	1µg/mL	1ng/mL
Astrocyte supplement (BrainXell, Madison, WI)	1000X	1X
Seeding supplement (BrainXell)	1000X	1X

Day 4 Media	Stock Concentration	Final concentration
DMEM/F12	1X	0.25X
Neurobasal	1X	0.25X
BrainPhys Media (STEMCELL Technologies #05790)	1X	0.5X
B27	50X	1X
N2	100X	1X
Glutamax	200mM	0.5mM
BDNF	10µg/mL	10ng/mL
GDNF	10µg/mL	10ng/mL
TGF-β1	1µg/mL	1ng/mL
Astrocyte supplement	1000X	1X
Day 4 supplement (BrainXell)	1000X	1X

Day 7 Supplement C Treatment	Stock Concentration	Final concentration
BrainPhys Media	1X	0.5X
B27	50X	1X
N2	100X	1X
Glutamax	200mM	0.5mM
Supplement C (BrainXell)	2000X	1X

Maintenance Media	Stock Concentration	Final concentration
BrainPhys Media	1X	0.5X
B27	50X	1X
N2	100X	1X
Glutamax	200mM	0.5mM
BDNF	10µg/mL	10ng/mL
GDNF	10µg/mL	10ng/mL
TGF-B1	1µg/mL	1ng/mL

Supplemental Table 3.2S: Media composition for MEA assay. Components and concentration of reagents used for media in the MEA assay.

Seeding Media	Stock Concentration	Final concentration
DMEM/F12	1X	.5X
Neurobasal	1X	.5X
B27	50X	1X
N2	100X	1X
Glutamax	200mM	.5mM
BDNF	10ug/mL	10ng/mL
GDNF	10ug/mL	10ng/mL
TGF-B1	1ug/mL	1ng/mL
Seeding supplement (BrainXell)	1000X	1X
Astrocyte supplement (BrainXell)	1000X	1X

Day 1 Media	Stock Concentration	Final concentration
DMEM/F12	1X	.25X
Neurobasal	1X	.25X
B27	50X	1X
N2	100X	1X
Glutamax	200mM	.5mM
BDNF	10ug/mL	10ng/mL
GDNF	10ug/mL	10ng/mL
TGF-B1	1ug/mL	1ng/mL
Astrocyte supplement	1000X	1X
Seeding supplement	1000X	1X
Geltrex	15mg/mL	15ug/mL

Day 4 Media	Stock Concentration	Final concentration
DMEM/F12	1X	.25X
Neurobasal	1X	.25X
B27	50X	1X
N2	100X	1X
Glutamax	200mM	.5mM
BDNF	10ug/mL	10ng/mL
GDNF	10ug/mL	10ng/mL
TGF-B1	1ug/mL	1ng/mL
Day 4 Supplment (BrainXell)	1000X	1X
Astrocyte supplement	1000X	1X

Day 7 and on Media	Stock Concentration	Final concentration
DMEM/F12	1X	.25X
Neurobasal	1X	.25X
B27	50X	1X
N2	100X	1X
Glutamax	200mM	.5mM

Supplemental Table 3.3S: Media composition for iPSC cortical neuron and iAstrocyte co-cultures. Components and concentration of reagents used for media in the iPSC derived neuron and astrocyte co-cultures.

Acknowledgments

This study was supported by the Rainwater Charitable Foundation (TMM and CMK and RJB) and NIH NS110890 (CMK). We would also like to thank the Human Cells, Tissues, and Organoids Core at Washington University in St. Louis for all iAstrocyte differentiation and culture. Antisense oligonucleotides used in these experiments were generously provided by Ionis Pharmaceuticals. Washington University in St. Louis has filed patents in conjunction with Ionis Pharmaceuticals regarding the use of Tau ASOs in neurodegenerative syndrome. Confocal images were taken on Nikon A1Rsi in the Washington University Center for Cellular Imaging (WUCCI) supported by Washington University School of Medicine, The Children's Discovery Institute of Washington University and St. Louis Children's Hospital (CDI-CORE-2015-505 and CDI-CORE-2019-813), and the Foundation for Barnes-Jewish Hospital (3770 and 4642).

Chapter 4:

Pre-trans-splicing molecules can alter tau isoform levels in primary astrocytes

Summary

Tauopathies are a group of diseases that are characterized by the accumulation of tau in insoluble neurofibrillary tangles and glial inclusions. Several primary tauopathies exhibit higher deposition of 4R tau; however, the mechanism of 4R tau toxicity remains unclear. Here, we piloted the use of pre-trans-splicing molecules (PTMs) to alter the ratios of 3R and 4R tau in primary astrocytes derived from human tau (hTau) expressing mice. We found that following increased expression of 4R tau, hTau astrocytes exhibit an increase in genes associated with neurotoxicity. Our data support the use of PTMs to alter the ratio of tau isoforms in specific cell types to better understand the impact of tau isoforms in disease.

Introduction

Tau is a microtubule-associated protein involved in many cellular functions, including axonal stabilization and synaptic plasticity. In tauopathies, tau becomes hyperphosphorylated and more prone to aggregation. These aggregates make up the neurofibrillary tangles that are found in tauopathies. While over 6 million American's have been diagnosed with Alzheimer's disease, the most common tauopathy, there is still no effective therapy.

The six isoforms of tau are created through the alternative splicing of the introns 2, 3, and 10 in the gene *MAPT* (Forrest, Kril, and Halliday 2019), all are expressed in the adult brain. The alternative splicing of exon 10 creates 3R or 4R tau (Connell et al. 2005), which is of particular interest in disease, as many tauopathies exhibit higher deposition of either 3R 4R tau within neurofibrillary tangles and in glial cells (Kovacs, Lee, and Trojanowski 2017). 4R tau may be the more toxic isoform. Studies have shown that an increase in 4R tau levels leads to increased phosphorylated tau deposition and altered behavior in a human tau-expressing mouse model (Schoch et al. 2016). Others have shown that overexpression of the 4R isoform led to increased neurodegeneration and impairments to learning and memory (Sealey et al. 2017). Additionally, overexpression of 3R tau led to deficits with axonal transport and issues with locomotion (Sealey et al. 2017; Richetin et al. 2020). These studies underscore the importance of understanding the impact of altering the ratios of tau isoforms on disease progression.

Several tools have been developed to understand better the impact of altering tau isoforms in disease. Antisense oligonucleotides (ASOs) are short strings of nucleic acids that can bind to

their target mRNA and alter the pattern of gene splicing (DeVos and Miller 2013). To date, both 3R to 4R tau splicing and 4R to 3R tau splicing ASOs have been created and used to test the impact of tau isoform alteration in a human tau expressing mouse (Schoch et al. 2016). ASOs have shown incredible promise as potential therapeutics in spinal muscular atrophy and amyotrophic lateral sclerosis (Schoch and Miller 2017). More recently, pre-trans-splicing molecules (PTMs) have been created as an alternative way to either increase or decrease the inclusion of exon 10 (Espíndola et al. 2018).

We performed experiments to identify if the previously developed PTMs can lead to similar splicing changes as the ASOs we have used in the past (Chapters 2 and 3). We identified that the 3R to 4R tau splicing PTMs could increase the ratio of 4R to 3R tau without altering total tau levels. We also found that increasing the levels of 4R tau using the PTMs led to similar genetic changes in primary astrocytes as seen when tau isoforms were altered using ASOs. These data provide crucial support for using PTMs to alter tau isoforms in a cell-specific manner to understand the role that cell types play in disease progression.

Materials and Methods

Plasmid preparation: The control, 3R to 4R and 4R to 3R tau splicing PTMs were kindly gifted by Elena Avale and Jean Marc Gallo. Upon receipt, the plasmids were purified and the transplicing sequence was isolated and verified using sanger sequencing. The purified plasmid was then cloned into an expression vector under control of a CMV promoter.

SH-SY5Y culture: SH-SY5Y human neuroblastoma cells were obtained from ATCC and cultured per supplier instructions. Briefly, the cells were grown in media containing DMEM, 10% FBS, and 1X penicillin-streptomycin) and sub-cultured every 4-7 days until the end of the experiment.

Primary astrocyte cultures: Cortices from hTau pups (P2-P4) were isolated as previously described (Schildge et al. 2013) and cultured until confluency on 100µg/mL poly-D-lysine (Corning CB-40210)-coated plates in primary astrocyte media (DMEM, 10% heat-inactivated fetal bovine serum (FBS), 1X penicillin-streptomycin) at 37°C, 5% CO2. The mixed glial cultures were purified by shaking at 270 rpm for 6 hours at 37°C to achieve a pure primary astrocyte culture.

Transfection of cells: Both SH-SY5Y and primary hTau astrocytes were plated onto 100µg/mL poly-D-lysine coated plates and cultured until reaching 70% confluency. Lipofectaimine LTX (ThermoFisher Cat #A12621) was used to transfect 1µg of the control PTM, 3R to 4R PTM, or 4R to 3R PTM into the cells in antibiotic-free media. 12 hours post-transfection, the media was

replaced with regular culture media, and the cells were allowed to grow for 7 days prior to collection and analysis.

Polymerase Chain Reaction (PCR): Primary astrocytes and SH-SY5Y cells were lysed using 500µL of QIAzol Lysis Reagent (Qiagen) and placed into Eppendorf tubes. Chloroform (100µL) was added to each sample, shaken vigorously for 5 seconds, and let to sit for 3 minutes at room temperature. The samples were centrifuged for 15 minutes at 12,000 RCF at 4°C. The aqueous layer was removed and combined with 1.5 times volume of 100% ethanol and together added to the RNeasy column for RNA purification according to the manufacturer's protocol. cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA Tau isoforms were detected by end-point PCR, 0.2 µg of reverse-transcribed RNA was amplified by PCR using Go Taq polymerase (Promega) with primers spanning exons 9–13 (9F: GTCAAGTCCAAGATCGGCTC, 13R: TGGTCTGTCTTGGCTTTGGC) as described previously (Avale 2013). The product was then separated using gel electrophoresis in 2% (W/V) agarose gel that was run at 50V for 2 hours in cold TAE buffer. Gapdh F: TGCCCCCATGTTGTGATG, Gapdh R: TGTGGTCATGAGCCCTTCC) amplified products were used as reference.

Quantitative real-time PCR: Primary astrocytes and SH-SY5Y cells were lysed using 500µL of QIAzol Lysis Reagent (Qiagen) and placed into Eppendorf tubes. Chloroform (100µL) was added to each sample, shaken vigorously for 5 seconds, and let to sit for 3 minutes at room temperature. The samples were centrifuged for 15 minutes at 12,000 RCF at 4°C. The aqueous layer was removed and combined with 1.5 times volume of 100% ethanol and together added to

the RNeasycolumn for RNA purification according to the manufacturer's protocol. cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA) and analyzed on theQuantStudio 12K Flex Real-Time PCR System using either the Power SYBR™ Green PCR Master Mix(ThermoFisher) the PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies, Coralville, IA). Expression levels were calculated by the ΔΔCt method, normalized to GAPDH and a biological reference sample. Primer sequences or identifiers (purchased from Integrated DNA Technologies) are detailed in Supplementary Table 4.1S.

Statistical analysis: All data were graphed as mean + SEM and analyzed using GraphPad Prism 9 statistical software (GraphPad Software, La Jolla, CA). Quantifiable cDNA levels of tau isoforms were evaluated using mixed effect analysis with multiple comparisons. Levels of mRNA expression in SH-SY5Y cells was analyzed using one -way ANOVA. Levels of mRNA in primary astrocytes were analyzed with a two-way ANOVA with multiple corrections. A value of $p<0.05$ was deemed significant for all analyses.

Results

Pre-trans splicing molecules can increase the ratio of 4R tau in SH-SHY5Y cells

We first wanted to identify if we would be able to alter the ratios of tau isoforms in SH-SY5Y cells. We transfected SH-SY5Y human neuroblastoma cells with either a control PTM, 3R to 4R PTM, or a 4R to 3R PTM for 7 days. We found that the control PTM and 4R to 3R PTM did not alter the levels of 4R tau in the cells (**Figure 4.1A**). However, the 3R to 4R PTM led to an increase in 4R tau levels (**Figure 4.1A**), this was not significant as identified through PCR, although there was a significant increase in 4R tau mRNA in SH-SY5Y cells that were transfected with the 3Rto 4R PTM as compared to the 4R to 3R PTM (**Figure 4.1B**). These data showed that pre-trans-splicing molecules could be used to alter tau isoforms in human tau expressing cells.

PTMs can alter tau isoform ratios in primary hTau astrocytes

After identifying that the tau splicing PTMs were able to alter the ratio of 3R:4R tau in SH-SY5Y cells, we wanted to see if we could identify similar results in hTau astrocytes. We found that hTau astrocytes that were transfected with the 3R to 4R PTM exhibited significantly higher levels of 4R tau mRNA than those that were transfected with the 4R to 3R tau splicing PTM (**Figure 4.2**). This result showed that the PTMs are able to alter even low levels of tau in astrocytes.

Altering tau splicing using PTMs in hTau astrocytes leads to similar genetic changes seen post tau splicing ASO treatment

Since we saw an increase in 4R tau mRNA in the hTau astrocytes that were transfected with the 3R to 4R tau splicing PTM, we next wondered if this increase would be accompanied by similar genetic changes to those seen in hTau astrocytes treated with the 3R to 4R tau splicing ASO. Interestingly, we saw similar trends in mRNA changes in the hTau astrocytes that had an increase in 4R tau levels using the PTMs as when tau splicing was altered using the ASOs (**Figure 4.3**). We saw a significant increase in the mRNA levels of *C3* as in hTau astrocytes transfected with the 3R to 4R PTM as compared to those that were transfected with either the control PTM or the 4R to 3R PTM (**Figure 4.3**). We also saw a significant decrease in mRNA levels of *Serpina3n*, *C3*, and *Srgn* in hTau astrocytes transfected with the 4R to 3R tau splicing PTM as compared to those that were treated with the 3R to 4R tau splicing PTM (**Figure 4.3**). While other genes examined showed a trend upwards in the 4R tau expressing hTau astrocytes, they were not significant (**Figure 4.3**). This may be due to the levels of 4R tau not being altered as much as they had been with the 3R to 4R tau splicing ASO. These data show that PTMs can be used to alter the levels of tau isoforms in hTau astrocytes and that this change leads to similar genetic changes seen previously with the use of the tau splicing ASOs.

Discussion

The impact that altering the levels of tau isoforms has on cellular function and disease progression is still not well understood. It has been shown that increased expression of 4R tau may lead to increased levels of phosphorylated and aggregated tau (Barron et al. 2020; Honson and Kuret 2008). Overexpression of 4R tau led to neuronal aneuploidy in drosophila which is similar to what is seen in some Alzheimer's disease postmortem brains (Malmanche et al. 2017). Others have shown that overexpression of both 3R or 4R tau in SH-SY5Y cells led to an increase in tau phosphorylation but that only 4R tau overexpression led to an increase in cell death (Delobel et al. 2003). These studies however are difficult to interpret as they rely on tau overexpression which has itself been shown to be detrimental to cellular function and lead to disease progression (Thies and Mandelkow 2007; David et al. 2005; Adams et al. 2009).

To study the impact of altering the ratio of tau isoforms on disease progression without altering total tau levels, we previously developed tau splicing ASOs which can bind to *MAPT* and lead to the inclusion or exclusion of exon 10 (Schoch et al. 2016). Using these ASOs, we showed that hTau mice treated with 3R to 4R tau splicing ASOs exhibited an increase in seizure severity, phosphorylated tau deposition and alteration to normal behavior as compared to those that were treated with the control ASO. More recently, pre-transplicing molecules which can also alter the ratio of tau isoforms without altering total tau levels were used in hTau mice. Interestingly, these results contradicted our previous findings where an increase in 4R tau was detrimental, here, it was found that an increase in 4R tau was beneficial as it reduced the levels of phosphorylated tau, and the mouses behavior was more like their WT counterparts than those that were treated

with a control PTM (Espíndola et al. 2018). These contrasting results show the importance of creating tools that allow for cell specific alteration of tau isoforms.

The ability to target specific cell types would allow for the creation of better therapeutics. ASOs are equally taken up by all cell types in the brain which makes it impossible to parse out the role that certain cells play in disease progression. The PTMs use by the Avale lab were targeted to neurons in the prefrontal cortex of hTau mice (Espíndola et al. 2018). In order identify if there is truly a difference between the use of ASOs and PTMs to alter tau splicing, it is necessary to create ASOs that can be targeted to specific cell types. IONIS pharmaceuticals has identified that conjugation of *MALAT1* to an ASO, led to increased uptake by cells that expressed Asgr1, the receptor of *MALAT1* (Youngsoo Kim et al. 2019). While this is promising, the expression of a liver receptor in cells of the CNS may create disruptions to cellular function. Therefore, future studies will focus on identifying receptors in the various cell types of the CNS that can be used to target ASOs specifically, allowing for better understanding of what occurs *in vivo* when tau splicing is altered in one particular cell type as opposed to all the cell types of the brain.

Figures

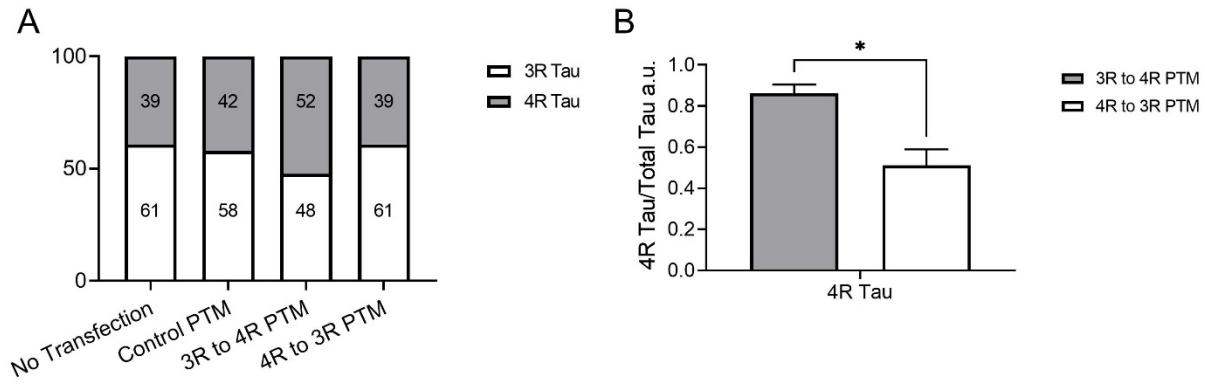


Figure 4.1 Pre-transplicing molecules increase the ratio of 4R tau in SH-SY5Y cells.

A) Ratio of tau isoforms in SH-SHY5 cells following transfection with the control PTM, 3R to 4R tau splicing PTM or the 4R to 3R tau splicing PTM relative to GAPDH levels. **B**) 4R tau mRNA levels in hTau astrocytes transfected with the 3R to 4R tau splicing PTM or the 4R to 3R tau splicing PTM, relative to GAPDH and total tau. Data are shown as mean + SEM, n=3 wells per treatment, *p<.05 by one way ANOVA.

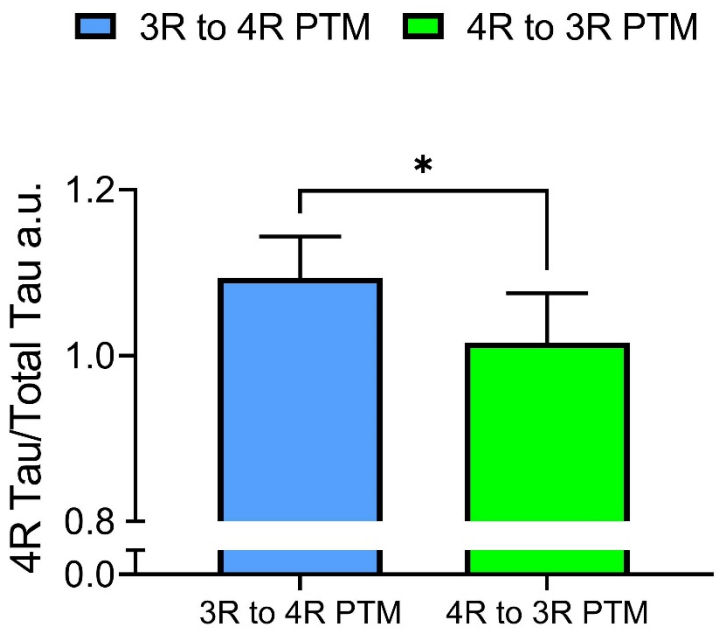


Figure 4.2: 3R to 4R tau splicing PTMs increase the amount of 4R tau mRNA in hTau astrocytes.

4R tau mRNA levels in hTau astrocytes transfected with the 3R to 4R tau splicing PTM or the 4R to 3R tau splicing PTM, relative to GAPDH and total tau. Data are shown as mean + SEM, n=3 wells per treatment, *p<.05 by one way ANOVA.

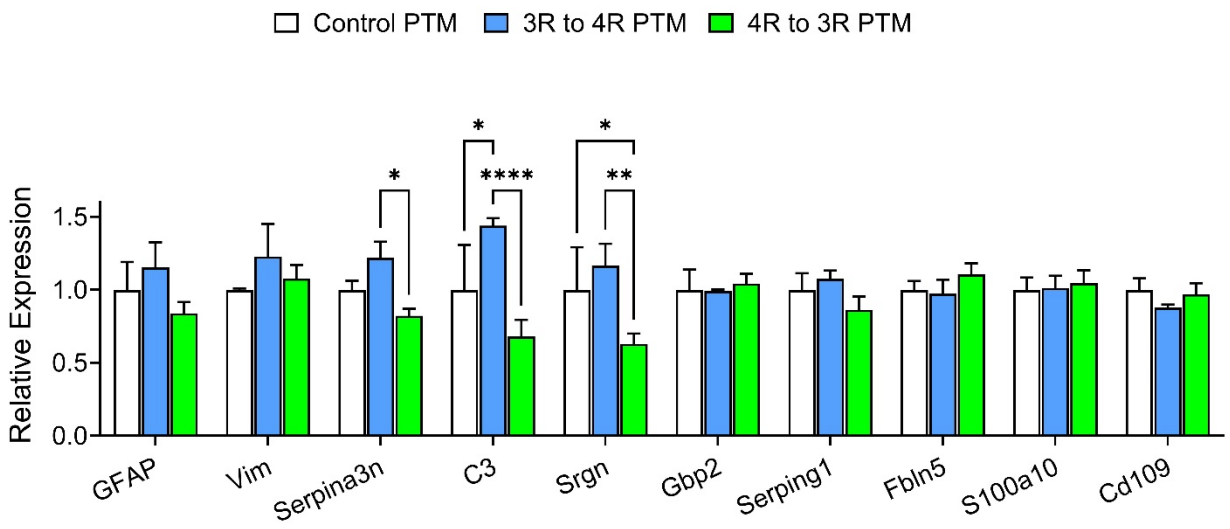


Figure 4.3: Changing tau isoform ratios with PTMs causes increased expression of neurotoxic genes.

Levels select genes in wildtype hTau astrocytes treated with control PTM, 3R to 4R tau splicing PTM or the 4R to 3R tau splicing PTM measured by qRT-PCR. Data are normalized to *GAPDH* relative to control PTM levels and shown as mean + SEM; n=3 wells/treatment; two-way ANOVA with Tukey's multiplecomparisons; *p<.05, **p<.01, ****p<0.0001.

Gene Name	Species	Reagents	Sequence (5' to 3') or Assay ID
Serpina3n	M	Sybr	F: CAGATCCCAGCCATCAAGAG
			R: CTGGCAGCTGGCTGGTTT
S100a10	M	Taqman	Mm.PT.58.6571055 NM_009112
Cd109	M	Taqman	Mm.PT.58.6710335 NM_153098
4R Tau	H	Sybr	F: GACTGGACGTTGCTAAGATC
			R: CATGCCAGACCTGAAGAATG
4R Tau Probe	H	Taqman	56-FAM/CCACTGAGAACCTGAAGCACCAGC/3IABkFQ
Total Tau	H	Sybr	F: AGAAGCAGGCATTGGAGAC
			R:TCTTCGTTTTACCATCAGCC
Total Tau Probe	H	Taqman	56-FAM/ACGGGACTGGAAGCGATGACAAAA/3IABkFQ
Gapdh	M	Sybr	F: TGCCCCCATGTTGTGATG
			R: TGTGGTCATGAGCCCTTCC
Gapdh Probe	M	Taqman	56- FAM/AATGCATCCTGCACCACCAACTGCTT/3IABkFQ
GFAP	M	Sybr	F: ACCGCATCACCATTCCTGTAC
			R: TGGCCTTCTGACACGGATTT
GFAP Probe	M	Taqman	56-FAM/TCCAGATCCGAGAAACCAGCCT/3IABkFQ
Fbln5	M	Taqman	Mm.PT.58.29865771 NM_011812
Serping1	M	Taqman	Mm.PT.58.30811631 NM_009776
Srgn	M	Taqman	Mm.PT.58.41483771 NM_011157
C3	M	Taqman	Mm.PT.58.17325540 NM_009778
Vim	M	Sybr	F: TCCACTTTCCGTTCAAGGTC
			R: AGAGAGAGGAAGCCGAAAGC

Supplemental Table 4.1S: Sequences of primers used.

Names and sequences of all primers and reagents used for qRT-PCR analysis. F, forward; R, reverse; M, Mouse H, Human.

Acknowledgments

Acknowledgments: This study was supported by the Rainwater Charitable Foundation (TMM. Pre-trans-splicing molecules used in these experiments were generously gifted by Elena Avale and Jean Marc Gallo at Kings College London. We would also like to thank Nathan Pomper for providing much needed guidance and advice on how to best approach the purification and packaging of the PTMs once received.

Chapter 5

Future Directions and Conclusions

Summary of Results

Neurodegenerative diseases impact over 30 million people worldwide, and there are still no effective therapeutics. The experiments described in this dissertation aim to expand our understanding of the impact of 4R tau expression in astrocytes and how it translates to disease progression. The results of these experiments show for the first time that even low levels of tau expression in astrocytes impact their ability to perform homeostatic functions and prevent neuronal toxicity, which can be mitigated by reducing the levels of either 4R tau or total tau in astrocytes alone. These findings provide support for the development of astrocyte-specific therapeutics to prevent neurodegenerative disease progression.

Increase in 4R tau levels in vivo and in vitro leads to astrocyte reactivity

Chapter 2 expanded upon our previously published study where we found that increasing the ratio of 4R to 3R tau using 3R to 4R tau splicing ASOs led to an increase in phosphorylated tau deposition, an increase in seizure severity, and abnormal murine behavior. Surprisingly, no overt neuronal phenotype was observed, which cause us to examine other cell types which may be responsible, namely astrocytes. We found that 4R tau appeared to co-localize with astrocytes that seemed to be reactive (**Figure 2.1A-H**). There was increased expression of *GFAP* and other genes associated with either a pan-reactive or neurotoxic phenotype in whole brain lysates isolated from hTau mice that had been treated with the 3R to 4R tau splicing ASOs than in those that were treated with a control ASO or the 4R to 3R tau splicing ASO (**Figure 2.1I)**. Given that neurons express tau at exponentially higher levels than other cell types in the brain, it was difficult to identify if tau deposition or expression in astrocytes was driving the phenotype that

was seen, if the ASOs used may have preferentially impacted astrocytic tau splicing, or if astrocytes were phagocytosing the 4R tau from the surrounding area to prevent further damage. Therefore, we began to perform our studies in a primary astrocyte model to better understand the impact of tau expression and the effect of altering the ratios of tau isoforms in astrocytes alone.

The primary astrocyte cultures that we created allowed us to identify how altering tau isoform ratios in astrocytes affected their homeostatic functions and reactivity. We determined that primary hTau astrocytes treated with the 3R to 4R tau splicing ASOs exhibited similar morphologic and genetic changes to those seen *in vivo* (**Figure 2.2A-I)**. These results led us to believe that the genetic changes seen in the hTau mice with higher 4R tau could be driven by astrocytic changes. As astrocyte reactivity is commonly associated with changes in homeostatic function, we next explored whether increased levels of 4R tau led to altered glutamate clearance and vulnerability to oxidative stress. We found that increased levels of 4R tau in astrocytes led to a decreased ability to remove excess glutamate from the media (**Figure 2.2J)** and that 4R tau expression led to a reduced expression of *Eaat2*, an astrocytic glutamate receptor (**Figure 2.4**). These findings provided further evidence that the increase in seizure severity we saw *in vivo* was due to dysfunctional astrocytes and suggested that the presence of 4R tau in astrocytes leads to a reduction in glutamate uptake ability and may cause neuronal hyper-excitability. As astrocytic ability to metabolize glutamate may be reduced following oxidative stress (Oksanen M, 2019), and oxidative stress leads to the progression of neurodegenerative disease, we wanted to identify if there would be an increase in cytotoxicity of astrocytes following exposure to hydrogen peroxide (H_2O_2). We found that following exposure to 100μM of H_2O_2; there was a higher level of cytotoxicity in the hTau astrocytes that had increased levels of 4R tau than in those treated

with the control ASO or the 4R to 3R tau splicing ASO, suggesting that the expression of 4R tau in astrocytes leads to their increased vulnerability to oxidative stress (**Figure 2.2K**). This vulnerability to oxidative stress also translated to increased neuronal death when cultured with 4R tau expressing astrocytes (**Figure 2.3**). We did not identify a change in neuronal survival in co-cultures that were not exposed to H_2O_2, similar to what we had seen *in vivo*, which suggests that 4R tau expression in astrocytes is detrimental to neuronal survival following secondary stressors as is common in neurodegenerative diseases. These results are novel in showing that 4R tau expression in astrocytes is sufficient to reduce glutamate uptake and leads to increased astrocytic vulnerability to oxidative stress, which leads to an increase in neuronal death. However, given that these experiments were done *in vitro*, there are some limitations to the findings.

As astrocytes in culture are different from those *in vivo,* it is possible that we are not accurately representing what happens in disease. To quantify cell-specific changes following 3R to 4R tau splicing, we can use mouse models that have cell type-specific expression of the GFP-tagged ribosomal protein, RPL10a. This will allow us to perform translating ribosome affinity purification (Hoye et al. 2017; Tryon et al. 2013) (TRAP) methods to isolate actively translated mRNAs from neurons using the hTau$^{+/-}$;mTau$^{-/-}$;SNAP25-L10a-GFP mouse line or astrocytes using the hTau$^{+/-}$;mTau$^{-/-}$;Aldh1-L10a-GFP mouse line. These mice can be used to determine how an ASO-mediated increase in 4R tau affects genetic expression in these distinct cell types, to elucidate the mechanisms of 4R tau-mediated toxicity in astrocytes and neurons to understand how best to target them to prevent the progression of disease.

4R tau expression in human astrocytes leads to similar changes as seen in primary cultures

Chapter 3 explored if increased expression in human astrocytes would lead to similar genetic and functional changes as previously seen in primary hTau astrocytes. We chose to create astrocytes from IVS 10+16 iPSCs, and their isogenic controls as the cells with the mutation express 4R tau directly post differentiation, unlike other iPSC lines (Verheyen et al. 2018). By using a cell line that has higher levels of 4R tau without treatment with ASOs or transfection with a 4R tau overexpression plasmid, it allowed us to identify if the increase in neurotoxic genes and reduced homeostatic functions seen in primary cultures was due to 4R tau expression itself and not a response to ASO toxicity. The isogenic control provided an additional layer of assurance that the changes we saw were due only to the increase in 4R tau levels.

IVS 10+16 iAstrocytes showed similar changes in upregulation of neurotoxic and pan-reactive genes seen in the hTau astrocytes treated with the 3R to 4R tau splicing ASO as compared to their isogenic controls (**Figure 3.1A)**. The upregulation of a similar set of genes (*Vim*, *C3*, *Serpina3n*) suggests that the complement and inflammatory pathways are involved in 4R tau-mediated astrocyte reactivity. Further, we found that 4R tau expression in human astrocytes led to an increased vulnerability to oxidative damage and that the astrocytes themselves were more prone to death than their isogenic controls (**Figure 3.1C**) at baseline and following treatment with hydrogen peroxide. While 4R tau expressing hTau astrocytes were not more prone to death at baseline, human astrocytes are much more sensitive than murine astrocytes (J. Li et al. 2020), so human astrocytes would likely be more prone to death if they are reactive and dysfunctional. Given that we saw a decreased ability to recycle glutamate in both murine in human 4R tau expressing astrocytes, we were interested in seeing if the presence of 4R tau would lead to neuronal hyper-excitability. We found that neurons co-cultured with the IVS 10+16 iAstrocytes

were hyper-excitable, exhibited hyper-synchrony, and were more prone to death than those cultured with the isogenic control (**Figure 3.2 and 3.5**).

We had previously seen that lowering levels of 4R tau was beneficial to astrocytic function in hTau astrocytes, and we hypothesized that this would be similar in human astrocytes. Unfortunately, the IVS 10+16 mutation is located right where the previously developed 4R to 3R tau splicing ASO binds, and therefore, it was not able to lower levels of 4R tau (**Supplementary figure 3.1S**). As our lab had shown the effectiveness of a total tau lowering ASO in preventing the progression of disease (DeVos et al. 2017), we proceeded with it for our experiments in place of the 4R to 3R tau splicing ASO. IVS 10+16 iAstrocytes treated with the total tau lowering ASO exhibited decreased expression of reactive and neurotoxic genes that had been previously upregulated, and many were not different from their isogenic control (**Figure 3.3A**). Glutamate uptake was increased, and vulnerability to oxidative stress was reduced following treatment with the total tau lowering ASO (**Figure 3.3B-C**). We also found that neuronal hyper-excitability, hyper-synchrony, and death were reduced when the neurons were cultured with the IVS 10+16 iAstrocytes treated with the tau lowering ASO compared to those treated with the control ASO (**Figure 3.4** and **3.5**) and were no different from their isogenic controls. These results strongly suggest that 4R tau expression in human astrocytes can create an environment that leads to neuronal death and disease progression. Future studies will focus on identifying if drugs that increase glutamate uptake may reduce neuronal hyper-excitability similar to what was seen with tau knockdown.

Some have suggested that lowering tau levels throughout the central nervous system may have deleterious effects as tau plays a crucial role in many cellular processes (Biundo et al. 2018). This finding supports the concept of creating ASOs that can be targeted to specific cell types, allowing for more potent therapeutics. IONIS pharmaceuticals has been studying how best to create cell-specific ASOs by conjugating ligands to the ASOs, which should allow for the ASO to be preferentially taken up by the cells that express the ligands receptor (Tanowitz et al. 2017; Youngsoo Kim et al. 2019). These have shown incredible promise, although this has not been tested in the central nervous system. Astrocytes express many receptors that can act as potential targets for ligand conjugated ASOs, such as the metabotropic glutamate receptors that are upregulated in many neurodegenerative diseases (Planas-Fontánez, Dreyfus, and Saitta 2020). This would allow for the use of the tau lowering ASOs in astrocytes without impacting total tau levels throughout the CNS which may prove to be beneficial. Alternatively, as many primary tauopathies are 4R tauopathies, optimizing the 4R to 3R tau splicing ASO can be powerful. These could also be modified for use as a personalized therapy by designing the ASOs through micro walking to identify which particular sequence would create the most impactful ASO for specific disease-causing mutations(Singh et al. 2010; Schoch and Miller 2017).

Pre-trans-splicing molecules (PTMs) may be used to alter tau splicing in astrocytes

Chapter 4 described preliminary results from experiments done to identify if PTMs can be used similarly to our previously used ASOs. We found that the 3R to 4R tau splicing PTM increased the ratio of 4R to 3R tau in hTau astrocytes, although at a lower rate than our ASOs (**Figure 4.2**), which also led to an increase in reactive gene expression (**Figure 4.3**). The increases in reactive genes were not all significant, but they were trending. This could be the PTMs did not increase

the ratio of 4R:3R as much as the ASOs. However, this does show that PTMs may be used to alter tau levels in a cell-specific manner. PTMs can be packaged into a cell-specific or a cre dependent viral vector and delivered to the central nervous system; making it possible to only impact specific cells. While viral vectors are not the ideal candidate for therapeutics, these experiments would allow us to see the impact of altering tau isoforms in certain cell types while cell-specific ASOs are being developed.

Future Directions

There are many future directions that can be taken to follow up on the experiments and findings described in this dissertation. They range from basic biological questions of how the alteration of tau isoforms using ASOs changes the half-life of tau in neurons and astrocytes to more translationally relevant ones on identifying if there is a link between 4R tau expression in astrocytes and the progression of tuberous sclerosis. The rationale and potential experiments are all described below.

Half-life and profiling of tau in neurons and astrocytes

Though tau is primarily an intracellular protein, new data have shown that tau is secreted into the extracellular space under normal and pathologic conditions (Karch, Jeng, and Goate 2013). In neurodegenerative disease, the amount of unphosphorylated and phosphorylated tau in the cerebrospinal fluid (CSF) is increased and can predict the magnitude of disease pathology (Toledo et al. 2013; Bateman et al. 2012; Fagan et al. 2014). In order to identify if this increase

was due to an increase in tau secretion from dying cells or decreased clearance, the half-life of tau was measured. Sato et al. identified that the half-life of tau was 6.74 ± 0.45 days in iPSC-derived neurons and 23 ± 6.4 days in the human CNS (Sato et al. 2018). They also identified that 4R tau has a faster turnover rate than 3R tau, although it remains unclear why. While this study provided critical information on the half-life of tau in iPSC neurons, it remains unclear if altering the ratios of tau isoforms has an impact on tau half-life. Future studies can use either the 3R to 4R or the 4R to 3R tau splicing ASOs in an hTau mouse model as well as in hTau mouse primary neurons and astrocytes to see how or if increasing the ratio of 3R:4R tau has an impact on tau turnover and half-life. These experiments would help to identify how effective these ASOs could be as a potential therapeutic.

Mass spectrometry is a powerful tool that allows for a more specific examination of how tau is altered in disease. It can identify modifications, truncations, and sites of phosphorylation (Barthélemy et al. 2019). As these all impact how tau functions and are altered in disease, it is essential to profile the tau found in astrocytes and neurons to understand the role that astrocytic tau plays in disease progression. To do this, future studies will involve the isolation and purification of both neurons and astrocytes from hTau mice that will then be treated with a control, 3R to 4R and a 4R to 3R tau splicing ASO. The cells would then be analyzed for isoform expression, sites of phosphorylation, truncation, and other post-translational modifications. These experiments will provide crucial information on how ASO treatment impacts tau other than altering its splicing patterns, giving a better idea of how best to target tau for future therapies.

Identifying how the presence of 4R tau in astrocytes leads to activation

Astrocyte reactivity and activation is a common hallmark of neurodegenerative disease (Haim et al. 2015). Our data suggest that 4R tau expressing astrocytes are reactive and neurotoxic, which may occur through a variety of pathways, including the complement cascade, the inflammatory pathway, and activation of the STAT3 pathway. Activation of the STAT3 pathway has been linked to astrocytic activation and decreased ability to recycle glutamate (R. Wang et al. 2018; Moidunny et al. 2016). We also found that 4R tau expressing astrocytes express lower levels of *EAAT2,* which has been correlated to increasing tau pathology (Simpson et al. 2010). Our results show that the presence of tau leads to decreased glutamate uptake ability. Still, future studies will need to focus on identifying how 4R tau expression in astrocytes leads to increased activation of STAT3 and if this plays a role in disease progression. These experiments can identify a potential avenue for therapeutics as many tauopathies and other neurodegenerative diseases exhibit a buildup of glutamate at the synapse (Kamat et al. 2016).

4R tau expressing astrocytes also exhibited an increased vulnerability to oxidative stress, which was reduced following lowering levels of either 4R tau or total tau. As astrocytes are involved in preventing neuronal damage by protecting them from oxidative stress (Y. Chen et al. 2020; González-Reyes et al. 2017), future experiments must focus on identifying how the presence of 4R tau in astrocytes leads to their increased vulnerability to oxidative stress.

Impact of 4R tau expression in astrocytes on sleep

Circadian rhythm disruption is common in a number of neurodegenerative diseases, including Alzheimer's disease (AD) and other tauopathies (Musiek 2015). It has recently been suggested

that tau phosphorylation increases and decreases following the circadian pattern (Guisle et al. 2020) and that both sleep deprivation and poor sleep can induce tau phosphorylation(Rothman et al. 2013; Di Meco, Joshi, and Praticò 2014; Musiek, Xiong, and Holtzman 2015). Additionally, it has been shown that tau expression in a drosophila model was sufficient to cause circadian abnormalities similar to those in tauopathy patients (Jaciuch et al. 2020).

Progressive supranuclear palsy (PSP) is a 4R tauopathy where patients experience significant sleep pattern disruptions (Abbott and Videnovic 2014). Additionally, 4R tau deposition or expression in tufted astrocytes is a hallmark of PSP (M. Yoshida 2014). We have identified that 4R tau deposition and expression leads to astrocytic dysfunction. This suggests that astrocytic tau may play a role in PSP progression. Given that there is emerging evidence linking disruption of the astrocytic circadian clock to neurodegenerative disease progression (McKee, Lananna, and Musiek 2020), it stands to reason that 4R tau deposition in astrocytes may impact sleep. Future studies can identify if human tau expressing mice that were treated with the 3R to 4R tau splicing ASO experience disruptions in their sleep/wake patterns, as compared to those treated with a control ASO and those treated with a 4R to 3R tau splicing ASO. Further, iPSC-derived astrocytes from PSP patients can be co-cultured with iPSC-derived neurons, and the impact of 4R tau on circadian rhythm can be analyzed using the microelectrode array (Ilaria et al. 2016). These experiments would also allow for testing of the tau lowering ASO and its potential use as a therapeutic in neurodegenerative disease patients that exhibit circadian rhythm dysfunction.

Interaction of Fyn and 4R tau in astrocytes

The interaction between the Src family non-receptor tyrosine kinase Fyn and tau has been shown to lead to the progression of disease (Briner, Götz, and Polanco 2020). Fyn interacts with tau at the N terminal and phosphorylates it at residue Y18 which leads to a greater affinity between Fyn and tau (Briner, Götz, and Polanco 2020; Alavi Naini and Soussi-Yanicostas 2015). Previously, the activation of Fyn by oligomeric amyloid-beta has been shown to impair synaptic plasticity (Spires-Jones, Attems, and Thal 2017). Tau accumulation at the synapse has been shown to be detrimental and associated with disease progression and may negatively impact synaptic plasticity (Tai et al. 2012; Sydow et al. 2011). These data suggest that a potential mechanism exists between the three in disease progression.

The level of interaction between Fyn and tau increases following tau phosphorylation, with FTD causing mutations leading to a 25-45 fold increase in interaction between 4R tau and Fyn (Bhaskar, Yen, and Lee 2005). Recent data have shown that reduction of Fyn levels, and consequently, its interaction with tau, led to a decrease in seizure severity (Putra et al. 2020). Interestingly, astrocytes express Fyn at approximately equal levels as neurons in mice and much higher levels than neurons in humans (Zhang et al. 2014, 2016). Our data show that 4R tau expression in astrocytes leads to neuronal hyper-excitability. Taking all of this into consideration, it is possible that Fyn may interact with 4R tau in astrocytes and cause the neuronal hyper-excitability that we found. Future studies can explore this further by identifying if Fyn does interact with tau in astrocytes and if altering Fyn levels in astrocytes alone may improve the seizure phenotype we see when 4R tau levels are increased. Alternatively, it is possible to see lowering the amount of 4R tau in astrocytes, and therefore its interaction with Fyn can lead to a reduction in seizure severity and neuronal hyper-excitability.

Tau isoform localizing in astrocytes

The localization of tau throughout the cell has been shown to play an important role in disease progression (Richetin et al. 2020; Bachmann et al. 2021; Tang et al. 2015). Future experiments can expand upon the work described here by taking advantage of the new imaging technology using super-resolution microscopy to better identify where and which type of tau is localizing throughout astrocytes and at the astrocytic end feet (Pizzarelli, Pediconi, and Di Angelantonio 2020). Other labs have pioneered the use of SEQUIN, which allows for the characterization of synaptic nanostructure and can be used in tandem with tau antibodies to better identify how exactly tau localizes at the synapse (Sauerbeck et al. 2020). These experiments would provide crucial information on how the 3R and 4R tau isoforms localize throughout different cell types, which could help to understand why 4R tau expression in astrocytes leads to neuronal hyper-excitability.

Role of astrocytic 4R tau expression in epilepsy

Epilepsy is a common neurological disorder characterized by the occurrence of unprovoked seizures (Beghi, Giussani, and Sander 2015; Binder and Steinhäuser 2021). It has long been considered that epilepsy may be an astrocytopathy as astrocytes have an established role in the removal of glutamate from the synapse to prevent aberrant neuronal activity (Perez-Catalan, Doe, and Ackerman 2021). We previously identified that altering the ratio of tau isoforms towards higher 4R tau expression led to an increase in seizure severity and latency (Schoch et al. 2016) and that lowering tau levels *in vivo* led to a decrease in seizures (DeVos et al. 2017). In this dissertation, we described that this might be due to 4R tau expression and deposition in

astrocytes. In order to parse out the involvement of 4R tau expressing astrocytes on the progression of epilepsy, future experiments can focus on identifying if anti-epileptic drugs lead to a similar result as lowering tau in astrocytes alone. These experiments would help show if even low levels of tau expression in astrocytes may be responsible for the progression of various neurologic and neurodegenerative diseases.

Impact of 4R tau expression in astrocytes in the progression of tuberous sclerosis

Tuberous sclerosis (TSC) is a rare childhood-onset disease that is characterized by the appearance of benign tubers in the brain and other vital organs, epilepsy, dermatological findings, and developmental delay owing to pathogenic genetic variants in the *TSC1* (hamartin) or *TSC2* (tuberin) genes (Olney et al. 2017; Alquezar et al. 2020). *TSC1* and *TSC2* are inhibitors of the mammalian target of rapamycin (mTOR). Mutations in *TSC1* and *TSC2* prevent them from functioning, leading to overactivation of mTOR (Curatolo and Moavero 2012) which leads to tuber formation throughout the body.

Changes in astrocytic glutamate clearance, potassium, and water homeostasis have all been associated with neuronal hyperexcitability and epilepsy, which occurs in 80-90% of all TSC patients (Binder and Steinhäuser 2021). It has long been considered that the seizures seen in TSC were caused by the tubers that form throughout the brain, which are made up of giant cells and have astrocytosis (Binder and Steinhäuser 2021), although this does not directly prove astrocyte involvement in disease. More recent data have identified that astrocytes in TSC patients exhibit reduced glutamate uptake ability (D. R. Miller et al. 2021) and that mouse models for TSC

exhibit similar disruption in glutamate and potassium clearance (Wong et al. 2003). Taken together, these data suggest that dysfunctional astrocytes may play a role in TSC disease.

Certain TSC patients may present with behavioral changes that are associated with behavioral variant frontotemporal dementia (bvFTD) (Olney et al. 2017; J. Liu et al. 2019). About 40% of all bvFTD cases also present with tau pathology (Bahia, Takada, and Deramecourt 2013). Recent data has identified that over activation of mTOR led to increased deposition of phosphorylated tau in the cytoplasm of cells which has been shown to lead to the progression of Alzheimer's disease (Tang et al. 2015), suggesting that there may be a link between TSC and tauopathies. We identified that an increase in 4R tau in mice led to increased seizure severity and phosphorylated tau deposition (Schoch et al. 2016) and that 4R expressing astrocytes exhibited lower levels of glutamate transporters, had decreased glutamate uptake ability, and caused increased neuronal excitability, all of which is seen in TSC. While it is still unknown if tau isoform expression plays a role in TSC, our data suggest that 4R tau expression in astrocytes may potentially be involved in TSC pathology and progression and is an avenue that should be explored further. Future studies can take advantage of the TSC mouse model that has already been bred onto a hTau mouse background and identify how altering the ratios of the tau isoforms impacts disease. Additionally, the *in vitro* system that we developed would be a powerful tool to see if modifying tau isoform levels in astrocytes derived from TSC patients would alter their glutamate uptake ability and if this would impact neuronal hyper-excitability.

Concluding Remarks

Despite being considered to be no more than a supporting cell of the brain for many years, astrocytes are now becoming widely studied, with their dysfunction being linked to neurodegenerative progression. However, we still do not truly understand the impact that tau expression has on astrocytes. The experiments described here show that alteration of the low levels of tau towards higher expression of 4R tau is sufficient to cause them to take on a neurotoxic genetic phenotype, increase their vulnerability to oxidative stress, and reduce their ability to properly maintain synaptic homeostasis thereby causing neuronal hyper-excitability and hyper-synchrony along with neuronal death. We found that by lowering either 4R tau or total tau levels in astrocytes alone, it was possible to rescue astrocytic function. These findings lay the foundation for creating cell specific therapeutics and suggest that altering tau in astrocytes may be a viable therapeutic target.

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Ph.D. Candidate in Neurosciences
(Expected December 2021)
Teaching Citation

Washington University in St. Louis
Bachelor of Arts, May 2014
Major: Biochemistry
Minor: Russian Language and Literature
Senior Thesis: Differential Expression of Alzheimer's Disease Risk Genes in Human Brain Tissue
Thesis Advisor: Alison Goate

Positions and Employment

2020 – 2020	Graduate Teaching Assistant, University College, Washington University in St.
2017 - 2017	Louis
	Graduate Teaching Assistant, Department of Neuroscience, Washington University in St. Louis
2016 – Present	Graduate Student Researcher, Washington University in St. Louis
2015 - 2016	Research Technician II, Department of Cardiology, Washington University in St. Louis
2014 - 2015	Research Technician I, Department of Psychiatry, Washington University in St. Louis
2013 - 2014	Undergraduate Research Assistant, Department of Psychiatry, Washington University in St.Louis
2012 - 2013	STARS Student Mentor, Department of Developmental Biology, Washington University in St.Louis
2011 - 2013	Undergraduate Research Assistant, Department of Developmental Biology, WashingtonUniversity in St. Louis
2009 - 2010	High School Research Assistant, Saint Louis University

Other Experience and Professional Memberships

2019 - Present	Member, American Neurological Association
2018	Shadowed Dr. Nupur Ghoshal as part of the Markey Pathway

Honors and Awards

2021	Tau Consortium Fellow, Tau Consortium
2020	Tau Consortium Fellow, Tau Consortium
2019	Winner of Poletsky Award for distinguished research in Aging and
2019	DementiaPoster award winner at the American Neurological
2019	Association meeting
2019	Tau Consortium Fellow, Tau Consortium
2019	Recipient of travel award to the American Neurological

Association meetingSelected to present at the Distinguished Alumni Seminar Poster Session

2017 - 2019	Lucille P. Markey Student, Washington University in St. Louis
2013 - 2013	Honorary Scholars Summer Research Grant Award, Washington University in St. Louis
2010 - 2014	Florence M. Moog Scholar, Washington University in St. Louis
2009 - 2009	Pfizer Award for Excellence in Research, Pfizer
2009 - 2009	Distinguished Student Speaker at Solae LLC., Solae LLC.

Publications

- *4R tau expression in astrocytes promotes their dysfunction and contributes to neurodegenerative diseaseprogression.* **Lubov A. Ezerskiy,** Kathleen M. Schoch, Chihiro Sato, Mariana Beltcheva, Frank Rigo, Ryan Martynowicz, Celeste M. Karch, Randall J. Bateman, Timothy M. Miller**.**. Under Revision JCI Insight.

- *Acute Trem2 reduction triggers increased phagocytosis, slowing amyloid deposition*. Kathleen M. Schoch, **Lubov A. Ezerskiy**, Michaela M. Morhaus, Riley N. Bannon, Andrew D. Sauerbeck, Mark Shabsovich, Paymaan Jafar-Nejad, Frank Rigo, Timothy M. Miller. Proc Natl Acad Sci U S A. 2021 Jul 6;118(27):e2100356118. doi: 10.1073/pnas.2100356118. PMID: 34187891; PMCID: PMC8271763.

- P Griffin, JM Dimitry, BV Lananna, ML Robinette, MR Cedeño, CJ Nadarajah, **LA Ezerskiy**, M Colonna,AQ Bauer, TP Burris, ES Musiek. *The circadian clock protein Rev-erb α regulates neuroinflammation*. Proceedings of the National Academy of Sciences Mar 2019, 116 (11) 5102-5107.

- CM Karch, **L Ezerskiy**, V Redaelli, AR Giovagnoli, P Tiraboschi, G Pelliccioni, P Pelliccioni, D Kapetis, ID'Amato, E Piccoli, F Tagliavini, G Rossi. Missense mutations in progranulin gene associated with frontotemporal lobar degeneration: study of pathogenetic features. Neurobiology of Aging. (2016)38:215.e1-12.

- CM Karch, **LA Ezerskiy**, S Bertelsen, Alzheimer's Disease Genetics Consortium (ADGC), AM Goate. *Alzheimer's disease risk polymorphisms regulate gene expression in the ZCWPW1 and the CELF1 loci.*PLOS One. (2016) 11(2): e0148717.

- R Martinez R, S Hsu, **LA Ezerskiy**, HJ Shu, S Mennerick, AM Goate, CM Karch. *Efficient production, banking and terminal differentiation of neural progenitor cells from human induced pluripotent stemcells.* Under Review, BMC Neuroscience

- Hahn JW, Jagwani S, Kim E, Rendell VR, He J, **Ezerskiy LA**, Wesselschmidt R, Coscia CJ, Belcheva MM. *Mu and kappa opioids modulate mouse embryonic stem cell-derived neural progenitor differentiationvia MAP kinases.* J Neurochem. (2010) 112(6):1431-41

- Ikeda H, Miyatake M, Koshikawa N, Ochiai K, Yamada K, Kiss A, Donlin MJ, Panneton WM, Churchill JD, Green M, Siddiqui AM, Leinweber AL, Crews NR, **Ezerskiy LA**, Rendell VR,

Belcheva MM, Coscia CJ. *Morphine modulation of thrombospondin levels in astrocytes and its implications for neurite outgrowthand synapse formation*. J Biol Chem. (2010) 285(49):38415-27.

Oral Presentations and Invited Talks

Poster Presentations:

- *Dysregulation of function in 4R tau expressing astrocytes. .* **Lubov A. Ezerskiy**, Kathleen M. Schoch, Frank Bennett, Frank Rigo, Timothy M. Miller. (July 2020) Glia in Health and Disease (Virtual) meeting.

- *An Increase in 4R Tau Levels in Astrocytes Leads to Dysregulation of Function*. **Lubov A. Ezerskiy**, Kathleen M. Schoch, Frank Bennett, Frank Rigo, Timothy M. Miller. (October 2019) American Neurological Association meeting.

- *Increase in 4R tau expression in astrocytes leads to dysregulation of function*. **Lubov A. Ezerskiy**, Kathleen M. Schoch, Frank Bennett, Frank Rigo, Timothy M. Miller. (June 2019) Keystone Meeting on Neural Environment in Disease: Glial Responses and Neuroinflammation.

- *Increase in 4R tau expression promotes astrocytic dysfunction*. **Lubov A. Ezerskiy**, Kathleen M. Schoch, Frank Bennett, Frank Rigo, Timothy M. Miller. (April 2019) Washington University Hope Center Retreat.

- *Increase in 4R tau expression promotes astrocytic dysfunction***. Lubov A. Ezerskiy**, Kathleen M. Schoch,Frank Bennett, Frank Rigo, Timothy M. Miller. (January 2019) Tau Consortium Investigators Meeting.

- *Identifying Tau Isoform Localization and its Role in Astrocyte Function*. **Lubov A. Ezerskiy**, Kathleen M. Schoch, Frank Bennett, Frank Rigo, Timothy M. Miller. (April 2018) Washington University Hope CenterRetreat.

- *Differential Expression of Alzheimer's Disease Risk Genes in Human Brain Tissue.* Poster Presentation.**Lubov Ezerskiy**, Celeste Karch, Alison Goate. Washington University in St. Louis Spring UndergraduateResearch Symposium, 2014.

- *CCR7 Chemokine GPCR Acts as a Negative Regulator of the Wnt/β-Catenin Pathway and CellProliferation in SW480 Colon Carcinoma Cells*. Poster Presentation. **Lubov Ezerskiy**, Mariana Beltcheva, Lilianna Solnica-Krezel. Washington University in St. Louis Fall Undergraduate Research Symposium, 2013.

- *Chemokine GPCR CCR7 Acts as a Negative Regulator of Wnt/β-Catenin Pathway and Cell Proliferation in SW480 Colon Carcinoma Cells*. Poster Presentation. **Lubov Ezerskiy**, Mariana Beltcheva, Lilianna Solnica-Krezel. Washington University in St. Louis Spring Undergraduate Research Symposium, 2013.

- *Chemokine Receptor CCR7 Acts as a Negative Regulator of Cell Proliferation and Wnt/β-*

catenin Pathway in Human Embryonic Stem Cells. Mariana Beltcheva, Sebastian Chung, **Lubov Ezerskiy** and Lilianna Solnica-Krezel Department of Developmental Biology, Washington University, School of Medicine, St. Louis, MO 63110, USA. Stem Cells in Cancer and Regenerative Medicine EMBL Heidelberg, Germany

Oral Presentations:

- *4R tau expression in astrocytes leads to their dysfunction and neuronal hyper-excitability*. **Lubov Ezerskiy** (2021) Tau Consortium Meeting.
- *Increase in 4R tau expression promotes astrocytic dysfunction*. **Lubov Ezerskiy**. (2020) Hope Center forNeurological Disorders Monday Noon Seminar.
- *4R tau expressing astrocytes exhibit aberrant dysfunction and activation*. Lubov Ezerskiy, Timothy Miller.(2019) Presented at the Markey Pathway retreat.
- *Identifying Tau Isoform Localization and its Role in Astrocyte Function*. **Lubov Ezerskiy**, Timothy Miller.(2018) Presented at Joint Tau Meeting, Washington University in St. Louis.
- *Identifying Tau Isoform Localization and its Role in Astrocyte Function*. **Lubov Ezerskiy**, Kathleen Schoch, Timothy Miller. (2017) Presented at Works in Progress Seminar, Washington University in St. Louis.
- *The Mechanism of Morphine Inhibition on Synapse Formation in Primary Astrocyte-Neuron Co-Cultures*. **Lubov Ezerskiy**. University of Missouri St. Louis STARS. 2009

Mentorship Experience

Graduate Students

2020	Benjamin Boros
2018	Shivani Aryal
2018	McKenna Wilhelm

Undergraduate Students

2019 - Present	Ryan Martynowicz
2019 - 2020	Michaela Morhaus
2015	Chimezie Ileje
2012-2013	Tony Wang

High School Students

2012 - 2013	Yash Patel and Priyanka Mahapatra