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Link(s) to article on publisher's website:

<http://dx.doi.org/doi:10.21954/ou.ro.00014014>

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Role of Cerebrovascular Cells in Tau Processing Following Traumatic Brain Injury

Maxwell Eisenbaum

A thesis submitted for the degree of Doctor of Philosophy in the discipline of Neuroscience

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Date of Submission: November 2021



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DECLARATION:

I hereby declare that the work presented in this thesis is my own, except for where stated. This work has not been submitted for any other degree of professional qualification.

Max Eisenbaum

PUBLICATIONS:

Ringland C, Schweig J, Paris D, Shackleton B, Lynch C, **Eisenbaum M**, Mullan M, Crawford F, Abdullah L, Bachmeier C. Apolipoprotein E isoforms differentially regulate matrix metalloproteinase 9 function in Alzheimer's disease. *Neurobiology of Aging* 2020; 95:56-68. doi: 10.1016/j.neurobiolaging.2020.06.018.

Lynch C, **Eisenbaum M**, Algamal M, Balbi M, Ferguson S, Mouzon B, Saltiel N, Ojo J, Diaz-Arrastia R, Mullan M, Crawford F, Bachmeier C. Impairment of cerebrovascular reactivity in response to hypercapnic challenge in a mouse model of repetitive mild traumatic brain injury. *Journal of Cerebral Blood Flow & Metabolism* 2020; 41(6):1362-1378. doi: 10.1177/0271678X20954015.

Ojo J, **Eisenbaum M**, Shackleton B, Lynch C, Joshi U, Saltiel N, Pearson A, Ringland C, Paris D, Mouzon B, Mullan M, Crawford F, Bachmeier C. Mural cell dysfunction leads to altered cerebrovascular tau uptake following repetitive head trauma. *Neurobiology of Disease* 2020; 150:105237. doi: 10.1016/j.nbd.2020.105237.

Ojo J, Reed J, Crynen G, Vallabhaneni P, Evans J, Shackleton B, **Eisenbaum M**, Ringland C, Edsell A, Mullan M, Crawford F, Bachmeier C. Molecular Pathobiology of the Cerebrovasculature in Aging and in Alzheimer's Disease Cases with Cerebral Amyloid Angiopathy. *Frontiers in Aging Neuroscience* 2021; 13(658605). doi: 10.3389/fnagi.2021.658605.

Ringland C, Schweig J, **Eisenbaum M**, Paris D, Ait-Ghezala G, Mullan M, Crawford F, Abdullah L, Bachmeier C. MMP9 modulation improves specific neurobehavioral deficits in a mouse model of Alzheimer's disease. *BMC Neuroscience* 2021; 22:39. doi: 10.1186/s12868-021-00643-2.

Eisenbaum M, Pearson A, Gratkowski A, Mouzon B, Mullan M, Crawford F, Ojo J, Bachmeier C. Influence of traumatic brain injury on extracellular tau elimination at the blood–brain barrier. *Fluids and Barriers of the CNS* 2021; 18:48. doi: 10.1186/s12987-021-00283-y.

Ojo J, Reed J, Crynen G, Vallabhaneni P, Evans J, Shackleton B, **Eisenbaum M**, Ringland C, Edsell A, Mullan M, Crawford F, Bachmeier C. APOE genotype dependent molecular abnormalities in the cerebrovasculature of Alzheimer's disease and age-matched non-demented brains. *Molecular Brain* 2021; 14:110. doi: 10.1186/s13041-021-00803-9

ACKNOWLEDGMENTS

It is a pleasure to thank the following people, without whom I would not have been able to complete this research.

I would first like to express my sincere gratitude to my supervisor Dr. Corbin Bachmeier for the continuous and unwavering support throughout the course of my Ph.D. studies. His patience and guidance were foundational for my growth and development as a scientist.

I'm extremely grateful for my secondary supervisor, Dr. Joseph Ojo. His practical suggestions and constructive criticism always kept me on the right path.

Of course, I'm deeply indebted to Dr. Fiona Crawford and Dr. Mike Mullan for their support. I am thankful for Dr. Crawford's encouragement to explore adjacent areas of research to gain new perspectives into my own field, and for Dr. Mullan's comprehensive knowledge and perpetual reminders to consider the broader implications of any observations made, either by myself or others.

I would like to say a big thank you to the other PIs at the Roskamp Institute, in particular Dr. Daniel Paris, Dr. Benoit Mouzon, and Dr. Laila Abdullah, for their individual expertise and help in various aspects of my project. I would like to acknowledge and thank Dr. Ben Shackleton for his prior research which provided the foundation for these studies. I would also like to thank the other current and past students and technicians that have helped me and who have made my overall experience enjoyable. In particular, Cillian Lynch, Utzav Joshi, Moustafa Algamal, Coral Hahn-Townsend, Ariane Menden, Charis Ringland, Camila Ortiz, Claire Huguenard, Alex Morin, Arissa Gratkowski, Anastasia Edsell, and Teresa Darcy.

In particular, I would like to thank Dr. Andrew Pearson for his insightful dialogue and lively discussions over the past four years. His considerable expertise and willing participation in challenging experiments reliably and consistently improved the quality of the science contained within this thesis.

I want to express my true gratitude to my family, and especially my parents, Wayne and Emily Eisenbaum, for their support of my education and of the choices I make in life.

Finally, it is my great privilege to thank my brilliant wife, Dr. Megan Spelman for her constant support and insight. Throughout the entirety of my scientific career, she has set a standard of excellence which I will always strive to match.

ABSTRACT

Repetitive exposure to mild traumatic brain injuries (r-mTBI) sustained through the participation in contact sports can lead to chronic post-concussive symptoms and the development of neurodegenerative diseases such as Alzheimer's disease and Chronic Traumatic Encephalopathy (CTE). A primary hallmark of CTE is the accumulation of pathogenic tau in neurons and astrocytes that surround small blood vessels in the brain. Chronic exposure to r-mTBI leads to elevated levels of extracellular tau and pathogenic tau accumulation in neurons, ultimately resulting in neuronal death. While the mechanisms responsible for pathogenic tau elimination from the brain are unclear, our prior work demonstrated that cells associated with the cerebrovasculature can interact with extracellular tau and may contribute to the removal of extracellular tau from the brain.

In this thesis, I examined the mechanisms through which the cerebrovascular cells eliminate extracellular tau from the brain and how those processes are impacted by r-mTBI. I demonstrated that brain vascular mural cells (pericytes and smooth muscle cells) progressively degenerate following exposure to r-mTBI consistent with what is observed in individuals with AD. This mural cell dysfunction impairs the ability of the cerebrovessels to interact with tau. Furthermore, I found that the cerebrovasculature can eliminate extracellular tau from the brain through caveolae-mediated endothelial transcytosis, which is impaired following chronic exposure to r-mTBI. The diminished tau transit across the blood-brain barrier following brain injury may be a contributing factor in the pathogenic tau accumulation observed in CTE.

A significant genetic risk factor for neurodegenerative diseases including AD and CTE is possession of the E4 isoform of Apolipoprotein E (ApoE). Astrocytes are the predominant source of ApoE in the brain, though there is very little understanding regarding their interactions with extracellular tau, particularly after exposure to head trauma. While the ApoE4 isoform has been associated with increased tau accumulation and cerebrovascular dysfunction after TBI, investigations into these associations are limited. The current studies found that while astrocytes internalize and release tau back into the extracellular space under normal conditions, these processes become dysfunctional following r-mTBI leading to astrocytic tau accumulation, which is further exacerbated by the ApoE4 isoform.

In summary, I identified the factors responsible for the elimination of extracellular tau across the BBB, which are impaired after head trauma. Therapeutic interventions that restore these processes may ameliorate the chronic accumulation of tau associated with neurodegenerative disease. These findings may be particularly important for individuals with the ApoE4 isoform, who are more susceptible to the pathophysiological sequelae of tau accumulation, particularly after exposure to r-mTBI.

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LIST OF ABBREVIATIONS:

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
Ang-1/2	angiopoietin-1/2
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
ApoE-TR	ApoE targeted replacement
APP	Amyloid precursor protein
AQP4	Aquaporin-4
ARTAG	Age-related tau astrogliopathy
A β	Amyloid Beta
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BM	Basement membrane
btau	biotin-labeled tau
CAA	Cerebral Amyloid Angiopathy
CBD	Corticobasal degeneration
CCI	Controlled Cortical Injury
CMA	Chaperone mediated autophagy
CNS	Central Nervous System
CSF	Cerebrospinal fluid
CTE	Chronic Traumatic Encephalopathy
CX3CR/L1	C-X3-C Motif Chemokine Receptor/Ligand 1
DAI	Diffuse axonal injury
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular matrix
EEA1	Early Endosome Antigen 1
ELISA	Enzyme-Linked Immunosorbent Assay
FAD	Familial Alzheimer's disease
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBMECs	Human brain microvascular endothelial cells
HBSS	Hanks Balanced Salt Solution
HBVP	Human brain vascular pericytes
HSPG	Heparin sulfate proteoglycan
IEP	isoelectric point
IPAD	Intramural Peri-Arterial drainage
ISF	Interstitial fluid
kDa	kilodalton
KO	Knockout
Lamp1	Lysosomal Associated Membrane Protein 1
LyD	Lucifer yellow dextran 10 kilodaltons

LRP1	Low density lipoprotein receptor-related protein 1
Mfsd2a	Major Facilitator Superfamily Domain containing 2A
MPER	Mammalian Protein Extraction Reagent
mTBI	mild traumatic brain Injury
muLEC	mural lymphatic endothelial cell
M β CD	Methyl- β -cyclodextrin
NFT	Neurofibrillary tangle
NVU	Neurovascular Unit
Papp	apparent permeability coefficient
PBS	Phosphate-buffered saline
PDGF-BB	Platelet derived growth factor BB
PDGFR β	Platelet derived growth factor receptor β
PFA	Paraformaldehyde
PHF	Paired helical filament
PMSF	phenylmethanesulfonyl fluoride
PSP	Progressive Supranuclear Palsy
p-tau	phosphorylated tau
r-mTBI	repetitive mild traumatic Brain injury
SD	Standard deviation
SEM	Standard error of the mean
TBI	Traumatic brain injury
TBS	Tris buffered saline
UPS	Ubiquitin proteasome system
WT	wild type
α SMA	α smooth muscle actin

CHAPTER 1: INTRODUCTION

1.1 TRAUMATIC BRAIN INJURY

1.1.1 Prevalence

Traumatic brain injuries can result from a violent blow or sudden trauma to the brain that disrupts normal brain function. According to the CDC, approximately 1 out of every 75 Americans will sustain a TBI each year (Dewan et al., 2019), though the true prevalence is likely significantly higher. They can be categorized into mild, moderate, and severe, though the classification strategies have been in constant flux for decades. Currently, the factors separating a mild TBI from moderate and severe TBI in humans include the duration of loss of consciousness/amnesia and a post injury Glasgow Coma Scale score between 13 and 15. Even within the category of mild traumatic brain injury (mTBI), there are ambiguous distinctions. There is still no conclusive diagnostic test to identify a mTBI, or a concussion, and the classification of mTBI does not take previous injury history, type, or severity into consideration (Mayer et al., 2017). Concussions do not require a loss of consciousness, exacerbating the difficulty of their diagnosis (Blennow et al., 2012). The lack of clarity within the mTBI category limits the prognostic abilities of doctors (Mayer et al., 2017), as these distinctions can have a crucial impact on recovery and treatment options.

1.1.2 Mild traumatic brain injury

Mild traumatic brain injury is commonly caused by falls (35%) and traffic accidents (17%) in the general population (Jullienne et al., 2016). Though these isolated incidents can severely hamper an individual's quality of life, there are subpopulations that are more susceptible to TBI. Those involved in contact sports or in the military have a higher risk of mTBI and recurrent mTBI, which can lead to chronic deficits that persist for weeks or months after the initial injury. TBI can result in focal and/or diffuse damage, though diffuse damage through acceleration force is more common in mTBI. This leads to mechanical harm to brain structures, including axonal stretching and shearing, termed diffuse axonal injury (DAI). DAI can cause microtubule buckling and collapse, resulting in a release of unbound tau into the cytosol (Blennow et al., 2012). While as many as 80-95% of adult individuals fully recover from these conditions within months (Mayer et al., 2017), a small but significant fraction of the population experiences chronic effects that can last from months to years after injury.

Due to the immunologically privileged nature of the CNS, the priority response is one of homeostatic maintenance, which may promote chronic pathogenesis through sustained neuroinflammation. This chronic secondary injury is biphasic (Puntambekar et al., 2018), induced by an overcorrection by the brain throughout the repair process, or as a direct result from the primary injury. During this vulnerable recovery period, additional TBIs can induce or exacerbate chronic pathology, which typically includes vascular dysfunction and tau pathology. Individuals that experience chronic vascular dysfunction through TBI early in life may be susceptible to an accelerated onset of aging related diseases and dementia (Jullienne et al., 2016). It is crucial to determine the underlying mechanism of chronic cerebrovascular-related pathology so cognitive impairments stemming from vascular breakdown can be ameliorated.

1.1.3 Chronic Traumatic Encephalopathy

Exposure to repetitive mild TBIs (r-mTBI) can further enhance the injury progression and may lead to a condition described as Chronic Traumatic Encephalopathy (CTE), first reported in boxers by Martland in 1928. The pathognomonic criteria for CTE are still under debate (McKee, 2020), and research into its pathologic progression is still in its infancy. Age, sport, type of injury, and the presence of co-morbid neurodegenerative or vascular disease may influence the presentation of the condition, which has demonstrated a need for a more comprehensive consensus on diagnostic criteria (Arena, Johnson, et al., 2020). Previously, CTE progression was based off a modified version of the Braak staging scheme for AD, using p-tau pathology (Figure 1.1) as a guideline (McKee et al., 2013).

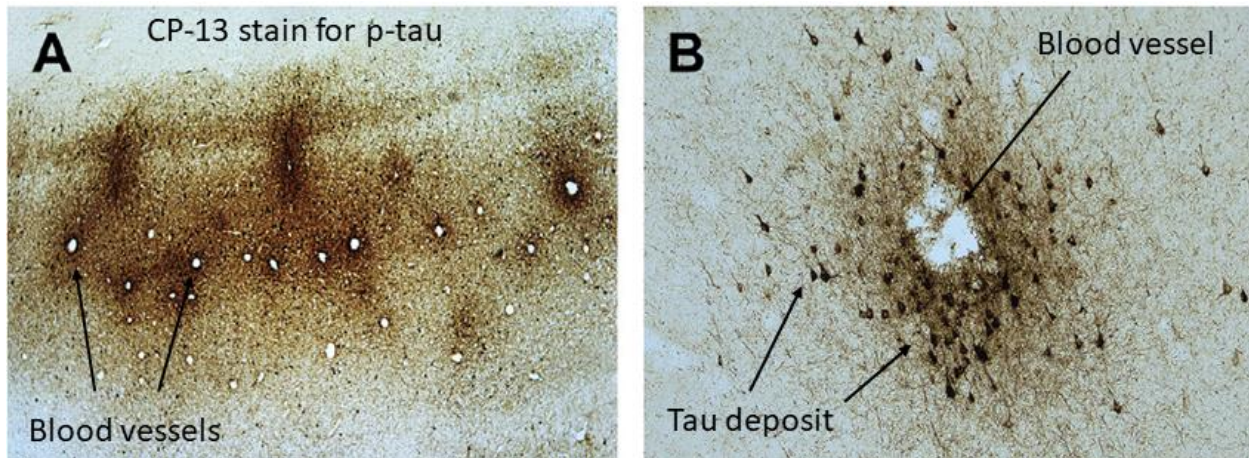


Figure 1.1 The pathognomonic lesion of CTE. The CTE lesion is comprised of pathological tau deposits within perivascular neurons and astrocytes at the depths of the cortical sulci. (A) Magnification 40x (B) Magnification 200x. Image adapted from Fig 2. of (McKee et al., 2016).

Recent consensus has differentiated CTE diagnosis into two stages, low and high (Bieniek et al., 2021). A pathognomonic lesion for CTE was recently characterized by perivascular p-tau aggregates in neurons focused in deeper cortical layers (Bieniek et al., 2021). This group of selected neuropathologists and CTE researchers concluded that the presence of astrocytic tau is not sufficient for a diagnosis of CTE, though they recognized that the role of glial tau accumulation is currently understudied (Bieniek et al., 2021). Other groups have argued that perivascular astroglial tau may be the only specific indicator of CTE (Arena, Johnson, et al., 2020), again demonstrating the need for further investigation into interactions between that occur between tau and the cerebrovasculature after r-mTBI.

It has been difficult to model CTE in mice, as their brain architecture does not contain folds of sulci, where the classical p-tau deposits that define CTE reside in humans. Despite this drawback, research into the consequences of repetitive brain injury in mice can still provide information on the impact to the blood brain barrier, and the initial progression of tau pathology. Finally, exposure to r-mTBI does not always result in the development in CTE. It is possible that contributing factors like age, or genetic risk factors (i.e., the APOE allele) may influence or predispose individuals to the progression of pathological neurodegeneration after TBI. A greater understanding of the interactions between the cerebrovasculature and tau after r-mTBI may offer novel opportunities for the development of more effective therapeutic interventions, as previous attempts to slow the pathological progression have been unsuccessful.

1.2 MICROTUBULE-ASSOCIATED PROTEIN TAU

1.2.1 Tau in health and disease

Characterized in the 1970's by Marc Kirschner (Weingarten et al., 1975), the microtubule associated protein tau has been studied extensively in the context of physiology and disease. It was originally identified as the microtubule polymerization protein that converts 6S dimers of tubulin into 36S rings (Weingarten et al., 1975) but has since been associated with a wide variety of physiological and pathogenic attributes. Tau is a universally conserved protein throughout the animal kingdom, though there are subtle differences in expression and isoforms (Bodea et al., 2016). While microtubule associated proteins contribute to processes ranging from microtubule motors to those associated with the centrosome, tau is primarily involved in binding to microtubules (Bodea et al., 2016).

Tau appears to play a prominent role in a group of neurodegenerative diseases called Tauopathies, including Alzheimer's Disease (AD) and Chronic Traumatic Encephalopathy (CTE), where the protein aggregates into fibrils and tangles in the brain. The mechanism of disease progression for many of these tauopathies is still unclear, as tau pathology is inconsistent across diseases, resulting in the emergence of a multitude of symptoms. Elucidating the molecular and cellular contributions to tau pathology in response to aging or trauma could yield new therapeutic targets. Previous therapeutic attempts have focused on individual species of tau, though recent research suggests lowering overall tau levels may be a more viable approach, at least until the true nature and physiological role of tau is elucidated (Chang 2021). The importance of understanding these underlying mechanisms increases each year, as the percentage of patients that are impacted with these neurodegenerative tauopathies grow, while therapeutics continue to fail.

The tau protein contains four distinct regions, composed of an N-terminal, proline-rich, microtubule binding, and C-terminal domain. There are six main isoforms ranging from 352 to 441 amino acids in length (Y. Wang & Mandelkow, 2016). Full length tau isoform has 2 N-terminal regions and 4 microtubule binding regions (2N4R), but other tau isoforms (Figure 1.2) can be found in humans with various combinations of

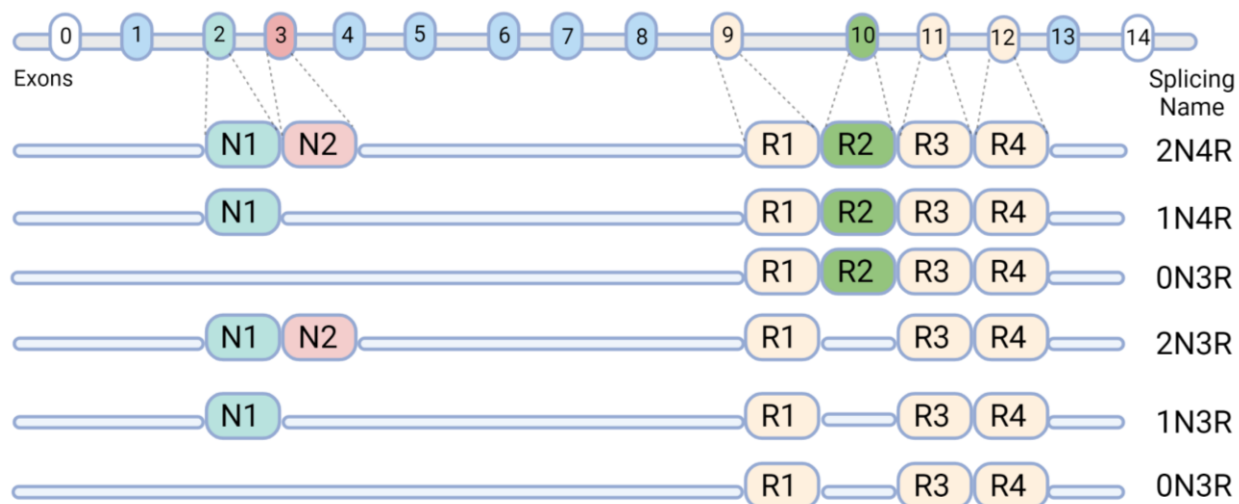


Figure 1.2 The human MAPT gene and the splice isoforms of tau in the human brain. Six major isoforms of tau are produced in the brain through alternative splicing of E2, E3 and E10 in the MAPT gene. Those isoforms differ by the presence of 0, 1 or 2 near-amino-terminal inserts (0N, 1N, or 2N, respectively) and the presence of R2 which results in either 3 or 4 carboxy-terminal repeat domains (3R or 4R respectively).

these regions. For example, during human brain development, tau with 3 microtubule binding regions is more prevalent than tau with 4. The MAPT gene that encodes tau, discovered in 1984 (Drubin et al., 1984), gives rise to the different isoforms of tau via differential splicing of exons 2, 3, and 10. Exons 2 and 3 correspond to N-terminal inserts, and exon 10 can be spliced in, which yields a 4R tau, or spliced out, which yields a 3R tau (Pérez et al., 2018). All six isoforms of tau can be expressed in human and murine brains, though in adult humans, 3R and 4R tau have balanced proportions, whereas adult mice express predominantly 4R tau (Hernández et al., 2020). As 3R has one less binding domain, it may play a stronger role in neuroplasticity or in response to injury than 4R tau (Castellani et al., 2019). Tau has two haplotypes as well, H1 and H2. Variable combinations of these haplotypes due to a 900 kb inversion polymorphism (Castellani et al., 2019) have been associated with altered risk for developing different neurodegenerative diseases.

Tau is an intrinsically disordered protein, a property which imbues it with the structural plasticity necessary to bind briefly and with specificity to multiple substrates. This innate ability is vital for a protein so prominent in the infrastructure of the axonal cytoskeleton (Baggett & Nath, 2018). Though tau is natively unfolded, it can adopt a paperclip-type structure, in which its terminal regions fold inward. The projection domain, an angled section of the protein comprised of the N-terminal domain, maintains axonal organization by creating a gap between axonal microtubules (Bodea et al., 2016). It extends away from where tau is bound to the microtubule and can interact with other cytoskeletal proteins and the plasma membrane (Scholz & Mandelkow, 2014).

Microtubules in neuronal axons are positioned such that their active polymerizing end is orientated towards the dendritic component, which tends to have a higher concentration of tau (Bodea et al., 2016). Initial studies investigating the function of tau suggested that it provided structural integrity to the axons by enhancing the assembly of the microtubules (Scholz & Mandelkow, 2014). Tau is also located in the dendrites and dendritic spines, contributing to neuronal plasticity (Bodea et al., 2016). Though axonal tau can be transported in both a retrograde and ante-retrograde manner, a barrier mechanism exists between the neuronal soma and the axon which inhibits free movement of tau. Tau can travel freely from the soma of the neuron to its axon, but it must be phosphorylated at specific sites to be able to re-enter the soma (Bodea et al., 2016). Microtubules tend to fluctuate between polymerization and depolymerization, allowing for rapid axonal remodeling (Al-Bassam et al., 2002).

Recent evidence has conflicted with tau's role as a microtubule stabilizer. The interaction of tau with microtubules in neuronal axons lasts around 40 milliseconds (Goedert et al., 2017). Compared with other MAPs, microtubules bound with tau have the highest turnover rate (Bodea et al., 2016). Neurons with tau genetically knocked out do not demonstrate dysfunctional neuronal processes that would be expected to rely on microtubule stability, including axonal transport (Yuan et al., 2008). Recent studies have suggested that tau may enable axonal microtubules to have elongated, stabilized labile domains, facilitating axonal plasticity (Qiang et al., 2018). When tau is knocked out, there is a compensatory upregulation of other microtubule associated proteins like MAP1B, which may play a more fundamental role in microtubule stability than tau (Chang et al., 2021). Tau's role in the CNS is still not understood, though it appears to play a role in processes ranging from insulin responsiveness and obesity to brain rhythm regulation (Chang et al., 2021). Tau likely modulates various physiological functions that may depend on cell specific and possible subcellular compartment specific environments (Chang et al., 2021). More research is needed to understand how tau influences central nervous system (CNS) processes before it can be targeted effectively and safely. Several key gaps in the literature that must be addressed include: the mechanisms by which non-neuronal cells interact with and eliminate extracellular tau, the mechanisms by which

neurodegenerative conditions impair tau clearance, and the pathways by which tau is eliminated from the CNS.

1.2.2 Tau Phosphorylation

Tau can undergo a variety of post-translational modifications (PTMs) which influence its form and function. Tau residue phosphorylation has been characterized extensively with respect to disease progression, though other PTMs may be relevant as well. Besides phosphorylation, tau can be oxidized, acetylated, glycosylated, nitrated, ubiquitinated, SUMOylated and truncated (Bodea et al., 2016). However, the majority of tau PTM research to this point has been focused on phosphorylation. Of the 85 residues that can be phosphorylated on full length tau, 20 sites are known to be phosphorylated physiologically (G. Ulrich et al., 2018), and the phosphorylation of 45 sites has been observed experimentally (Zempel & Mandelkow, 2014). Under physiological conditions, there is an equilibrium between phosphorylation and dephosphorylation of tau that dynamically modulates processes including cargo transport and the outgrowth of neurites (Albayram et al., 2016), however under neurodegenerative conditions, this equilibrium can become destabilized. An increase in phosphorylation can affect the ability of tau to bind microtubules, which eventually leads to an accumulation of unbound tau in the neuronal cytosol. Tau phosphorylation of specific serine and threonine residues is associated with many neurodegenerative diseases including AD and TBI. This is characterized by an increase in number of phosphate groups attached to specific residues, estimated at a three-fold increase rather than sporadic phosphorylation of any residue capable of phosphorylation (Castellani et al., 2019). Still, late neurodegenerative disease states typically contain tau that is phosphorylated at almost 40 residues, demonstrating that physiological phosphorylation through a variety of phosphatases and kinases is likely dysregulated (Castellani et al., 2019).

Elevated tau phosphorylation can induce a shift in the distal axon initial segment, which dysregulates the tau diffusion barrier, leading to tau retention and mis-localization, resulting in a reduction in neuronal excitability (Ittner & Ittner, 2018). Recent simulations of molecular dynamics have provided evidence that hyperphosphorylation may facilitate a favorable molecular environment for tau aggregation (Sonawane & Chinnathambi, 2018). This phosphorylation state could shift the attraction and repulsion states between tau domains, which may expose regions that enable subsequent misfolding and aggregation (Sonawane & Chinnathambi, 2018). Tau phosphorylation at specific sites may imbue the protein with resistance to various clearance methods, which could result in an accumulation of the protein. Phosphorylated tau has been linked to neurodegenerative diseases for decades now, but more investigation is necessary to determine the specific driver of tau aggregation and pathology.

In the CSF, elevated levels of tau phosphorylation at threonine 181 and particularly at threonine 217 (Barthélemy et al., 2020) have a higher predictive prognosis for AD patient decline than CSF A β 42 levels. Studies investigating the efficacy of using these phosphorylated tau sites as biomarkers is still ongoing, and the underlying mechanisms of why these sites are specifically associated with a disease state are also under investigation. Tau has been shown to bind to both DNA and RNA, and evidence suggests that this binding occurs in a protective manner (G. Ulrich et al., 2018). DNA damage was shown to induce tau nuclear translocation and dephosphorylation of nuclear tau, possibly to increase tau binding to DNA for protection (G. Ulrich et al., 2018). The primary phosphorylation site that became dephosphorylated during this process was at threonine 181, indicating that this site, residing in the proline rich region of tau, may be critical for DNA binding. Global increases in phosphorylation at this site would suggest that tau-mediated DNA protection is disrupted (G. Ulrich et al., 2018).

Emerging studies have suggested that a cis-p-tau isoform, phosphorylated at threonine 231, may have a higher propensity towards toxicity than its trans-p-tau isoform (Nakamura et al., 2012). The cis-p-tau isoform is more stable and aggregation-prone than its trans-p-tau counterpart (Nakamura et al., 2012). Its ability to bind microtubules is reduced and it demonstrates a higher resistance to dephosphorylation than trans-p-tau (Albayram et al., 2016). A prolyl isomerase, Pin1, converts cis-p-tau back to trans-p-tau, and can play a role in the regulation of phosphorylation of tau as well (Albayram et al., 2016). Cis-p-tau accumulation is correlated with the progression of mild cognitive impairment, AD, and CTE, and could be the driver of hyperphosphorylated tau induced toxicity in disease (Albayram et al., 2016). A recent study investigating the link between bipolar disorder and tauopathies suggested that cis p-tau₂₃₁ is at least partially responsible for the disease progression, which was reversed in vitro with lithium treatment (Naserkhaki et al., 2019). Mouse models of TBI have demonstrated that cis-p-tau specifically is increased after injury (Kondo et al., 2015) and antibodies that target cis-p-tau are able to ameliorate pathological effects, lending further support to the theory that the toxicity of phosphorylated tau is stereoisomer specific (Albayram et al., 2016).

Though AD and CTE both present with tau hyperphosphorylation, the aggregated species of hyperphosphorylated tau tend to accumulate in different regions and cell types, and exhibit some differences in phosphorylated sites, demonstrating a distinction between the two neurodegenerative diseases. In CTE, hyperphosphorylated tau tangles tend to accumulate in perivascular cerebral sulci folds, unlike AD, indicating unique pathological features between the two conditions (McKee et al., 2013). Studying the intersection between, and contribution of TBI to AD pathogenesis is common, so it is critical to be cognizant of the disconnect between their pathologies, especially with regards to tau.

Finally, the identification of pathological tau by its phosphorylation status is not always straightforward. Due to the biochemical resistance of paired helical filaments to phosphatase activity compared to soluble tau, tau in the form of paired helical filaments found at autopsy are associated with elevated levels of phosphorylation (Y. Wang & Mandelkow, 2016). Additionally, phosphorylation at these sites appears to be residue specific, as some sites buried within the filament may have more shelter from phosphatase activity than other residues. This observation may contribute to the perception that hyperphosphorylated tau at pathological sites was the driver of PHF formation. Decades ago, a study investigating adult tau in biopsies compared with autopsies, demonstrated a disconnect with what is typically found during post-mortem pathology, and what is present physiologically (Matsuo et al., 1994). The tau detected in a biopsy of healthy brain tissue contained tau phosphorylated at disease-relevant sites including S202/T205 (recognized by the AT8 antibody) and S396/S404 (recognized by the PHF1 antibody) (Matsuo et al., 1994). When that same tissue was re-examined after a brief period, replicating the post-mortem interval before an autopsy, those disease-relevant sites were efficiently dephosphorylated by resident phosphatases (Matsuo et al., 1994). This suggests that these tau species may have a physiological role, and their presence may not be linked directly to disease pathology. It is important to elucidate any differential pathogenicity of specific residue hyperphosphorylation before therapeutics are developed to target specific sites. In support of this, tau toxicity may not require residue specific phosphorylation but could deliver its pathogenicity through conformational change.

1.2.3 Tau conformational change

While neurofibrillary tangles have long been the target of therapeutics, recent evidence has pointed to the soluble, low molecular weight tau aggregates (oligomers) as the most toxic species. As seen with other pathogenic proteins including A β , oligomeric tau has largely become the focus of new therapeutic efforts.

Monomeric tau undergoes a conformational change, where the repeat regions align into a beta pleated sheet (Kaniyappan et al., 2017). Cross link patterns commonly occur between repeat domains 1 and 2, resulting in a stable, distinct species of tau (Mirbaha et al., 2018). As with A β , this initial step to aggregation is driven by the formation of disulfide bridges within the protein, favoring the formation of beta sheets (Guerrero-Muñoz et al., 2015). This conformational change is sensitive to the environment and pH but can also be driven via binding to specific substrates, alternative splicing of the MAPT gene, and post-translation truncation of the tau monomer (Guerrero-Muñoz et al., 2015).

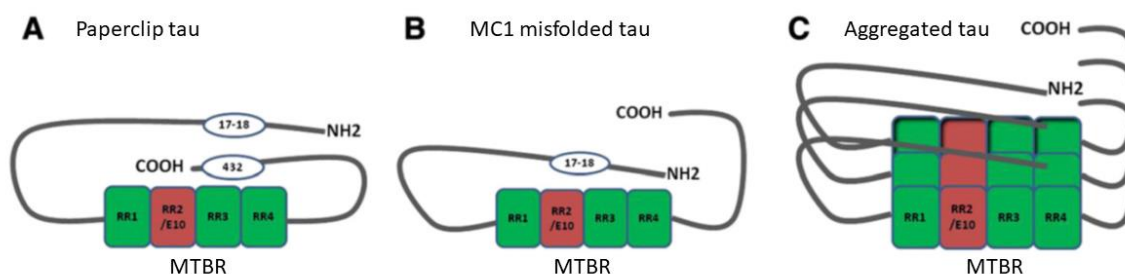


Figure 1.3 Conformations of tau. The paperclip conformation (A) describes when the C-terminus folds in proximity to the MTBR, and the N-terminus folds back towards the C-terminus, but still distant from the MTBR. The Alz-50 conformation (B) occurs during the initiation of tau aggregation, where the C-terminus is no longer adjacent to the MTBR, and the N-terminus can interact with this region. Tau monomers with this conformation bind together (C) and form tau aggregates. Adapted from Fig 2. of (Himmelstein et al., 2012).

Tau is typically not prone to aggregation. In its native state, the intrinsically disordered protein can fold both of its terminal regions back onto itself to cover its microtubule binding regions (MTBR), preventing aggregation (Zhou et al., 2018). This paperclip conformation shields critical regions for tau aggregation, including the hexapeptide VQIINK and VQIVYK sequences, in the repeat domains of the MTBR in a hairpin structure (Figure 1.3). Truncation of either of the terminal regions may expose the MTBR, which could make the tau protein more prone to aggregation (Zhou et al., 2018). Various proteases including calpain, chymotrypsin, and caspases 3 and 6 can interact with and cleave tau at different residues, resulting in the generation of truncated species (Zhou et al., 2018). Full length tau may be more resilient to aggregation than truncated tau for this reason. Evidence supports this theory, as tau truncated at serine 421, recognized by the C3 antibody, has been shown to promote aggregation (Zhou et al., 2018). Mutations can also predispose tau to misfolding and are associated with a variety of different tauopathies. For example, the autosomal dominant point mutation in the MAPT gene encoding tau that causes frontotemporal dementia with Parkinsonism linked to chromosome-17, at P301S/P301L (either a serine or leucine replacement in the R2 domain) gives tau a propensity to shift into a beta sheet structure, which leads to accelerated aggregation in mice expressing this mutation (J. E. Gerson et al., 2016). These point mutations occur near the V sequences and simulated models predict that this mutation would destabilize the R2 region and expose these aggregation prone sequences (Mirbaha et al., 2017).

1.2.4 Tau prion-like propagation

Monomeric tau has been functionally classified by some groups as inert or seed competent (Mirbaha et al., 2017). It is likely that inert monomeric tau must overcome a defined energy or kinetic barrier to become seed competent, which may be the initial driver of aggregation in the brain (Mirbaha et al., 2017). Once initiated, the hydrophobic interactions in the repeat domains drive the secondary structures to shift from an α -helix to a β -sheet formation (Dan & Chen, 2019). Conformationally misfolded tau species may

be heterogeneous, as *in vitro* evidence suggests there are multiple intermediate structures that are seed competent (Mirbaha et al., 2017). It has been suggested that the region of conformational initiation in the brain can contribute to the formation of different tau strains, referring to the observed distinct structural conformations of tau. However, a study introducing isolated human AD, PSP (progressive supranuclear palsy) and CBD (corticobasal degeneration) tau strains into the same mouse brain region indicated that all strains are capable of driving neuronal aggregates in each region, though there is a difference in potency (Narasimhan et al., 2017). Differential exposure of V sequences and other core elements may contribute to this diversity as well, as only one of the V sequences is vital to the core fibril structure in AD (Mirbaha et al., 2017). A study of various strains of seed competent tau suggested that individual conformational structures will give rise to a limited number of fibril formations, specific to each strain (Sharma et al., 2018). Seed competent tau species derived from AD brains were able to form only a single fibril structure when injected into a tauopathy mouse model, whereas the seed competent tau species from CBD brains were able to form multiple different types of fibrils after injection (Sharma et al., 2018). Growing research suggests that the cellular environmental conditions, where the tau monomeric species originally shifted to a seed competent state, may be a critical factor in determining the subsequent pathology (Sharma et al., 2018). The presence of 3 or 4 repeat regions in tau, as well as the effect of any post translational modifications or phosphorylation may also contribute to this heterogeneous population of conformational species. Furthermore, an asymmetry exists between 3R and 4R tau seeding, as 4R tau can seed 3R and 4R, but 3R can only seed itself (Kumar & Udgaonkar, 2018). Filaments formed primarily of 3R tau are typically twisted paired helical filaments, while straight filaments are typically formed from 4R tau aggregates (J. E. Gerson et al., 2016). These isoform specific properties are observed in prion disease as well (Kumar & Udgaonkar, 2018), supporting the existence of an array of conformational tau varieties, which is consistent with the different tau formations observed in various tauopathies. In a study comparing the characteristics of various tau strains *in vitro*, Sanders et al. observed that each unique strain seeded naive tau into distinct conformations that were maintained indefinitely through subsequent rounds of seeding (Sanders et al., 2014). The ability to corrupt the template of a new protein in a sequence specific manner such as this has previously only been observed in prion proteins. Due to tau's stability, replicable phenotype in living systems, and the ability to isolate it in a strain specific manner, Sanders proposed that tau should be considered a true prion (Sanders et al., 2014). At this stage, the characteristics of what constitutes a prion are under debate (Jucker & Walker, 2018), but tau does have many of the prerequisite properties.

1.2.5 Tau oligomerization

Oligomeric tau species are heterogeneous, and their levels significantly increase in preclinical models of TBI, even in the acute stage (Gerson et al., 2016). They can be found physiologically in a wide range of lengths and conformations and are thought to be formed through different mechanisms (Gerson et al., 2016). Different antibodies raised against oligomers including T22 and TOMA can be used for detection. In addition, these antibodies have shown some *in vitro* efficacy in cellular protection from oligomeric toxicity (Gerson et al., 2016). Not all oligomers follow the same formation trajectory towards a filament, as some may generate annular protofibrils at their end stage, which are pore-like structures. These protofibrils can impact the cell membrane permeability (J. E. Gerson et al., 2016). Their formation appears to be disease and cell specific, suggesting specific conditional requirements for their genesis (Lasagna-Reeves et al., 2012). Variants in the seed protein could lead to different tau oligomeric species, and

chaperones may impact their formation as well (J. E. Gerson et al., 2016). Conformational differences in tau oligomers can impact their toxicity and their eventual filamentous form.

There are multiple theories for the formation of tau oligomers, as *in vitro* and *in vivo* formation of the aggregates may occur through different mechanisms. Evidence suggests that a beta sheet in a second tau monomer will align in an antiparallel manner to form a dimer. Dimerization of tau appears to be the rate limiting step, and exists in a transient manner, leading to the subsequent addition of monomers after formation (Congdon et al., 2008). *In vitro*, tau seeding typically occurs through a template assisted growth mechanism. A filament forms from a tau oligomer, as subsequent monomeric tau proteins are added to the end of the filament. However, *in vivo* it has been proposed that monomeric tau will assemble into oligomers first instead, and the oligomers will eventually combine to form filaments (J. E. Gerson et al., 2016). It is possible that both mechanisms could play a role in tau filament formation, as a variety of oligomeric conformations can be found in the same patient (J. E. Gerson et al., 2016). If tau oligomers are truly more toxic than fibrils, the aggregation of oligomers into fibrils may be a protective mechanism *in vivo* to sequester the pathogenic tau into a more benign fibril. Tau oligomers generated *in vivo* are 5 times more toxic and have a much higher seeding capacity than recombinant tau oligomers generated *in vitro* (Lasagna-Reeves et al., 2012). This has been observed in other pathogenic proteins as well and post translational modifications and other factors may create this disparity. Recombinant aggregated A β generated *in vitro* required a concentration three orders of magnitude higher to induce seeding *in vivo* than isolated A β aggregates (Jucker & Walker, 2018).

Much of the tau aggregation research has focused on how the inherent characteristics of tau can lead to aggregation, and yet tau cannot aggregate *in vitro* without the addition of a cofactor such as heparin or RNA. When the cofactor used to aggregate tau into fibrils *in vitro* is removed by a digestion agent, the fibrils depolymerize, and the disassociated monomers show no evidence of seed competence compared to naïve tau (Fichou et al., 2018). Fibrils formed *in vivo* are much more resilient to specific digestion agents, but after multiple rounds of seeding recombinant fibril formation, the monomeric tau derived from brain homogenate loses the ability to maintain a stable fibril in the presence of cofactor digestion (Fichou et al., 2018). These results suggest that there may be an unknown cofactor or combination of cofactors in the neuron that play a significant role in tau aggregation and fibril formation. This unknown polyanionic cofactor could be influenced by neuronal health or inflammation, and may be cell type or disease specific, giving it therapeutic potential, as it could be targeted without directly affecting tau. Primarily, the authors from that study suggested that tau fibrils formed *in vitro* differ from fibrils formed *in vivo* in their stability, structure, components, and breakdown products, as fibrils formed *in vitro* break down into conformationally inert monomers (Fichou et al., 2018). This discrepancy between aggregated tau species could account for observed inconsistencies in investigations of tau propagation and neurotoxicity and indicates that experiments utilizing recombinant tau oligomers may not fully capture the physiological relevance of aggregated tau *in vivo*.

For decades, tau tangles were thought to be the pathogenic driver of disease, just as amyloid plaques were thought to be the toxic disease component in AD, but recent evidence has suggested that these final stage protein products may not be the best therapeutic target. As strategies to clear A β plaques have failed in clinical trials, it may be necessary to target the proteins earlier in the aggregation process. High A β levels may help facilitate tau aggregation, but the existence of other tauopathies have demonstrated that A β is not required for tau to become pathogenic. Furthermore, pathological tau development may extend throughout an entire lifetime. Braak and Tredici found pre-tangle tau material and abnormally phosphorylated tau in 38/42 cases of otherwise healthy individuals between the ages of 4 and 29 years

old (Braak & del Tredici, 2011). Young, healthy neurons may have the capacity to withstand initial tau aggregation by sequestering the tau into fibrillary deposits (Lasagna-Reeves et al., 2012). This tau might not be toxic in the acute stage as neurons have demonstrated the ability to survive with NFTs for decades (Lasagna-Reeves et al., 2012), but it could eventually result in chronic dysfunction, suggesting that AD may develop more slowly, and over a much more chronic time-period, than previously thought (Braak & del Tredici, 2011).

Despite the slow progression of pathogenic tau development, the soluble oligomeric species are likely to be the primary toxic component. In AD, tau oligomers have been identified in the majority of pre- and post-synaptic compartments of neurons (Guerrero-Muñoz et al., 2015), and can undergo ante- and retro-grade movement between neuronal compartments (J. W. Wu et al., 2013), indicating that they may play a significant role in trans-synaptic spreading. Evidence suggests that resources should be shifted towards targeting oligomeric tau species, and how the clearance of such species from the brain may be disrupted in disease.

1.2.6 Tau secretion

Tauopathies are characterized primarily by intracellular inclusions of pathological tau, resulting in the consensus that extracellular tau was simply a byproduct of cell death. Correlations between neurodegeneration and elevated tau levels in the CSF lead researchers to believe that neuronal apoptosis or injury was the primary source of tau found in the extracellular spaces of the brain (Hampel et al., 2010). Recently, studies have investigated the possibility of a physiological mechanism for tau secretion. While some cell-to-cell spreading of tau may occur through tunneling nanotubes (Tardivel et al., 2016), other

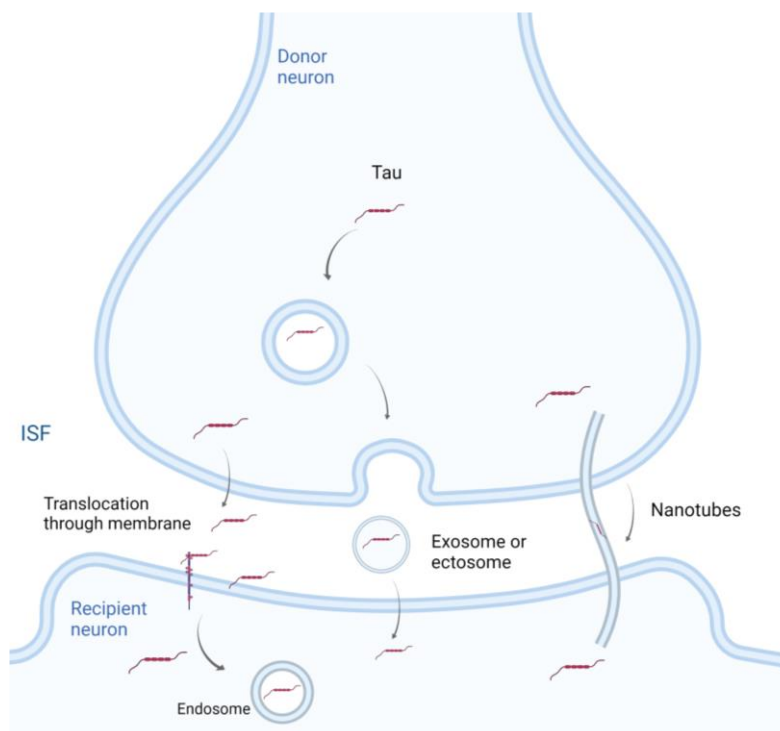


Figure 1.4 Mechanisms of tau secretion. Tau can be secreted from a donor neuron directly into the ISF by translocation through the plasma membrane and internalized from the ISF by a recipient neuron. Tau encapsulated within exosomes or ectosomes can be secreted from a donor neuron, and can subsequently fuse with a recipient neuron, where tau is then deposited into the cytosol. Tau can also transfer from a donor neuron to a recipient neuron through tunneling nanotubes.

reports have suggested multiple mechanisms of tau release to the extracellular space. This release can occur through multiple pathways, including translocation through the membrane, or vesicular release in exosomes and ectosomes (Figure 1.4). Tau can undergo both passive and active release (Sato et al., 2018), suggesting internal machinery within the cell that regulates this process. Neuronal secretion of tau has important implications when investigating the mechanisms of tau aggregation and propagation.

There are various mediators of free tau release, including regional neuronal excitation (Pooler et al., 2013) which can result in elevated levels of ISF tau within hours depending on the degree of activation (Yamada et al., 2014). Tau phosphorylation appears to enhance release as well (Katsinelos et al., 2018). As tau becomes phosphorylated, its ability to bind microtubules decreases, leading to an abundance of free tau in the cytosol. Though extracellular tau is largely dephosphorylated (Pooler et al., 2013), the degree of phosphorylation is correlated with increased secretion (Katsinelos et al., 2018). It is possible that altered tau phosphorylation may be a driving force for secretion, as tau dephosphorylation has been shown to enhance secretion as well (Mohamed et al., 2014). Additionally, interfering with the degradation machinery within the cell by inhibiting lysosomal function leads to the unconventional secretion of α -synuclein (Rodriguez et al., 2017), and had a similar effect on tau (Mohamed et al., 2014), indicating multiple physiological triggers that are common in neurodegenerative diseases can facilitate tau secretion. It is likely that there are multiple pathways by which tau can be released (Mohamed et al., 2014), working simultaneously in response to different stimuli.

In addition to stimulated tau release, evidence suggests that tau undergoes constitutive secretion via translocation through the plasma membrane (Chai et al., 2012). The microtubule binding domain of tau can bind to the plasma membrane surface (Chai et al., 2012), and mediated by short amphipathic helices that rest on the surface of the PM. Diffusion of tau, along with its direct binding capacity, allows free tau to pool at the membrane without requiring intracellular transportation. Recent studies have provided evidence that this plasma membrane-associated tau is secreted through unconventional protein secretion type I, a pathway previously established in fibroblast growth factor 2 (Katsinelos et al., 2018). Fibroblast growth factor 2 shares some properties in common with tau, including its lack of a secretory signal, which lead researchers to investigate whether tau secretion occurred by the same mechanism. Tau has been shown to bind to and utilize ubiquitin proteasome system (UPS) type I machinery at the inner leaflet of the plasma membrane, which opens a pore in the plasma membrane allowing tau to pass through and bind to sulfated proteoglycans on the extracellular plasma membrane surface (Katsinelos et al., 2018). This is supported by the evidence that an increase in membrane permeabilization subsequently increases the release of tau (Mohamed et al., 2014). Though full-length tau can be secreted directly, protein modifications alter the secretion process. Aggregated, phosphorylated tau can get sequestered at microdomains in the plasma membrane, which may enhance cellular secretion (Merezhko et al., 2018). In addition, evidence suggests interactions between tau and the plasma membrane may facilitate tau aggregation before secretion (Merezhko et al., 2018). It is possible that the mechanism for unconventional tau secretion through the plasma membrane into the extracellular space requires tau aggregation as an intermediate step (Merezhko et al., 2018). This could be one of the mechanisms for tau aggregation, and it would implicate elevated intracellular tau concentrations with the subsequent development of tau aggregates.

An alternative pathway involved in tau secretion uses vesicle-trafficking (Saman et al., 2012). Between 2% (Y. Wang et al., 2017) and 7% (Katsinelos et al., 2018) of extracellular tau is encapsulated within small extracellular vesicles with a lipid bilayer called exosomes. Exosomes have been found in most bodily fluids, including urine, saliva, blood, and CSF, and can be released by most cell types including neurons,

astrocytes, and microglia (Quek & Hill, 2017). These nanoscale vesicles reflect the parent cell's lipid, protein, and nucleic acid composition. They are derived from the endosome system as part of the macroautophagy pathway. Proteins are sorted into intraluminal vesicles of multivesicular bodies and are subsequently fused with the lysosome for degradation or fused to the plasma membrane to be released as exosomes (Quek & Hill, 2017). Under physiological conditions, only a small fraction of tau may avoid autophagy to be released in exosomes (Guix et al., 2018). Other pathogenic proteins including PrP, A β , α -synuclein and SOD1 have been seen in exosomes as well (Quek & Hill, 2017), suggesting a role in neurodegenerative disease. As indicated above, neuronal activity can stimulate the release of tau in exosomes, indicating it is a regulated process (Y. Wang et al., 2017), though it may play a protective role as well. While some tau is trafficked to the lysosome and degraded through autophagy (Guix et al., 2018), elevated levels may overwhelm the autophagic system, requiring exosomal release of excess tau. Neurons may preferentially release aggregated and hyperphosphorylated tau in exosomes as a physiological protective mechanism.

Tau has also been associated with vesicles shed directly from the PM, called ectosomes, which are larger than exosomes and are formed by budding (Dujardin et al., 2014). This could be mediated in part by tau's propensity to bind to the plasma membrane. Shedding of ectosomes is triggered by membrane activation, a mechanism which can be influenced by common neurodegenerative disease conditions including increased intracellular calcium levels and oxidative stress (Dujardin et al., 2014). Tau bound to the inner leaflet of the plasma membrane may be encapsulated into an ectosome in response to a stimulus. Tau associated with ectosomes may be different than the tau encapsulated within exosomes. Exosomal tau had likely been trafficked to be either degraded or released from the cell, whereas ectosomal tau may not have been targeted for degradation. It has been suggested that the exosomal pathway for tau release is activated once tau accumulates (Dujardin et al., 2014) indicating that physiological release of tau may occur through UPS I or ectosomal budding, but under pathological conditions, exosomal release could help relieve some of the cellular tau burden.

Each secretory pathway could be responsible for different variants of tau and may be responsible for distinct stages of tau clearance. More importantly, these recently discovered mechanisms of tau secretion demonstrate the relevance of extracellular tau, particularly in diseased states where these processes may be disrupted. In fact, extracellular tau levels are elevated in tauopathies and can be used as a biomarker to predict the progression of cognitive decline in AD (Degerman Gunnarsson et al., 2016). Although the composition of ISF tau is unclear, a recent *in vivo* study using microdialysis reported that a significant amount of extracellular tau is comprised of a combination of truncated tau fragments of various sizes and seed competent phosphorylated species (Barini et al., 2022). The authors suggested that the aggregated phosphorylated species of tau, not the fragments, were driving seeding competence (Barini et al., 2022). At this time, further studies are required to determine whether these pathogenic species are encapsulated within exosomes, and how they interact with other cells in the CNS (Barini et al., 2022). After TBI, increased levels of extracellular tau are observed (Magnoni et al., 2012), and have been associated with adverse clinical outcomes, indicating the importance of investigating extracellular tau elimination, in both normal and disease conditions.

1.3 ESTABLISHED MECHANISMS OF TAU ELIMINATION FROM THE BRAIN

Once secreted or released from the neuron into the interstitial fluid, proteins like tau or A β can be eliminated from the CNS through several different overlapping pathways. The mechanisms for the clearance of extracellular A β have been studied extensively. In contrast, mechanisms of extracellular tau clearance have not been fully characterized (Table 1.1). Despite the limited research into tau clearance, several mechanisms for tau clearance have been investigated, including cellular enzymatic degradation, ISF bulk flow, and perivascular drainage.

Clearance System	A β	Tau
Blood-brain barrier clearance	Majority of A β clearance LRP1, ApoE, ABCB1 mediated efflux	Contribution unknown Mechanism unknown
Degradation clearance		
Intracellular	Proteasome Autophagy-lysosome Endosome-lysosome Proteases	Proteasome Autophagy-lysosome Endosome-lysosome Proteases
Extracellular	Proteases Glial phagocytosis	Unknown Glial phagocytosis suspected
ISF bulk flow		
CSF sink	Contributes to eA β clearance	Contribution unknown
Perivascular drainage	Contributes to eA β clearance Facilitates BBB clearance	Contribution suspected

Table 1.1 Clearance mechanisms of tau. Adapted from (Tarasoff-Conway et al., 2015a)

1.3.1 Tau elimination through enzymatic degradation

The degree to which CNS cells internalize and process tau is still under investigation, though it is generally understood that neurons and microglia actively take in extracellular tau. The predominant methods used by cells to eliminate proteins from the CNS incorporate enzymatic protein digestion, through a combination of autophagic and proteasomal degradation. The exact contributions of autophagy and the proteasome towards tau degradation may be dependent on factors including the CNS cell type or the PTM and conformation status of tau. Despite the uncertainty, either of these enzymatic elimination mechanisms could be potential therapeutic targets for tauopathies.

Autophagy is responsible for the sequestration and degradation of many long lived, pathogenic-prone proteins including A β and tau (Menzies et al., 2017) and faulty autophagy has been implicated in neurodegenerative diseases including AD (Krüger et al., 2012). This process occurs by encapsulating cytoplasmic proteins in a double-membraned vesicle called an autophagosome, which is transported along microtubules towards the lysosome for degradation (Menzies et al., 2017). Mouse models with disrupted autophagy demonstrate an accumulation of protein aggregates (Menzies et al., 2017). As with A β (Mawuenyega et al., 2010), it is possible that the accumulation of tau in the brain is due to impaired clearance, rather than enhanced expression. Most of the autophagy-mediated clearance is carried out by bulk degradation, though targeted mechanisms like selective macroautophagy can traffic aggregated

proteins to the lysosome. Tau can be targeted through cargo recognition machinery and is subsequently degraded through both endosomal microautophagy and chaperone-mediated autophagy (Scrive et al., 2018).

Autophagy mediated degradation may be more resilient to conformational changes in tau than proteasome degradation, as autophagy does not depend on a complicated protein-complex insertion mechanism, in contrast to the proteasome (Krüger et al., 2012). Evidence does suggest that autophagy can exhibit a preferential degradation of such proteins (Krüger et al., 2012), however, this mechanism could be susceptible to tau hyperphosphorylation. Disruption of axonal transport due to tau hyperphosphorylation may disrupt autophagosome trafficking and lead to an accumulation of tau in neurons (Menzies et al., 2017). This can result in the delivery of these tau containing vesicles to the plasma membrane, where they are released into the extracellular environment (Menzies et al., 2017). Mouse models of AD have shown that tau can also bind lysosomes directly and degrade membrane integrity, leading to a reduction in degradation efficiency, which has been confirmed in AD patients (Menzies et al., 2017).

Though autophagy does contribute to intracellular tau degradation, evidence suggests it may be species dependent. Small variations in tau including pathogenic point mutations like P301L or the tau mutation A152T can lead to a shift in the specific autophagy pathway activated and can diminish the efficacy of tau degradation as well (Caballero et al., 2018). However, upregulating autophagy in a zebrafish A152T mutated tau model was neuroprotective, suggesting that pathogenic tau degradation can be a critical step in tau-mediated neurodegeneration (Chang et al., 2021). Mutations in tau can reduce the binding affinity of endosomal microautophagy, which can lead to a disruption in degradation pathway homeostasis as the cell attempts to overcompensate, resulting in a susceptibility to additional stressors (Caballero et al., 2018). Chaperone mediated autophagy (CMA) in tau via two CMA binding motifs in the microtubule binding domain delivers tau to the lysosome. Other mutations in tau can lead to incomplete lysosomal degradation, resulting in truncated tau fragments which easily aggregate (M. J. Lee et al., 2013). At this stage, the role of lysosomal degradation in pathogenic tau elimination is not fully understood

An alternative enzymatic mechanism for tau elimination involves the ubiquitin-proteasome system (UPS) which targets pathogenic proteins using a ubiquitin tag, then uses the proteasome to degrade the proteins into smaller peptides. Typically, the proteasome is responsible for shorter-lived proteins than those degraded through autophagy. Proteasome inhibitors can induce an accumulation of tau, indicating that the proteasome plays a role in tau degradation (David et al., 2002), though at this stage, proteasomal degradation of tau *in vivo* is controversial (M. J. Lee et al., 2013). While the proteasome may be able to degrade tau without requiring the use of a ubiquitin tag, the majority of tau turnover during physiologic conditions was suggested to be through the ubiquitin mediated pathway (M. J. Lee et al., 2013). Evidence suggests that the proteasome is capable of degrading soluble tau species, but the complex does not interact with larger insoluble deposits (M. J. Lee et al., 2013). It has been suggested that the proteasome may be more efficient in degrading monomeric tau clearance, but aggregated species could interfere with the machinery, requiring degradation by autophagy (M. J. Lee et al., 2013). Additionally, tau phosphorylation appears to inhibit proteasome degradation to some degree (Krüger et al., 2012).

Alterations in ubiquitin have been correlated with neurodegenerative diseases, and ubiquitin itself co-localizes with tau, accumulating in PHFs (M. J. Lee et al., 2013). Soluble PHF-tau isn't degraded by the proteasome, but it can inhibit proteasome activity by binding to the proteasome 20S core particle (M. J. Lee et al., 2013). Small ubiquitin-like modifiers (SUMO) triggered by tau phosphorylation can tag tau for proteasome degradation (Deger et al., 2015). Evidence suggests that SUMOylation preferentially targets

free pools of soluble tau, which could help maintain physiologic tau levels and encourage sufficient turnover (Deger et al., 2015). SUMOylation and ubiquitination activity is elevated in brain regions with neurodegeneration, indicating that unhealthy cells can still target pathogenic proteins for degradation (Deger et al., 2015). Tau oligomers that are hyperphosphorylated are also correlated with increased ubiquitination in AD, indicating altered proteasome activity (Deger et al., 2015). It is possible that these proteins are tagged for the UPS pathway, but the proteasome itself is dysfunctional. This could be due to misfolded proteins blocking the proteasome machinery, or some other stressor disturbing UPS activity in the proteasome complex. As levels of the tau oligomers rise, they could exacerbate the proteasome deficiency, resulting in neurotoxicity. Oligomeric tau species may have inherent mechanistic or structural safeguards against degradation, which could be conserved among other toxic aggregates like A β and α -synuclein, limiting current therapeutic efforts (Deger et al., 2015). Further investigation into the mechanisms used by CNS resident cells to degrade tau are required, though some progress has been made in both neurons and microglia.

1.3.2 Neuronal elimination of extracellular tau

Neurons have shown the capacity to interact with and internalize tau (Evans et al., 2018), though their ability to degrade pathogenic species efficiently is questionable. Neurons may use multiple mechanisms to internalize different tau species, including the recently identified receptor mediated pathways, such as muscarinic M1 and M3 receptors (Morozova et al., 2019), or the low-density lipoprotein receptor-related protein 1 (LRP1) (Rauch et al., 2020). While LRP1 appear to bind to and facilitate tau endocytosis, its role in neuronal processing of tau is still under investigation (Cooper et al., 2021).

Monomeric tau can enter neurons through bulk endocytosis and dynamin mediated endocytosis (Evans et al., 2018) in a rapid manner, potentially mediated via LRP1. A recent report suggested that LRP1 mediated endocytosis of monomeric tau results in lysosomal degradation of tau, though this elimination pathway may become pathogenic if the internalized tau is misfolded (Cooper et al., 2021). Once internalized into the endosome, the low pH environment combined with compact molecular packing within the compartment could facilitate the aggregation of misfolded monomeric tau (Michel et al., 2014). Furthermore, internalized misfolded tau may induce endosomal and lysosomal leakage into the cytosol, which could drive pathogenic seeding of tau aggregation (Michel et al., 2014) in a naïve neuron.

Aggregated tau appears to enter neurons through a distinct pathway. Though aggregates have been demonstrated to enter HEK293 cells through macropinocytosis in an actin-driven manner (Holmes et al., 2013), recent evidence has suggested that this pathway does not apply to neurons (Evans et al., 2018). Instead, neurons may take in aggregates through endocytosis (Evans et al., 2018) in a method independent of actin. Wu et al. found that soluble tau aggregates are internalized through dynamic-driven endocytosis, indicating that multiple overlapping mechanisms may be responsible for tau internalization, which may be dependent on the conformation or isotope (J. W. Wu et al., 2013). Oligomers may induce the formation of a pore on the cell membrane as well, disrupting the structural integrity and enabling entry (Ait-Bouziad et al., 2017). The repeat domains R2 and R3 of tau can interact with the neuronal membrane. They might extract resident phospholipids and form a stable complex, which facilitates the endocytosis of tau into the endosomal pathway (Ait-Bouziad et al., 2017).

Both monomers and aggregates appear to be internalized quickly, indicting the presence of receptors or carrier molecules that can interact with tau (Evans et al., 2018). Tau monomers and tau aggregates can bind to HSPGs, but it was proposed that only aggregates can be internalized through that mechanism (Holmes et al., 2013). A β and prion proteins are both internalized by binding to HSPGs which have been

secreted onto the extracellular matrix, demonstrating a precedent for this type of internalization pathway (Stopschinski et al., 2018). The tau aggregates bind to HSPGs through specific glycosaminoglycan architecture with sulfated residues in separated patches (Stopschinski et al., 2018). Tau binding requires sulfates in the N- and 6-O positions, which are different from those required for α -synuclein and A β aggregate internalization (Stopschinski et al., 2018). It appears that the quaternary structure of tau is responsible for regulating its internalization (Rauch et al., 2018). Evidence suggests that neurons may preferentially internalize smaller species of tau, which appears to be consistent with other cell types (Rauch et al., 2018). Neurons may have difficulty internalizing or interacting with fibrils, which implicates misfolded and oligomeric tau as the species responsible for pathogenic seeding (Rauch et al., 2018). Internalized exogenous tau aggregates appear to proceed into the endosome pathway, but similar to misfolded tau, oligomers appear to permeate into the cytosol before lysosomal degradation (Ait-Bouziad et al., 2017). The current literature indicates that while neurons are capable of enzymatically eliminating exogenous monomeric tau, they may not play a significant role in the elimination of pathogenic extracellular tau.

1.3.3 Glial elimination of tau

Microglia, the resident macrophages of the CNS, are responsible for maintaining homeostasis in the brain by regulating the inflammatory environment and removing toxic material. Due to the phagocytic capabilities of microglia, their capacity for tau clearance has been investigated. Microglial activation has been directly correlated with tau pathology (Maphis et al., 2015), even preceding it in a P301S mouse model of tauopathy (Maphis et al., 2015), indicating a possible mechanism for microglial regulation of tau. Microglia can internalize oligomeric tau more efficiently than neurons, demonstrating their potential for tau clearance (Asai et al., 2015). Microglia express the receptor CX3CR1, which is used to communicate to the ligand CX3CL1 released by neurons. This signaling pathway is used to inhibit the microglial release of proinflammatory cytokines (Amro et al., 2021). Tau has been shown to bind directly to CX3CR1, triggering tau internalization into microglia (Bolós et al., 2017), but consequently disrupting the neuronal ability to suppress microglia neuroinflammation via the CX3C1 pathway. It has been demonstrated that even though there are higher levels of microglial activation in AD, they are less efficient in the phagocytosis of tau, indicating that their ability to remove foreign material in disease conditions could be compromised (Bolós et al., 2017).

In fact, activated microglia may contribute to tau pathology, through internalization and secretion of tau to neighboring neuronal networks. Evidence suggests that activated microglia can induce disease relevant AT8 tau phosphorylation, tau aggregation, and even tau propagation (Maphis et al., 2015). Microglia display a high degree of exocytic activity, which enables them to release material that they phagocytose in exosomes (Asai et al., 2015). This enables a large degree of protein turnover and could help the microglia avoid disrupted lysosomal activity in a similar manner to neurons. However, tau phagocytosis and the subsequent release in exosomes may contribute to tau pathological progression. Indeed, it was previously reported that both microglial depletion and reduction of exosome synthesis ameliorated tau propagation (Asai et al., 2015). Moreover, suppression of activated microglia in P301S mice lead to a significant increase in lifespan (Asai et al., 2015). Misfolded tau is engulfed and degraded by microglia in a delayed manner relative to physiological tau (Hopp et al., 2018). Hopp and colleagues noted that internalized misfolded tau was still present in microglia after 6 days (Hopp et al., 2018). It was suggested that microglia may internalize full length tau and partially degrade it, leaving it seed competent before it is released back into the extracellular environment where it can seed neurons (Hopp et al., 2018).

Misfolded tau may block the degradation machinery in microglia, leaving them less efficient at protein clearance (Hopp et al., 2018). Activated microglia migrate to areas of the brain with elevated levels of hyperphosphorylated tau, but their inability to effectively remove the tau may drive further pathogenesis. This reduced ability to degrade tau was not affected by inflammation, indicating that modulating inflammatory molecules may not be an effective method to stimulate tau clearance by microglia (Hopp et al., 2018). Microglia deficient in TREM2, a receptor that regulates extracellular A β , are especially inept at tau clearance (Bemiller et al., 2017). It appears that microglia are not capable of handling the burden of removing extracellular tau from the brain (Majerova et al., 2014). Additionally, their activation in disease states may contribute to the propagation of pathogenic tau to distant brain regions.

A second type of glia found in the CNS, astrocytes, modulate neuronal synaptic activity and play a critical role in the maintenance of neuronal circuits and health. Astrocyte end-feet are a structural component of the blood brain barrier, where they facilitate fluid movement and solute elimination from the CNS. Recent investigations have indicated that astrocytes may interact with extracellular tau (Perea et al., 2019) as well, though the mechanisms by which this occurs are unclear. Astrocyte accumulation of tau pathology has been observed in several primary tauopathies, including age-related tau astroglipathy (ARTAG), which has not been fully characterized to this point, though it has been linked with prior exposure to TBI (Kovacs, 2020). Furthermore, observations of astrocytic tau pathology have uncovered significant heterogeneity amongst the isoforms of tau that accumulate, the affected brain regions, and the phenotypic presentation of the tau containing astrocytes (Reid 2020). Interestingly, astrocytic tau pathology is predominantly observed in primary tauopathies, as it does not occur in secondary tauopathies like AD (Garwood et al., 2017).

A distinguishing feature between CTE and other tauopathies is the accumulation of perivascular astrocytic tau pathology in the sulci (Arena, Smith, et al., 2020), which may develop in response to mechanical damage to the BBB after exposure to a TBI (Kovacs, 2020). The current literature indicates that astrocytes do not produce much of their own tau, if any, (Kovacs, 2020), but instead phagocytose extracellular tau released by neurons. The mechanisms and consequences of astrocytic tau internalization are still under investigation. As the field of extracellular tau research is still in its infancy, very little is understood with respect to the role that glial cells play in either tau clearance or propagation. However, existing evidence suggests that neither neurons, nor microglial play a significant role in reducing pathological extracellular tau levels in the CNS, suggesting other mechanisms or cellular interactions may be responsible for extracellular tau elimination.

1.3.4 Tau elimination in extracellular brain fluids.

Outside of the CNS, a lymphatic system exists that is responsible for the clearance of cellular debris. Recent evidence has supported the existence of a similar system confined to the CNS, though its exact mechanisms are still up for debate. To maintain homeostasis in the CNS, solute elimination must approximate solute synthesis. Solutes, proteins, and other debris in the brain that are not metabolized by cells, are removed from the parenchyma via the blood-brain barrier or perivascular pathways. These efflux mechanisms are important for preventing the buildup of toxic proteins that cannot be efficiently removed through cellular processes. An accumulation of unremoved waste products due to the disruption of these clearance systems have been associated with neurodegenerative disease, sleep deprivation, and traumatic brain injury. Understanding this system could lead to new therapeutics to facilitate and enhance solute elimination from the brain.

There are several mechanisms by which extracellular solutes can be eliminated from the brain fluids. Bulk flow is one solute elimination mechanism which carries all components within the fluids of the brain at the same speed, akin to the flow of blood in the circulatory system. Every parenchymal cell is within 10 microns of a microvessel, so diffusion was proposed as a mechanism by which water-soluble solutes are transported to the perivascular space (Hladky & Barrand, 2018). Once a solute reaches the perivascular space, it is either transported through the BBB to the blood, or it may flow into the CSF, where it subsequently drains into peripheral lymph and is processed by the liver (Jessen et al., 2015). Because the blood-brain barrier is so effective at restricting solute permeability, most solutes require active secretion through facilitated transport systems. As an example, extracellular A β is eliminated from the CNS via endothelial LRP1-mediated endocytosis and subsequent efflux into the blood, which occurs rapidly and efficiently (Tarasoff-Conway et al., 2015a). Large solutes like tau that do not have known specific BBB transporters, along with other waste products, may not be secreted efficiently through the BBB and would require a different method of bulk clearance. However, full length and truncated tau species may cross the BBB in a bi-directional manner, though the mechanism is unclear (Banks et al., 2016). More investigations are required to understand the nature and extent of tau transcytosis across the BBB.

Proteins in the ISF can be directly cleared to the CSF through bulk flow (Tarasoff-Conway et al., 2015a). CSF from the subarachnoid space is proposed to flow into the parenchyma through a perivascular space, where it mixes with the ISF and collects solutes, returning them along a perivascular route back into the CSF. Much of this bulk flow and fluid exchange occurs courtesy of fluid filled canals called the Virchow-Robin Space (VRS), which are located on the periphery of the capillaries, arteries and veins that penetrate the parenchyma. There are several proposed theories for how this fluid movement is regulated. One contributor is the constant production of CSF in the choroid plexus, which creates positive pressure to drive movement (Silva et al., 2021). An additional explanation was suggested in the wake of a foundational study showing that the interstitial space expands during sleep, which enables a greater exchange between the ISF and CSF, allowing metabolic waste to be removed (Hladky & Barrand, 2014). Tau ISF levels during wakefulness increase by 90% in mice, which may be due to a combination of elevated release via neuronal activity, and reduced clearance due to contraction of the interstitial space (Holth et al., 2019). Alterations in the sleep-wake cycle can lead to the accumulation of tau in the ISF and the CSF. During sleep deprivation, levels of tau in the ISF of mice increased 2-fold, and in human CSF, tau levels increased by 50% (Holth et al., 2019). There has been some doubt cast into whether this sleep induced expansion occurs (Gakuba et al., 2018), and other mechanisms driving fluid movement have been suggested. The Monroe-Kellie dogma (Hladky & Barrand, 2014), which requires the volume of the parenchyma and the vasculature to be constant, proposes that the cardiac cycle can drive pulsations along the vasculature. This arterial pulsatility has been proposed to be a driver of perivascular solute flow. It has been demonstrated the CSF is able to enter the ISF from the perivascular space (Tarasoff-Conway et al., 2015a) following the same direction as blood flow. CSF has been proposed to flow via arterial pulsation, while ISF flows via bulk flow (Tarasoff-Conway et al., 2015a).

Recently, new studies have provided evidence for alternative mechanisms driving these clearance pathways (Figure 1.5). Iliff and colleagues reported on a series of water channels termed the glymphatic system in 2012, proposing that CSF enters from the periaxonal space, circulates through the parenchyma, and is eliminated back to the surface of the parenchyma via a perivenous route (Iliff et al., 2012). Carare and colleagues proposed an Intramural Peri-Arterial Drainage (IPAD) pathway where the fluid in the parenchyma entered via a route within the periaxonal space, underwent convective circulation, and re-

entered a parallel route within the periarterial space to drain back to the surface of the parenchyma against the flow of blood (Carare et al., 2008).

1.3.5 Glymphatic elimination of tau

It has been proposed that the glymphatic system uses water influx via the water channel aquaporin-4 (AQP4) polarized to perivascular astrocytic end-feet to drive CSF-ISF exchange (Iliff et al., 2012). This flow results in a convective flux, flushing solutes away from arteries and towards veins in the parenchyma. After they are taken up through the perivenous space, they are cleared from the CNS through the lymphatic vessels in the neck. The glymphatic theory was derived by injecting markers into the cisterna magna which were seen in the periarterial space, but markers injected into the parenchyma were only seen around veins (Iliff et al., 2012). Initially studied using A β tracers (Iliff et al., 2012), these researchers have proposed that interstitial tau is cleared via the glymphatic system (Iliff et al., 2014). They found that interstitial monomeric tau was removed from the parenchyma via the perivascular space (Iliff et al., 2014). Genetic deletion of AQP-4 significantly reduced solute clearance in the parenchyma, which was exacerbated by TBI (Iliff et al., 2014), resulting in an increase in phosphorylated tau species in those mice. A β clearance was not impacted as much by glymphatic reduction as other solutes, which may be a result

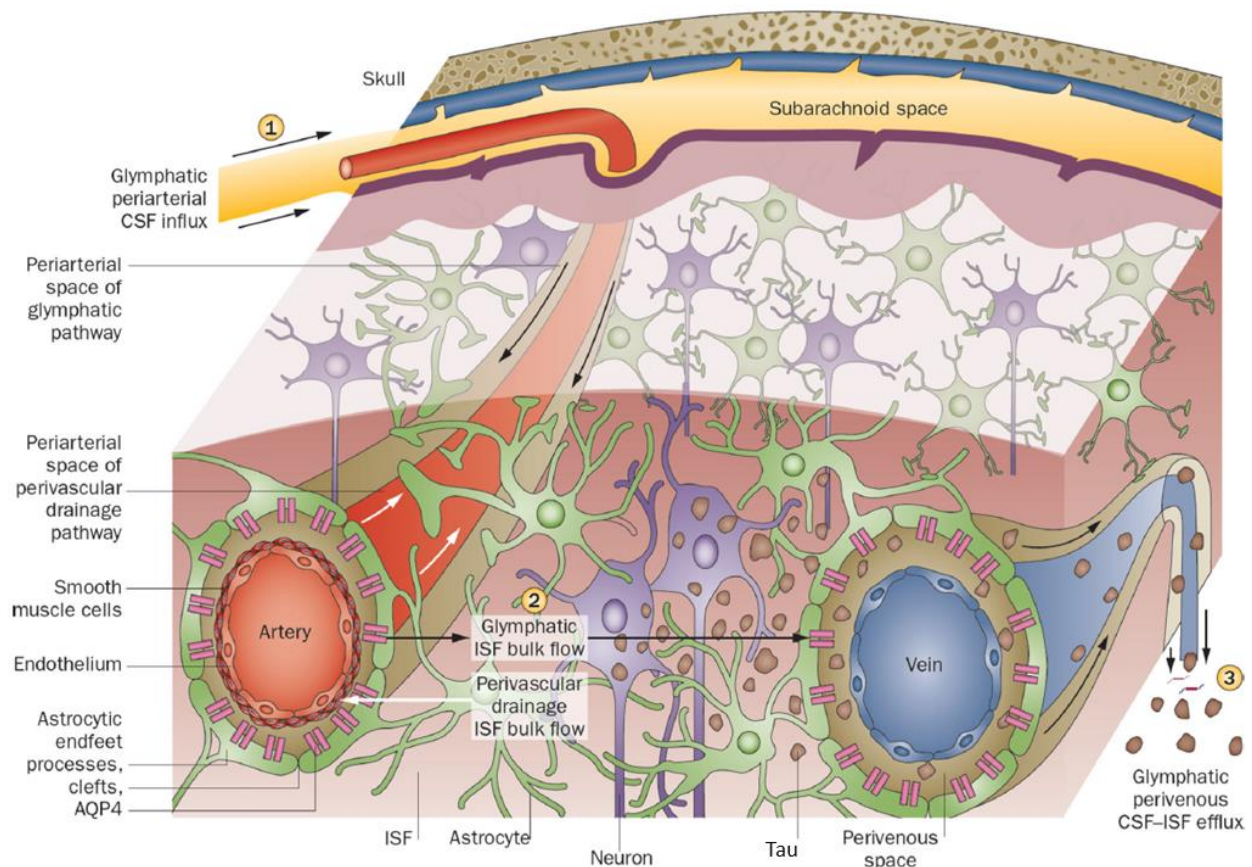


Figure 1.5 Perivascular clearance via Glymphatic and Intramural Peri-Arterial drainage. Perivascular drainage channels remove extracellular waste from the ISF to the CSF. From there, waste is either carried to the subarachnoid space, or is eventually absorbed into the lymphatic system. The glymphatic pathway (black arrows) flows from the periarterial space, through the ISF, to the perivenous space, using the perivenous channel for efflux. The Intramural Peri-Arterial drainage pathway (IPAD) (white arrows) uses the periarterial drainage channel to clear waste from the ISF to the CSF in the opposite direction of blood flow. Adapted from Figure 1 of (Tarasoff-Conway et al., 2015).

of the existence of alternative A β -specific BBB transporters like LRP1 (Iliff et al., 2012). However, injection of soluble A β impaired glymphatic flow, indicating that an increase in such a solute might propagate a feedforward mechanism whereby the extracellular accumulation may impair further clearance (W. Peng et al., 2016). Given that tau does not have any known transporter, glymphatic impairment would significantly impact tau clearance, resulting in extracellular tau deposition over time. Iliff et al. notes that if a transporter for tau at the blood-brain barrier were to be discovered, glymphatic impairment would still reduce tau clearance (Iliff et al., 2014). This proposed drainage pathway distributes solutes along the BBB and generates solute interactions with efflux machinery, so an impairment in this solute delivery system would diminish net BBB efflux of extracellular waste products (Iliff et al., 2012).

The other proposed mechanism by which extracellular solutes are thought to move throughout the brain resides primarily within the peri-arterial space. Carare et al. found that tracers injected into the CNS all exited within the arterial walls formed by the mural cell basement membrane (Albargothy et al., 2018), instead of draining into the peri-venous space, as proposed by the glymphatic pathway (Iliff et al., 2012). The IPAD pathway proposes that the CSF flows along the pial-glial periarterial BM into the parenchyma, mixes with the ISF, collecting solutes, then returns to an alternate layer in the peri-arterial space, where it is carried to the surface of the parenchyma. An example of the relevance of this proposed pathway is the observation that in AD, often A β appears to get trapped in this periarterial space around cortical blood vessels and leptomeninges, leading to the emergence of CAA (Albargothy et al., 2018). In CTE, pathological tau is typically trapped in the neurons and glia adjacent to the perivascular space (McKee et al., 2013), suggesting that tau may follow a similar elimination pathway as A β , and is taken up by the surrounding cells. Though it is important to identify the precise location of the drainage route, both proposed pathways result in the drainage of extracellular waste products like tau to the surface of the parenchyma and may play complementary roles to each other.

Once tau or other solutes reach the surface of the brain from the deep parenchyma, a proposed network of meningeal lymphatic vessels may be responsible for scavenging and removal. This system, recently termed a dural lymphatic system (Patel et al., 2019), is located in the dura of the meninges, and travels adjacent to the superior sagittal and transverse perisinusoid space (Bower & Hogan, 2018). A newly discovered mural lymphatic endothelial cell (muLEC), exclusive to the meninges may be the driver of this lymphatic process (Bower & Hogan, 2018). Though they are perivascular, they don't express known mural cell markers. MuLECs can internalize tracers up to 150 kDa through endocytosis, which may enable them to scavenge monomeric and low molecular weight oligomeric tau from the ISF and CSF (van Lessen et al., 2017).

CSF cleared through this pathway eventually drains to the deep cervical lymph nodes (Bower & Hogan, 2018). Extracellular tau was observed to be trafficked through this system, and by using transgenic mice that had their dural lymphatic system disrupted, researchers were able to demonstrate delayed tau clearance from the brain to the periphery (Patel et al., 2019). This mechanism appeared to impair tau clearance to a greater extent than other solutes with similar molecular weights, indicating that additional research is needed into the specific interactions between tau and the components of the CNS lymphatic system (Patel et al., 2019). This observation is supported by investigations of tau efflux to the periphery which suggest that the CNS sequesters tau to some degree (Banks et al., 2016). As tau clearance was not completely inhibited following the transgenic removal of the dural lymphatics, it appears that glymphatic drainage may not be the only mechanism for extracellular tau elimination from the CNS (Patel et al., 2019). At this point, evidence suggests that extracellular tau diffuses to the perivascular space, where the glymphatic and/or IPAD system carries it from the deep parenchyma to the surface of the brain. There,

the dural lymphatic system exports whatever extracellular tau is remaining to the periphery, where it descends into the cervical lymph nodes. Interestingly, while A β can accumulate around leptomeninges, tau does not. This observation suggests a potential mechanism of tau elimination at the brain vasculature, prior to reaching the meningeal lymphatic system.

1.4 BRAIN VASCULATURE

In addition to the mechanisms just described, the brain vasculature can have a profound influence on solute disposition and elimination in the brain. The cerebrovasculature provides the oxygen and nutrients required for neuronal function, but these specialized vessels also play a significant role in the preservation of CNS homeostasis by acting as a gateway for the passage of molecules between the brain and the blood. This is possible due to a unique series of physical properties and transportation machinery within brain endothelial cells, that owe their existence, in large part, to the microenvironment generated by the adjacent perivascular mural cells and astrocytes. As discussed, a significant amount of proteinaceous waste is flushed towards the cerebrovasculature, which is responsible for the elimination of this material to the periphery. The disruption and dysfunction of the cerebrovessels can impair this waste elimination, commonly resulting in the progression of neurodegenerative diseases.

1.4.1 Blood-brain barrier

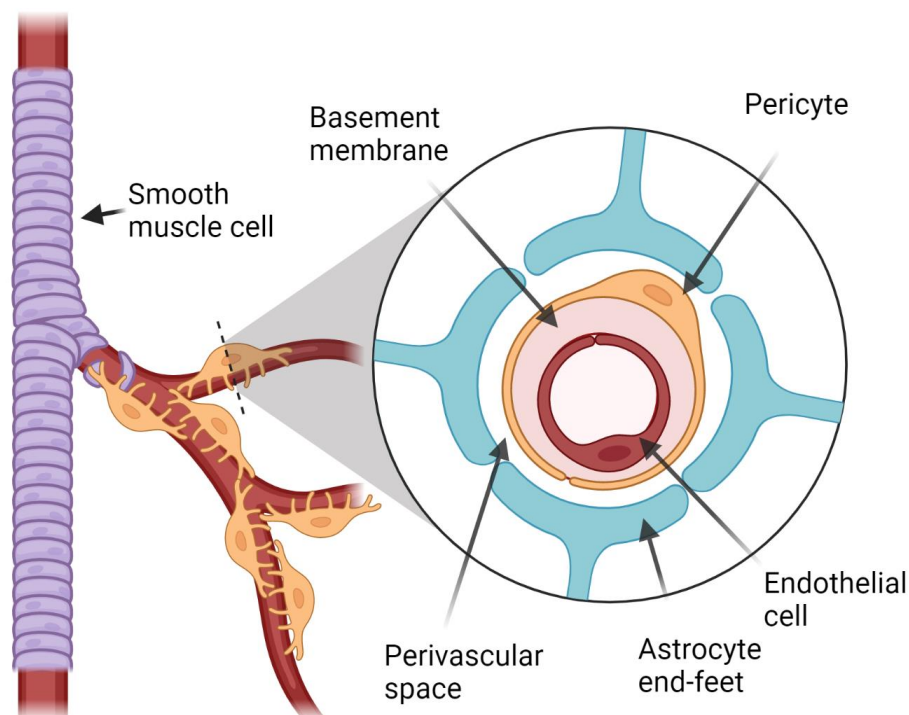


Figure 1.6 Blood-brain barrier. The BBB found in capillaries is comprised of endothelial cells (red), which share a basement membrane (pink) with pericytes (orange). Astrocyte end-feet (blue) encircle this structure. In larger vessels, pericytes are mostly replaced by smooth muscle cells (purple), which occupy most of the vascular wall.

The greatest exchange of solutes between the brain and the periphery occurs at the blood-brain barrier. It is a composition of cells and cellular components, including endothelial cells, mural cells, astrocytes, and basal lamina (Figure 1.6). The BBB is restrictive compared to peripheral blood vessels, with comparatively lower levels of transcytosis across endothelial cells, but it is not an impermeable physical entity. The influx and efflux of ions and molecules between the blood and the parenchyma are regulated via junctional proteins, transporters, and transcytosis machinery. Tight junctions bind adjacent endothelial cells together to prevent solute leakage, which ensures that charged molecules and larger proteins like tau and A β can't permeate across the BBB without a transport system. While some transporters are non-specific with respect to their substrate, other substrate specific transporters actively facilitate the removal of molecules from the CNS (Profaci et al., 2020).

The BBB is highly dynamic under physiological conditions, and responds to both intrinsic and extrinsic signals to maintain a homeostatic environment in the CNS. The BBB is also influenced by various other factors including the circadian rhythm and aging. BBB dysfunction can be caused by insults to cerebrovascular cells (e.g., mural cells or astrocytes), vascular trauma, or the even differential genetic expression of certain proteins like ApoE, which may accelerate the BBB's age-related changes and potentially increase susceptibility to neurodegenerative diseases (Banks et al., 2021).

1.4.2 Mural cell physiology

Mural cells, the umbrella term for pericytes and smooth muscle cells, ensheath much of the vasculature in the body. They were originally discovered by Charles Rouget but were formally named in 1923 by Karl Zimmerman. Pericyte processes cover over 90% of the 600 km of vasculature in the human brain (Winkler et al., 2014), a much higher percentage than in the periphery, and play a vital role in maintaining brain homeostasis. They contribute to cerebral blood flow, the regulation of BBB integrity, neuroinflammation, angiogenesis, the phagocytosis of small molecules, and have demonstrated stem cell characteristics (Sweeney et al., 2016).

There is an ongoing debate about how to accurately describe mural cells due to their heterogeneous morphology and characteristics. Berthiaume recently attempted to elucidate the specific subgroups of brain mural cells based on location and expression of receptors (Berthiaume, Hartmann, et al., 2018; Grant et al., 2019). Pericytes located on pre-capillary arterioles and post-capillary venules express the contractile protein α SMA (α smooth muscle actin) and are believed to contribute to the regulation of cerebral blood flow (Sweeney et al., 2016). These ensheathing pericytes are in proximity to neurons, which allows them to respond to energy deficits in a rapid manner (Berthiaume, Hartmann, et al., 2018). Mural cells in this region have the capacity to quickly switch phenotypes between contractile cells and secretive cells, enabling them to adapt to volatile vascular conditions (Jullienne et al., 2016). Pericytes in capillaries express extremely low levels of α SMA, but they do express a variety of membrane transporters, indicating their role in blood-brain barrier maintenance (Berthiaume, Hartmann, et al., 2018). These mesh-like pericytes extend long processes that combine to cover most of the capillary bed (Berthiaume, Hartmann, et al., 2018). In contrast to the fast reactions of upstream mural cells, pericytes in the capillaries may provide continuing equilibrium over longer periods of time (Berthiaume, Grant, et al., 2018).

Mural cells also express a wide variety of endocytic and phagocytic receptors including the Fc receptor, CD36, CD38 and LRP1 (Rustenhoven et al., 2017) enabling them to scavenge and internalize compounds from the blood and parenchyma. Their perivascular location makes them uniquely suited to monitor and maintain BBB integrity. Pericytes have demonstrated the capacity for multiple internalization methods including phagocytosis, receptor-mediated endocytosis, and pinocytosis (Thomas, 1999). Phagocytosis is

upregulated in pericytes during acute cerebral ischemia, where their typical markers like CD13, platelet derived growth factor receptor β (PDGFR β) and α SMA are reduced, while microglial markers including Iba1 are enhanced in pericytes (Rustenhoven et al., 2017). It's possible that pericytes switch towards a more microglial phenotype during times of BBB disruption as observed following injury, to facilitate the clearance of necrotic cell components or blood-derived toxins. The perivascular location of mural cells puts them in direct contact with the proposed glymphatic drainage pathway of extracellular solutes and waste. The confluence of their location and phagocytic capabilities results in a significant role in extracellular solute elimination from the CNS.

1.4.3 Mural cells in neurodegenerative disease

Mural cell health is critical to maintain and preserve the homeostatic functions of a wide array of CNS processes, and conditions resulting in their dysfunction can lead to consequential neuronal decline. Mural cell degeneration has been observed in a variety of neurodegenerative diseases ranging from AD to ALS (Winkler et al., 2014). The loss of pericytes in disease can compromise BBB integrity, and could provide an environment favorable to the accumulation of blood derived toxic metabolites in the parenchyma. Fibrin deposits that occurred due to diminished pericyte coverage leads to demyelination, axonal retraction, and oligodendrocyte cell death (Montagne et al., 2018). Plasmin and thrombin can leak through the permeable BBB as well, leading to neuronal injury and memory impairment, respectively (Sweeney et al., 2018). Pericyte maintenance of the BBB to prevent the entry of the blood-derived components is crucial for WM and GM homeostasis. An additional consequence of mural cell loss is a diminished capacity of mural cells to regulate cerebral blood flow at the macrovascular and microvascular level (Sweeney et al., 2016). This can result in an impairment of blood vessels to deliver oxygen and nutrients in response to local brain requirements, which may attenuate neuronal activity. Many vascular risk factors including type 2 diabetes and high blood pressure are correlated with increased susceptibility to AD (Henstridge et al., 2019), suggesting a possible link between vascular dysfunction and neurodegeneration.

Prolonged exposure to high concentrations of A β is toxic to pericytes (el Ali et al., 2014), resulting in the shedding of PDGFR β , followed by cell death (Sagare et al., 2015). Elevated levels of soluble PDGFR β in the CSF correlates with BBB dysfunction in individuals with mild cognitive impairment (Sweeney et al., 2018). This mural cell damage occurred prior to detectable changes in CSF tau and A β , suggesting that dysfunctional mural cells may play an early role in the progression of cognitive impairment that could be contributing to the accumulation of those proteins (Nation et al., 2019). AD patients with pericyte degeneration showed evidence of extensive phagocytosis of blood derived proteins, which can lead to neuroinflammation and further neuronal damage (Sweeney et al., 2018). In fact, AD patients not only have diminished pericyte coverage, but reduced microvasculature as well, which may limit the availability of oxygen and glucose to neurons resulting in cognitive impairment (Sweeney et al., 2018).

Pericyte death in the presence of A β could lead to further plaque formation and neurodegeneration, promoting the progression of the disease state. In AD patients, hippocampal A β load was negatively correlated with pericyte coverage (Winkler et al., 2014), indicating a link between pericyte death and disease progression. This is supported by investigations of sporadic AD, where A β accumulation occurs because of insufficient clearance from the brain, not overproduction (Winkler et al., 2014). The 2-hit vascular hypothesis for AD proposes that vascular damage leads to the dysfunction of the BBB, resulting in subsequent accumulation of A β (Sweeney et al., 2018). Some evidence has suggested that mural cell health may influence tau pathology as well. The presence of tau pathology emerged in a mouse model of AD only when the mice were crossed with a mural cell deficient genotype (Sagare et al., 2013). Tau may

also contribute to vascular dysfunction. This has been investigated in hTau mice, which are generated through a targeted replacement of murine tau with the human tau gene, and P301S mice, which have a point mutation in their targeted replaced humanized tau gene conferring additional propensity for tau to misfold (Bennett et al., 2018). Both transgenic mouse lines demonstrated structural changes and alterations in their microvasculature (Bennett et al., 2018). It is possible that pathogenic tau species could impact endothelial cell physiology, resulting in pericyte dysfunction. Pericyte loss appears to correlate with BBB breakdown, nutrient deprivation, vascular instability, and white matter damage, in addition to pathogenic protein accumulation. These degenerative mechanisms have been observed in the context of chronic TBI, suggesting that pericyte health may play a role in the progression of TBI pathology.

1.4.4 TBI on mural cell status

Cerebral microvascular injury is observed in most preclinical and clinical cases of mTBI. Directly after TBI, the BBB becomes permeable, and toxic components, including serum-derived albumin, leak into the parenchyma (Jassam et al., 2017). Blood derived solutes can activate neuroinflammation, as the microglia attempt to clear out the pathogenic components (Jassam et al., 2017). This damage to the BBB has been associated with a reduction in small vessel density, complexity (Obenaus et al., 2017) and cerebral blood flow (Kenney et al., 2016), leading to insufficient energy delivery to adjacent neurons. This can contribute to post-concussive symptoms and may leave the brain susceptible to further injury if the vasculature is not restored to physiologic conditions. Preclinical studies into the mechanisms underlying this observed vascular degeneration have provided preliminary evidence that exposure to TBI can cause acute and chronic dysfunction in vascular brain mural cells.

The disruption of normal pericyte function after TBI can have a variety of consequences. Evidence suggests that pericytes contribute to neurogenesis through cross-talk with neural stem cells (Choi et al., 2016). This cross talk can be inhibited post-TBI as pericytes are damaged, which could prevent neuronal regeneration and repair (Choi et al., 2016). Though TBI can lead to mural cell loss, it can also result in mural cells with a dysfunctional phenotype with retracted processes, elevated transport machinery, swollen cell bodies and alterations in intracellular organelles (Castejón, 2011). TBI can initiate pericyte loss and disrupted expression of PDGFR β in an acute stage, but there may be a biphasic response where pericytes and expression of PDGFR β increase in the days following a single CCI injury (Zehendner et al., 2015). However, in this CCI mouse model of TBI, the newly formed pericytes had an altered morphology and some were not associated with vessels (Zehendner et al., 2015). Some are likely migrating pericytes which establish new microenvironments that promote vessel formation in response to injury and help to restore nutrients to compromised brain regions (Dore-Duffy et al., 2000). The long-term consequences of these mural cell alterations in status are currently unclear, and may be exacerbated by additional injuries, or aging.

Endothelial cell response to BBB disruption could have indirect effects on chronic pericyte health. In multiple preclinical studies evaluating endothelial health after TBI induced BBB disruption, long-term phenotype changes to endothelial cells were observed (Jullienne et al., 2016). This could have implications for signaling between endothelial cells and mural cells, either through PDGFBB release by brain endothelia or the interactions of other growth factors. The disruption of this PDGFR β -PDGFBB signaling pathway was seen 7 days after fluid percussion injury in mice, though this signaling pathway appeared to recover slowly after injury (Bhowmick et al., 2019). The disrupted endothelial-mural cell crosstalk can manifest through other growth factors as well, though it is currently unclear how each signaling pathway influences the others. TBI can induce the acute endothelial secretion of Ang-2, a Tie2 antagonist, which inhibits endothelial Tie2 activation by pericyte-derived Ang-1 (Nourhaghighi et al., 2003). This leads to pericyte

detachment from the vessel wall and BBB leakiness (Gu et al., 2016). When endothelial secreted Ang-2 binds to Tie2 receptors it upregulates endothelial expression of factors like MMP-9, which degrades the extracellular matrix, and upregulates the expression of caveolin-1 (Q. Wang et al., 2020). Elevated levels of caveolin-1 in endothelia are commonly seen in the early post injury phase after TBI (Nag et al., 2017), though this increase can be prevented by the administration of exogenous Ang-1 (Gu et al., 2016). Ang-2 expression may promote early BBB repair, and subsequent endothelial growth and angiogenesis (Nourhaghighi et al., 2003). Ang-1 expression increases within hours or days after injury, as the BBB is repaired, and new vessels mature (Dore-Duffy et al., 2007; Gong et al., 2011). That said, the chronic impact of TBI or repetitive m-TBI on PDGFR β -PDGFBB signaling or the Ang-1/Tie2 axis is unclear.

If components of the NVU are impaired through disease or injury, solute clearance may be impacted. Atherosclerosis and arterial wall stiffening would diminish solute clearance through glymphatic impairment, leading to an accumulation of toxic proteins like tau. In mouse models of tauopathy, perivascular PHF tau accumulates in the perivascular space, which can damage the wall of the blood vessel (Merlini et al., 2016). This is supported by the evidence that BBB damage can be ameliorated through tau depletion in tauopathy mouse models (Blair et al., 2015). An interaction between extracellular pathogenic tau and perivascular mural cell damage appears to be linked to Braak staging (Merlini et al., 2016). This is exemplified in CTE, where deposits of perivascular tau are found in compromised areas. It was proposed that disease-associated arterial stiffness and mural cell loss may decrease the ability of medium sized arteries to cushion the force generated by cardiac pulsatile blood flow waves on smaller vessels, resulting in microvessel damage (Merlini et al., 2016). Microvascular pathology could impair perivascular clearance, resulting in an accumulation in perivascular tau, and BBB disruption (Merlini et al., 2016), which could be exacerbated by genetic factors including the ApoE4 genotype (Abrahamson & Ikonomic, 2020). This implicates vascular health in extracellular tau efflux from the deep brain parenchyma.

1.5 ApoE

1.5.1 ApoE characteristics

Apolipoprotein E (ApoE) was originally identified in the 1970s (Shore & Shore, 1973) and has been the subject of considerable investigation since the early 1990s when it was identified as a strong modulator of AD risk (Strittmatter et al., 1993). It is produced by many of the CNS resident cells including microglia, neurons, and vascular mural cells, though the majority is secreted by astrocytes. Human ApoE is a 34 kDa glycoprotein (Yamazaki et al., 2019), that can be found in 3 isoforms which differ from each other at only two residues out of the 299 total amino acids. These amino acid substitutions that constitute either ApoE2 (Cys112 and Cys158), ApoE3 (Cys112 and Arg158), or ApoE4 (Arg112 and Arg158) have significant consequences on the form and function of the protein. With regards to AD, a single copy of the ϵ 4 allele increases risk by 3-fold, with two ϵ 4 alleles increasing AD risk by 8-fold (Yamazaki et al., 2019). ApoE2 confers a protective effect against AD, as a recent study found that compared to individuals with two ϵ 4 alleles, those with two copies of ϵ 2 allele had their AD odds reduced by 99.6% (Reiman et al., 2020). ApoE plays a critical role in the CNS as the primary lipid and cholesterol transporter (Chen et al., 2021), though it can bind to and transport various disease-relevant ligands and receptors as well, in an isoform-dependent manner. The vast majority of ApoE interactions with disease relevant proteins have been conducted on A β , due to the significant role ApoE isoform expression plays in AD risk, which have generally

determined that expression of ApoE4 is linked with A β aggregation and diminished A β clearance from the brain.

1.5.2 ApoE and astrocytes

Astrocytes are responsible for a variety of roles in the CNS including the metabolic support of neurons, and the modulation of synaptic transmission. As the predominant source of ApoE in the CNS, the effects of ApoE isoform expression in astrocytes have been investigated to examine how the ApoE isoforms influence astrocyte function in physiology and disease conditions. For example, ApoE isoforms differentially influence the ability of astrocytes to phagocytose neurons, with the expression of ApoE2 enhancing the rate of synapse pruning, while ApoE4 expression diminishes this ability in astrocytes (Chung et al., 2016). Astrocytes may also contribute to solute elimination in the CNS. Their expression of the water channel AQP4 plays a fundamental role in driving the glymphatic system, and astrocytes provide key contributions to the BBB including the regulation of blood flow and maintenance of BBB integrity, but studies have also investigated whether astrocytes are capable of direct phagocytosis of solutes. Early studies found that astrocytes may be able to phagocytose and degrade A β *in vitro* (Wyss-Coray et al., 2003). Perivascular astrocytes are believed to clear extracellular A β into the perivascular space from the CNS though there have been conflicting findings when investigating this *in vivo* (Spanos & Liddelow, 2020). The expression and presence of extracellular ApoE may influence astrocyte interactions with extracellular solutes like A β in multiple ways. It is possible that extracellular ApoE particles compete with A β for astrocyte uptake in an isoform dependent manner, though these interactions likely depend on the aggregation state of A β , the lipidation of ApoE, and the concentrations of each, among other factors (Kanekiyo et al., 2014). However, ApoE isoform expression in astrocytes can differentially modulate astrocyte receptor localization, including the astrocyte surface expression of LRP1, with ApoE4 expression yielding the lowest LRP1 surface expression in astrocytes (Prasad & Rao, 2018). Additionally, ApoE expression can modulate the intracellular trafficking machinery within astrocytes, potentially leading to defective clearance of A β in astrocytes expressing ApoE4 (Prasad & Rao, 2018). At this stage, the contribution of perivascular astrocytes to pathogenic solute elimination, and the effects of ApoE isoform expression on these processes are still uncertain.

1.5.3 ApoE and the NVU

ApoE has been shown to modulate disease pathology independently of its role in cholesterol transport or by directly facilitating the clearance of pathogenic proteins like A β . Recent studies have demonstrated that ApoE is involved in signaling pathways at the BBB in an isoform dependent manner, and that ApoE4 expression leads to BBB breakdown via this pathway. Astrocytes that express ApoE4 secrete a dysfunctional basement membrane compared to that which is secreted by astrocytes expressing the ApoE3 isoform, which could lead to reduced elimination of solutes at the BBB (Keable et al., 2020). Astrocyte-secreted ApoE2 and ApoE3 bind to LRP1 on the surface of pericytes, which inhibits cyclophilin A (CypA) function (Bell et al., 2012). Alternatively, ApoE4 interactions with LRP1 on pericytes lead to the activation of the CypA signaling cascade in pericytes, resulting in elevated matrix metalloproteinase 9 activity that degrades the basement membrane and tight junctions at the BBB (Bell et al., 2012). This BBB breakdown can lead to neuronal loss and accelerated neurodegeneration, potentially facilitating or positively reinforcing other neurodegenerative processes occurring simultaneously, including neuroinflammation and pathogenic protein accumulation (Montagne et al., 2020). As ApoE4 expression appears to accelerate vascular dysfunction and neurodegeneration, it is possible that it could influence

neurovascular processing and elimination of extracellular solutes, particularly in a compromised environment as seen in r-mTBI.

1.5.4 ApoE and TBI

Exposure to repetitive concussions does not always lead to the development of CTE, suggesting there may be additional contributing factors, including genes that may predispose an individual towards neurodegeneration. Some studies have suggested that the ApoE4 isoform may exacerbate TBI-related pathologies, and in fact, expression of the ApoE4 genotype has been linked to a higher incidence of CTE in athletes (Stern et al., 2013). Exposure to repetitive blast mTBI in mice has been linked with higher p-tau levels in ApoE4 than in ApoE3 mice (Cao et al., 2017), which could be due to dysfunctional phosphorylation activity (Cao et al., 2017) or altered microglia homeostasis (Y. Shi et al., 2019). TBI has been associated with BBB dysfunction, and ApoE4 has been demonstrated to contribute to BBB breakdown, leading to investigations into whether ApoE4 can exacerbate BBB impairment after TBI (Main et al., 2018). The authors found that ApoE helps repair the BBB after TBI, but expression of ApoE4 showed impaired restoration compared to ApoE3, potentially due to delayed mural cell repopulation (Main et al., 2018), as PDGFR β expression was reduced for a more chronic time-period in ApoE4 compared to ApoE3 after TBI (Main et al., 2018). ApoE was shown to modulate spontaneous repair of the BBB in response to TBI, with ApoE4 disrupting this process (Main et al., 2018). To date, studies investigating the impact of ApoE isoform on TBI have been inconclusive (Muza et al., 2019), and while it does appear to influence tau pathology (Y. Shi et al., 2017, 2019), the mechanism by which this occurs remains unclear.

1.5.5 ApoE and Tau

ApoE binds tau in an isoform dependent manner *in vitro*, with ApoE3 showing a much stronger interaction with tau than ApoE4 (Huynh et al., 2017). As ApoE plays an isoform dependent role in A β binding and clearance, it is possible that ApoE might modulate tau in a similar manner, though ApoE's binding site for tau is distinct from its binding site to A β . Investigations into the correlation between ApoE isoform expression and Braak staging have revealed significant associations, though they are not as straightforward as with A β . ApoE4 is associated with greater tauopathy when it is in the presence of A β pathology, but in the absence of A β pathology, ApoE2 expression is linked to increased tau pathology (N. Zhao et al., 2018). In fact, ApoE2 has a stronger binding affinity to tau than ApoE3 (N. Zhao et al., 2018), though this enhanced binding affinity in ApoE2 relative to ApoE3 was negated when the ApoE was lipidated (N. Zhao et al., 2018). ApoE2 expression is associated with increased risk for primary tauopathies including PSP and CBD, and may increase the severity of PSP, though the mechanism for this is still unclear (N. Zhao et al., 2018).

ApoE may form an extracellular complex with tau in an isoform-dependent manner, which could facilitate clearance or cellular uptake. ApoE may also bind tau and modulate tau aggregation in the cytoplasm, which could facilitate or hinder enzymatic processing or intracellular trafficking. The concentration of ApoE may impact tau, as ApoE competitively inhibits astrocyte uptake of A β at higher concentrations. Physiologically, ApoE isoforms have differential concentrations in the ISF with E2 > E3 > E4 (J. D. Ulrich et al., 2013). It is currently unclear whether a higher concentration of extracellular ApoE would lead to enhanced ApoE/tau complexes to facilitate cellular uptake, or if it could competitively inhibit receptor mediated internalization of tau through LRP1, as demonstrated previously in neurons (Rauch et al., 2020). Though previous studies have demonstrated that ApoE isoforms differentially bind to tau *in vitro*, this may not translate into a physiologically relevant phenomenon. Regardless of ApoE lipidation status, which was

not fully captured into in the previously discussed *in vitro* studies, there is very little evidence of tau/ApoE complexes observed *in vivo*. This is consistent with investigations into physiological A β /ApoE complexes which suggest these interactions may be rare, if not irrelevant (Verghese et al., 2013). It is possible that ApoE isoforms contribute to tau-mediated neurotoxicity through indirect mechanisms, which have not been fully explored to date, but may include altered subcellular trafficking, or other cell specific neurotoxic effects.

ApoE may influence tau pathology in a cell specific manner, as depletion of microglia in a mouse model of tauopathy expressing ApoE4 was able to prevent neurodegeneration suggesting that ApoE4 expression by microglia in the presence of tauopathy may play a detrimental role with regards to microglial homeostasis (Y. Shi et al., 2019). Similar findings were seen with depletion of astrocytic ApoE4 in the context of tau mediated neurodegeneration (C. Wang et al., 2021), suggesting a gain of toxicity effect in CNS glia which may vary amongst CNS resident cells. There is some debate as to how ApoE exerts its toxic effect, as a clinical study found that healthy older ApoE4 carriers had higher levels of neuroinflammation which were associated with lower AD pathology (Albrecht et al., 2021). The authors of this study suggested that ApoE4 initially triggers neuroinflammation to prevent accumulation of A β in the initial stages of disease, however this effect was not seen with ApoE3 (Albrecht et al., 2021). More research is required to clarify the mechanisms by which ApoE isoforms may contribute to the development and exacerbation of neurodegenerative disease. In particular, it is critical to understand the interactions between ApoE isoforms and tau, and the mechanisms through which those interactions contribute to the elimination of tau from the CNS.

1.6 AIMS

Despite limited therapeutic options available to those who have sustained a TBI, there has been a lack of investigation into the mechanisms underlying the vascular decline and progressive tau pathology commonly observed in the chronic stages after injury. Identifying the physiology that regulates the intersection between these two phenomena, and how this interaction is influenced by TBI, may shed light on potential therapeutic targets. There is not a clear understanding of how tau interacts with any of the components of the NVU, or whether they could contribute to tau elimination. Despite the evidence linking negative outcomes after TBI to the expression of the ApoE4 isoform, investigations into the potential interactions between ApoE4, tau, and the NVU are sparse. Therefore, to address these limitations, this thesis will investigate the role of each of the cellular components of the NVU on the elimination of extracellular tau, and how those interactions may be impacted in the chronic stages after r-mTBI. My hypothesis is that brain vascular mural cells are a pathway for processing and eliminating tau from extracellular brain fluids and disruption of these cells after TBI leads to tau pathology and neurodegeneration. This hypothesis will be investigated in the following aims:

To determine the impact of TBI on mural cell function and processing of extracellular tau

- Evaluate mural cell expression in isolated vasculature from human TBI brain specimens
- Evaluate the post-injury time course of mural cell expression and function in a mouse model of r-mTBI
- Evaluate tau internalization and degradation in r-mTBI cerebrovasculature

To determine the impact of TBI on tau elimination across the BBB

- Determine the elimination profile of extracellular tau from the brain in the context of r-mTBI
- Evaluate the impact of r-mTBI on cerebrovascular transcytosis
- Evaluate endothelial transcytosis of tau

To determine the contribution of astrocytes and ApoE isoforms on extracellular tau elimination following TBI

- Investigate the influence of ApoE genotype on tau elimination from the brain following r-mTBI
- Evaluate tau degradation pathways in astrocytes, and the effect of ApoE genotype and TBI on these processes
- Assess the influence of ApoE genotype on the sub-cellular trafficking of tau in astrocytes

CHAPTER 2: ROLE OF MURAL CELLS IN EXTRACELLULAR TAU ELIMINATION AFTER TBI

2.1 INTRODUCTION

Mural cell dysfunction has been reported in several neurodegenerative diseases, including AD and ALS (Winkler et al., 2014). In AD, mural cell loss is linked to blood brain barrier breakdown, and the accumulation of pathogenic proteins. Furthermore, mural cells are known to play a role in solute elimination from the brain, through uptake and degradation. These cells are involved in the regulation of endothelial solute transport across the BBB and some reports have suggested that they may facilitate the transport of solutes across the BBB as well (Henstridge et al., 2019), though the mechanism of the latter is unclear. Healthy pericytes contribute to the removal of A β , but in AD mural cells accumulate the pathogenic protein (Ma et al., 2018), which is associated with the development of CAA in the perivascular space. While brain pericytes have shown the capacity to eliminate pathogenic solutes, their interaction with tau has not been investigated. Our group found preliminary evidence that mural cells interact with tau to a greater extent than other cells in the NVU (endothelial cells, astrocytes), though the nature of that interaction is unclear (Ojo et al., 2021). Our team also found an elevation in total tau levels in PDGFR β ^{+/−} transgenic mice, which are characterized by a diminished mural cell population, compared to WT littermates (Ojo et al., 2021). These results suggest that mural cells may facilitate tau elimination from the CNS, and mural cell disruption could lead to an accumulation of extracellular tau. For this reason, I focused my investigation on mural cell interactions with tau.

While our previous preliminary studies and the work of others indicated that mural cell status may be impacted by TBI (Bhowmick et al., 2019; Choi et al., 2016), the mechanism by which this occurs is unclear. In neurodegenerative disorders including AD there is a loss of mural cells (Sagare et al., 2013), but there has been a lack of studies investigating whether TBI induces a similar loss of mural cells or a progressive dysfunctionality in these cells. Perivascular tau pathology has been correlated with a downregulation of mural cell markers including α -SMA in both human (Merlini et al., 2016) and mouse (Blair et al., 2015) tissue, suggesting an interaction between mural cells and extracellular tau, which may be relevant to disease progression. As CTE is characterized by an accumulation of tau in perivascular locations, I focused on whether r-mTBI could result in mural cell dysfunction and contribute to tau deposition around the brain vasculature. In particular, I investigated whether r-mTBI could influence the capacity for the cerebrovasculature to interact with extracellular tau. Attempts to replicate the human pathognomonic lesion of CTE in mice have been challenging (Smith et al., 2021), potentially due to differences in the brain architecture between humans and mice, including the murine lack of sulcal folds where the perivascular tau pathology is found in humans. However, our group had previously found that a 24-hit mouse model of r-mTBI developed cerebrovascular abnormalities (Lynch et al., 2016), which corresponded with cerebrovascular damage, diminished cerebrovascular reactivity and neurobehavioral impairment (Lynch et al., 2021). This extended injury model, adapted from a previously characterized 5-hit model by our group (Mouzon et al., 2012; Mouzon et al., 2014), has demonstrated white matter neuroinflammation and an accumulation both total tau, and oligomeric tau, at chronic time points post injury (Ojo et al., 2016). As our group observed a comparable accumulation of total tau in the mural cell depleted PDGFR β ^{+/−} transgenic mice, I investigated whether cerebrovessels isolated from r-mTBI mice and mural cell depleted mice had a diminished capacity to process tau.

To interrogate pathobiological changes to mural cells, I use two important mural cell specific markers - PDGFR β and α -SMA. As discussed in Chapter 1, endothelial cells release the ligand PDGF-BB to signal to mural cells through PDGFR β receptors to modulate several critical cellular functions (Winkler et al., 2014), and disruptions in this pathway can lead to cellular dysfunction or death. The contractile protein α -SMA is implicated in vasomotion and perivascular fluid movement and has been shown to be disrupted in areas of perivascular tau accumulation (Merlini et al., 2016). I investigated whether the chronic stages of r-mTBI could influence the expression of these key mural cell markers, as well as the ligand PDGF-BB, and compared any changes to those observed in the PDGFR β transgenic mice and a transgenic mouse model of AD (PSAPP mice) which has previously been associated with changes in mural cell markers (Sagare et al., 2013). Additionally, I used flow cytometry to determine cell density estimates for mural cells to confirm evidence of mural cell loss in our mouse model of r-mTBI and finally, to demonstrate translational relevance, I probed these same mural cell markers (PDGFR β and α -SMA) in cerebrovessels isolated from human AD and TBI brain specimens.

Despite results suggesting that mural cells contribute to the clearance of A β , including aggregated A β , the mechanism by which this occurs is unclear. Though mural cells demonstrate phagocytic capabilities after BBB injury (Castejón, 2011), there is a lack of understanding about whether the internalized material is enzymatically degraded. Herein, I focused on investigating whether mural cells were capable of degrading tau, either through the proteasome, or through autophagy. Additionally, as cellular dysfunction can influence degradation pathways, I investigated whether exposure to r-mTBI could impact the mural cell proteasome or autophagic activity. Neurodegenerative conditions can lead to dysfunctional proteasome machinery in neuronal cells (Deger et al., 2015), which results in the accumulation of tau. There is still debate in the literature regarding the mechanisms responsible for tau degradation under various conditions, though autophagy is believed to play a role in neuronal tau removal (Caballero et al., 2018).

As mural cells play a vital role in the elimination of extracellular solutes from the CNS, and their ability to fulfill this role may be impaired under neurodegenerative conditions, advancing our current understanding of the impact of r-mTBI on mural cell health is critical as this could reveal potential therapeutic targets. In particular, knowledge of the mechanisms involved in the interactions between mural cells and extracellular tau, and how those interactions are influenced by r-mTBI could uncover novel routes of extracellular tau elimination, especially under conditions in which the accumulation of extracellular tau may facilitate the progression of neurodegenerative disease. Therefore, this chapter will investigate the physiological role of mural cells in extracellular tau elimination, both *in vitro* and *ex vivo*, and how this process is impacted by r-mTBI.

I hypothesize that mural cells are involved in the elimination of extracellular tau, which is impaired after r-mTBI. The following sub-hypothesis will be interrogated:

- TBI results in progressive mural cell marker decline
- Mural cell marker decline leads to diminished mural cell uptake of tau
- Degenerative mural cells exhibit impaired tau degradation.

2.2 METHODS

2.2.1 Materials

Primary human brain vascular pericytes (HBVP) (cat#1200), primary human vascular smooth muscle cells (SMC) (cat#1100), and associated culture reagents were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). The proteasome inhibitor MG-132 (cat#ab141003), and mouse PDGF-BB ELISA (cat#ab224879) were purchased from Abcam (Cambridge, MA, USA). Poly-L-lysine solution (cat#P4707), Autophagy inhibitors 3-MA (cat#189490-100 MG), Spautin-1 (cat#567569), antibodies for LC3-II/I (cat#ABC929), GAPDH (cat#AB2302), and the live-dead discriminator Propidium iodide (cat#P-4864), and Hanks' balanced salt solution (HBSS) (cat#H8264) were purchased from MilliporeSigma (St. Louis, MO, USA). The CYTO-ID autophagy detection kit (cat#ENZ-51031-0050) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Lucifer yellow dextran (10 kDa) and the human tau enzyme linked immunosorbent assay (ELISA) (cat#KHB0041) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Mammalian protein extraction reagent (M-PER) (cat#78505), Halt enzyme inhibitor cocktails (cat#78442), and the bicinchoninic acid (BCA) protein assay (cat#23225) were purchased from ThermoFisher Scientific (Waltham, MA, USA). The ELISA kits for human (cat#LS-F13051) and mouse (cat#LS-F21849) α smooth muscle cell actin (α SMC-actin) were purchased from LifeSpan BioSciences, Inc. (Seattle, WA, USA). The ELISA kits for human (cat#EHPDGFRB) and mouse (cat#MBS919047) PDGFR β (platelet-derived growth factor receptor beta) were purchased from ThermoFisher Scientific (Waltham, MA, USA) and MyBioSource, Inc. (San Diego, CA, USA), respectively. Suc-Leu-Leu-Val-Tyr-AMC (cat#10008119) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Recombinant human tau-441 (rhtau) and fluorescein-labeled A β (1-42) (cat#A-1119) were purchased from rPeptide (Watkinsville GA, USA) (cat#T-1001-2). Recombinant biotin-labeled human tau-441 (cat#T08-54BN) was purchased from SignalChem (Richmond, BC, Canada). Antibodies for N- aminopeptidase, CD13 (cat#558744), and the brain endothelial cell marker, CD31 (cat#561410), were purchased from BD Biosciences (San Jose, CA, USA).

2.2.2 Animals

Both male and female mice [Human tau (hTau) (cat# 005491) and wild-type (C57BL/6) (cat# 000664)] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All studies used a mix of male and female mice. The hTau mice express six isoforms of human tau on a C57BL/6 background, but do not express murine tau, as previously described (Andorfer et al., 2003). The hTau genotype was confirmed after purchase using PCR from a tail snip via a third party (Transnetyx, Cordova, TN, USA). The mice were between 12 and 14 weeks old at the start of the study, housed in standard cages under 12-hour light/12-hour dark schedule at ambient temperature. Transgenic PS1/APPsw (PSAPP) mice overexpressing the "Swedish" mutation APP695 and mutant presenilin-1 (M146L) on a C57BL/6 background were bred in house and were used as a model of AD. The mural cell-depleted animals, PDGFR β (+/-), were kindly provided by Dr. Richard Daneman (University of California, San Diego, La Jolla, CA). Wild type mice littermates with a C57BL/6 background were used as controls and bred in house. All studies used mice housed 3 per cage under standard laboratory conditions (23 \pm 1°C, 50 \pm 5% humidity, and a 12-hour light/dark cycle) with free access to food and water throughout the study. All procedures involving mice were performed in accordance with Office of Laboratory Animal Welfare and National Institutes of Health

guidelines under a protocol approved by the Roskamp Institute Institutional Animal Care and Use Committee.

2.2.3 Human brain tissue

Human brain specimens were acquired from Dr. Thomas Beach, Director of the Brain and Body Donation Program at the Sun Health Research Institute (Sun City, AZ, USA). Intact frozen human cortex samples from the inferior frontal gyrus (500 mg) were obtained from the autopsied brains of 1) control subjects (no history of brain injury or AD), 2) TBI subjects (established history of a single head injury), 3) AD subjects, and 4) TBI-AD subjects (history of a single head injury and AD), though the TBI brains did not have the pathognomonic lesion of CTE. Additional information on these samples can be found in Table 2.1. ApoE genotype status was designated as a carrier of at least one copy of either the $\epsilon 2$, $\epsilon 3$, or $\epsilon 4$ allele. Where an individual had an $\epsilon 2$ and an $\epsilon 3$ allele, they were described as ApoE2 in Table 2.1, and a similar distinction was applied for the $\epsilon 4$ allele.

2.2.4 Injury Protocol

A mouse model of closed head injury was used to investigate the effects of r-mTBI on mural cell status. Mice were anesthetized with 1.5 L/min of oxygen and 3% isoflurane for 3 minutes, and the injury site was shaved. The mice were then transferred to a stereotaxic frame (Just For Mice Stereotaxic Instrument, Stoelting, Wood Dale, Illinois) mounted with an electromagnetic controlled impact device (Impact One Stereotaxic Motorized Impactor, Richmond, Illinois). The animals were positioned on a heating pad to maintain their body temperature at 37°C. The head was positioned and secured in the device to prevent lateral movement during the impact delivery. A 5 mm blunt metal impactor tip was positioned midway in relation to the sagittal suture using a NeuroLab controller. The tip was and then retracted and the strike depth was adjusted to 1.0 mm. The scalp was gently stretched by hand to ensure the rod delivered an adequate impact and the proper angle, and the impact was delivered at 5 meters per second with a dwell time of 200 milliseconds, providing a force of 72 N. The mice were then allowed to recover on a heating pad set at 37°C until they became ambulatory, to prevent hypothermia. Mice in the repetitive TBI group received 2 impacts per week, approximately every 72 hours, for 3 months. Sham mice were exposed to isoflurane for the same frequency and duration as the TBI mice but did not receive the brain injury. Mice

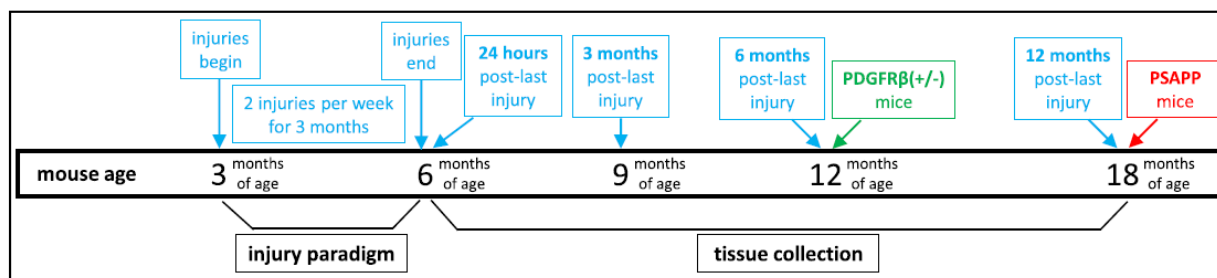


Figure 2.1 Timeline of the brain injury paradigm and tissue collection for the mouse studies. Human tau (hTau) mice at 3 months of age received 2 injuries per week for 3 months. As a control, sham animals did not receive any brain injuries, but were exposed to anesthesia for the same length of time as the injured mice and under the same paradigm (2 exposures per week for 3 months). Mice were euthanatized and tissue was collected at 24 h, 3 months, 6 months, and 12 months after the final brain injury or anesthesia exposure (blue boxes). Moreover, transgenic mural cell-depleted animals, PDGFR β (+/-) (green box), and a mouse model of Alzheimer's disease, PSAPP (red box), were euthanatized and tissue collected at 12 months and 18 months of age, respectively. Of note, neither the PDGFR β (+/-) nor PSAPP animals were administered any brain injuries.

were euthanized at post injury time points of 24 hours, 3 months, 6 months, and 12 months. Additional information on the injury timeline can be found in Figure 2.1.

2.2.5 Peptide preparation

Lyophilized A β peptides were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) using a standard process to limit aggregation as previously described (Bachmeier et al., 2013) to minimize the formation of β -sheet structures. Briefly, 1 mg of lyophilized pellet was resuspended in 1ml of HFIP and air dried for 1 hour before further drying in a speed-vac centrifuge for 30 minutes. The resulting film was re-suspended in 100% dimethyl sulfoxide (DMSO) to a concentration of 1 mM and store at -80°C. Recombinant human tau-441 (50 μ g) was dissolved in 1 ml HBSS and stored at -80°C.

2.2.6 Isolation of cerebrovasculature.

Parenchyma and cerebrovasculature from mouse and human brains were isolated using a protocol as previous described (Bachmeier et al., 2014). Freshly extracted mouse brains were collected (minus the cerebellum), the meninges and other outer vessels were removed with a dry cotton swab, and then minced with a blade in a Dounce homogenizer on ice. For human brains, ~ 450 mg human brain tissue was used. HBSS was added in fivefold excess of the brain volume, then the minced brain material was stirred with the spatula to break up larger material. After 6-8 passes with a Teflon pestle, a sample of the brain homogenate was collected as a representation of the whole brain. This homogenate fraction was stored in an equal volume of lysis buffer consisting of M-PER (Pierce Biotechnology, Rockford, IL, USA) supplemented with phenylmethanesulfonylfluoride (PMSF) (1 mM) and Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). An equal volume of 40% dextran solution was added to the brain homogenate for a final concentration of 20% dextran, and subsequently centrifuged at 6000g for 15 min at 4°C. This results in a compact mass at the top of the solution (parenchyma) and a pellet at the bottom of the container (cerebrovasculature) separated by a clear dextran interface (non-cell associated fraction). The parenchyma and dextran were collected and added to an equal volume of HBSS, then centrifuged at 6000g for 10 min at 4°C. A sample of dextran was collected, then the parenchyma was resuspended in HBSS and centrifuged for 5 min at 4°C to remove any residual dextran. This parenchymal pellet was collected in lysis buffer. The remaining pellet of cerebrovasculature after the parenchyma and dextran were removed, was washed with ice cold HBSS and collected in lysis buffer. All samples were stored at -80°C until further analysis.

2.2.7 Tau uptake and mural cell marker expression in isolated cerebrovessels

Freshly extracted brains from r-mTBI, PDGFR β +/-, and PSAPP mice were immediately fractionated to isolate the cerebrovasculature. These vessels were incubated with 5 ng/ml of recombinant human tau-441 (rPeptide, Watkinsville, GA, USA) for 1 hour at 37°C. They were then washed with ice cold HBSS and collected in lysis buffer. Lysates of the cerebrovascular tissue were probed for tau and mural cell markers. The levels of human tau were measured using a tau (total) human kit. Levels of the mural cell markers were evaluated using an enzyme-linked immunosorbent assay and normalized to total protein using the BCA protein assay. The mural cell markers, α SMA and PDGFR β , were assessed by ELISA and normalized to total protein using the BCA protein assay.

2.2.8 PDGF-BB expression in tissue homogenate

Mouse and human cortical tissue were processed as described, and an aliquot of the homogenate was collected using lysis buffer as described in Chapter 2, prior to cerebrovascular separation. Cortical homogenate was analyzed for PDGF-BB levels by ELISA and normalized to total protein content using the BCA protein assay.

2.2.9 Dextran and A β 42 uptake in isolated cerebrovessels

Freshly extracted brains from r-mTBI mice at 12 months post-last injury were immediately fractionated to isolate the cerebrovasculature. These vessels were incubated for one hour at 37°C with either 10 μ M of 10 kDa dextran tagged with Lucifer yellow, or 2 μ M of fluorescein-labeled A β 42. They were then washed with ice cold HBSS and collected in lysis buffer. The uptake of each solute was measured by fluorescence.

2.2.10 Cerebrovascular flow cytometry

To evaluate whether r-mTBI can impact the mural cells and endothelial cells in the cerebrovasculature, the cerebrovasculature was collected from r-sham and r-mTBI mice (hTau) at 6 months post injury and processed as previously described (Crouch & Doetsch, 2018). Briefly, the cerebrovascular pellet (obtained as described above) supernatant was removed, then the pellet was resuspended in 1 mg/ml Collagenase/dispase (Sigma-Aldrich) solution and incubated for 1 hr at 37° C with gentle agitation. After enzymatic digestion, the tissue was pelleted by centrifugation at 6000 rpm for 3 min, then the supernatant was discarded. The pellet was resuspended in 1 mg/ml DNase solution (Worthington) and subjected to mechanical digestion via trituration with a pipette to achieve a single cell suspension. The tissue was pelleted by centrifugation at 6000 rpm for 3 min, then the supernatant was discarded, and the resulting pellet was stained with antibodies against the cell surface markers: mural cell specific CD13 (BD Biosciences, San Jose USA) using anti-CD13-FITC (1:200) and the brain endothelial cell marker CD31 (BD Biosciences, San Jose USA) using anti-CD31-PE-CY7 (1:500). For live cell discrimination, the viability dye propidium iodide (Sigma Aldrich) was added to the antibody cocktail. Cells were stained for on ice for 30 minutes in the dark, then pelleted and resuspended in 1% BSA in HBSS. Analysis of cells was conducted using Attune® NxT Acoustic Focusing Flow Cytometer (Thermo Fisher Scientific, Waltham, MA USA). Analysis of flow cytometry was conducted using Attune® NxT software version 2.7 (Thermo Fisher Scientific, Waltham, MA USA).

2.2.11 Proteasome activity assay

Human brain vascular pericytes (HBVP) were seeded in a black clear bottom 96 well plate pretreated with poly-L-lysine in 100 μ L of culture medium at 10,000 cells per well and grown in Pericyte media (PM) for 48 hours until confluence. Cells were then treated with various doses of MG-132 (10 μ M, 1 μ M, 0.1 μ M) or vehicle in media for 24 hours at 37°C. The media was aspirated, and cells were washed twice with ice cold PBS. Afterwards, 90 μ L of lysis buffer was added to each well, and cells were incubated for 30 minutes until lysis occurred. To evaluate 20S proteasome activity, 10 μ L of 1 mM Suc-LLVY-AMC, which fluoresces in response to 20S proteasome activity, was added to each well. For the isolated cerebrovasculature, lysates were added directly to the 96 well plate, and Suc-LLVY-AMC was added to each well. All samples were analyzed for fluorescence (λ_{ex} = 380 nm, λ_{em} = 460 nm) every 5 minutes for 1 hour.

2.2.12 Autophagy activity assay

HBVP were seeded in a black clear bottom 96 well plate as described above and allowed to grow until confluency. Culture media was removed, and cells were treated for 24 hours at 37°C with the autophagy inducer Rapamycin (500 nM) and the lysosomal pH neutralizer Chloroquine (100 µM) (vehicle) in the presence or absence of autophagy inhibitors 1 mM 3-MA or 10 µM Spautin-1. Media was removed and cells were incubated for 30 minutes at 37°C with a 1:500 Cyto-ID green stock solution and 1:1000 DAPI mix (Enzo Life Sciences) in a mixture of 1x assay buffer containing 5% FBS. Cells were washed twice with assay buffer then analyzed for DAPI ($\lambda_{\text{ex}} = 480 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$) and Cyto-ID ($\lambda_{\text{ex}} = 340 \text{ nm}$, $\lambda_{\text{em}} = 480 \text{ nm}$). The Cyto-ID fluorescence intensity measuring autophagosome formation was normalized to the total cell count as assessed using DAPI.

2.2.13 Cerebrovascular tau processing

Tau degradation was evaluated in cerebrovessels isolated from r-mTBI wild-type mice 6 months post injury. Freshly isolated cerebrovessels were treated with 50 ng/ml of rhTau-441, or biotin-labeled tau in the presence or absence of the proteasome inhibitor MG-132 (10 µM) for 24 hours at 37°C. Following the treatment period, the extracellular media was removed and the cerebrovessels were washed with ice-cold PBS. Cell lysates were collected using lysis buffer and Western blotting analysis was used for all unlabeled tau analysis. Cell lysates were denatured at 95°C by boiling in Laemmli buffer (Bio-Rad). Samples were subsequently resolved on 4% to 12% gradient NuPage Novex Bis-Tris precast polyacrylamide gel. After electro transferring, nitrocellulose membranes were blocked in 5% milk and then probed with primary antibodies (1:1000) overnight. After washing, HRP-conjugated secondaries were applied and signal intensity ratios were quantified by chemiluminescence imaging with the ChemiDoc TM XRS as previously described (Ojo et al., 2016). Monomeric tau was analyzed using Da9, before being normalized to GAPDH (1:1000). Autophagic flux was measured using an antibody for LC3 II/I and normalized to total protein using GAPDH. Biotin-labeled tau accumulation was analyzed for total tau content by modified biotin ELISA. The ELISA was performed according to the manufacturer's protocol, using stock biotin-labeled tau as the standard and excluding the 1-hour incubation step with a biotin-conjugated primary antibody. The samples were normalized to total protein content using the BCA assay.

2.2.14 Pericyte response to proteasome and autophagy inhibitor treatment

HBVP were seeded in at 24 well plate pretreated with poly-L-lysine and grown to confluence. Cells were then treated with either MG-132 (10 µM), Spautin-1 (10 µM), or a vehicle in media for 24 hours at 37°C. The media was aspirated, and cells were washed twice with ice cold PBS. Cells were lysed with lysis buffer, analyzed for tau using the hTau ELISA, and normalized to total protein using the BCA protein assay.

2.2.14 Statistical Analysis

Quantitative data were plotted as mean \pm standard error of the mean. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc). The Brown-Forsythe and Bartlett's tests were performed to ensure homogeneity of variance and the Shapiro-Wilk test was completed to assess normality. Statistical significance was evaluated using a two-way ANOVA with Bonferroni's post hoc test with multiple comparisons as indicated in the figure legends. For data that did not reflect a normal/Gaussian distribution, the Kruskal-Wallis test was utilized followed by Dunn's multiple comparisons test. Correlation analyses were evaluated using Pearson's correlation coefficient. For all analyses, a p value ≤ 0.05 was considered statistically significant.

2.3. RESULTS

2.3.1 Mural cell uptake of tau ex vivo

Given that mural cells are involved in extracellular solute elimination at the BBB, and our preliminary evidence demonstrated a strong interaction between mural cells and tau *in vitro*, I studied whether these interactions were influenced by r-mTBI. To investigate this, isolated cerebrovessels from r-sham and r-mTBI mice at various timepoints after injury were exposed to tau *ex vivo*, and tau uptake was quantified using ELISA. At 24 hours and 3 months post last injury there was no significant difference in tau uptake of the cerebrovasculature between r-mTBI and r-sham cerebrovessels (Figure 2.2A). There was a significant decrease in the tau uptake of mice exposed to r-mTBI at the more chronic time points of 6 months (25%) and 12 months (30%) post last injury (Figure 2.2A). To investigate whether a similar phenomenon occurred in other mouse models of neurodegeneration, tau uptake was also probed in the isolated cerebrovessels of PSAPP and PDGFR β (+/-) mice and compared with their respective wild-type litter mates. In PSAPP mice, cerebrovascular tau uptake was diminished by 40% compared to wild-type mice, and PDGFR β (+/-) mice demonstrated a 30% decrease in tau uptake compared to wild-type littermates (Figure 2.2B). To investigate whether this diminished uptake was limited to tau, the uptake of two other solutes, fluorescein-labeled A β (1-42) and lucifer yellow dextran (10 kDa), was examined in 12-month post injury r-mTBI and r-sham vessels. The r-mTBI cerebrovessels showed a 20% decrease in A β (1-42) uptake, while there was no difference between r-mTBI and r-sham in the uptake of dextran (Figure 2.2C).

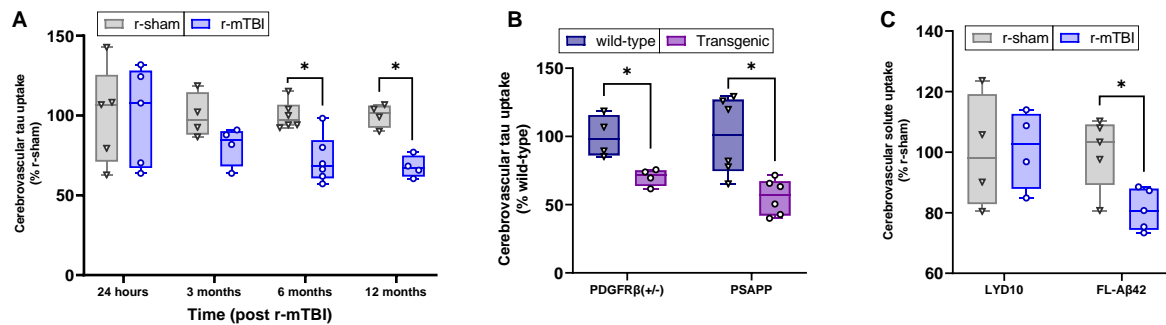


Figure 2.2 Tau uptake in freshly isolated cerebrovasculature from (A) r-mTBI mice (24 h, 3 months, 6 months, and 12 months post injury), and (B) PDGFR β (+/-) mice, PSAPP mice, and respective wild-type littermates. Cerebrovessels were exposed to 5 ng/ml recombinant human tau (rhtau-441) for 1 h at 37 °C. Lysates were analyzed for tau content by ELISA and normalized to total protein using the BCA assay. The sample sizes for the r-sham and r-mTBI groups were the same for each timepoint: 24 h (n = 5), 3 months (n = 4), 6 months (n = 6), and 12 months (n = 4). Sample sizes were the same for PDGFR β (+/-) and wild-type littermates (n = 4 each) and PSAPP and wild-type littermates (n = 6 each). (C) Solute uptake was measured in freshly isolated cerebrovasculature from r-mTBI mice at 12 months post injury. Cerebrovessels were exposed to lucifer yellow dextran (10 μ M) or fluorescein-labeled A β 1-42 (2 μ M) for 1 h at 37 °C. Lysates were analyzed for fluorescence and normalized to total protein using the BCA assay. The sample sizes for r-sham and r-mTBI groups were the same for each probe: lucifer yellow dextran (n = 4), fluorescein labeled A β 1-42 (n = 5). Values represent mean \pm SEM and are expressed as a percentage of each respective r-sham group or wild-type littermate. *P < 0.05 compared to each respective r-sham as determined by two-way ANOVA and Bonferroni's multiple comparisons test.

2.3.2 Mural cell marker expression after chronic repetitive mild TBI, 24 hours, 3 months, 6 months, and 12 months post injury compared with 18-month-old PSAPP mice and PDGFR β (+/-) mice.

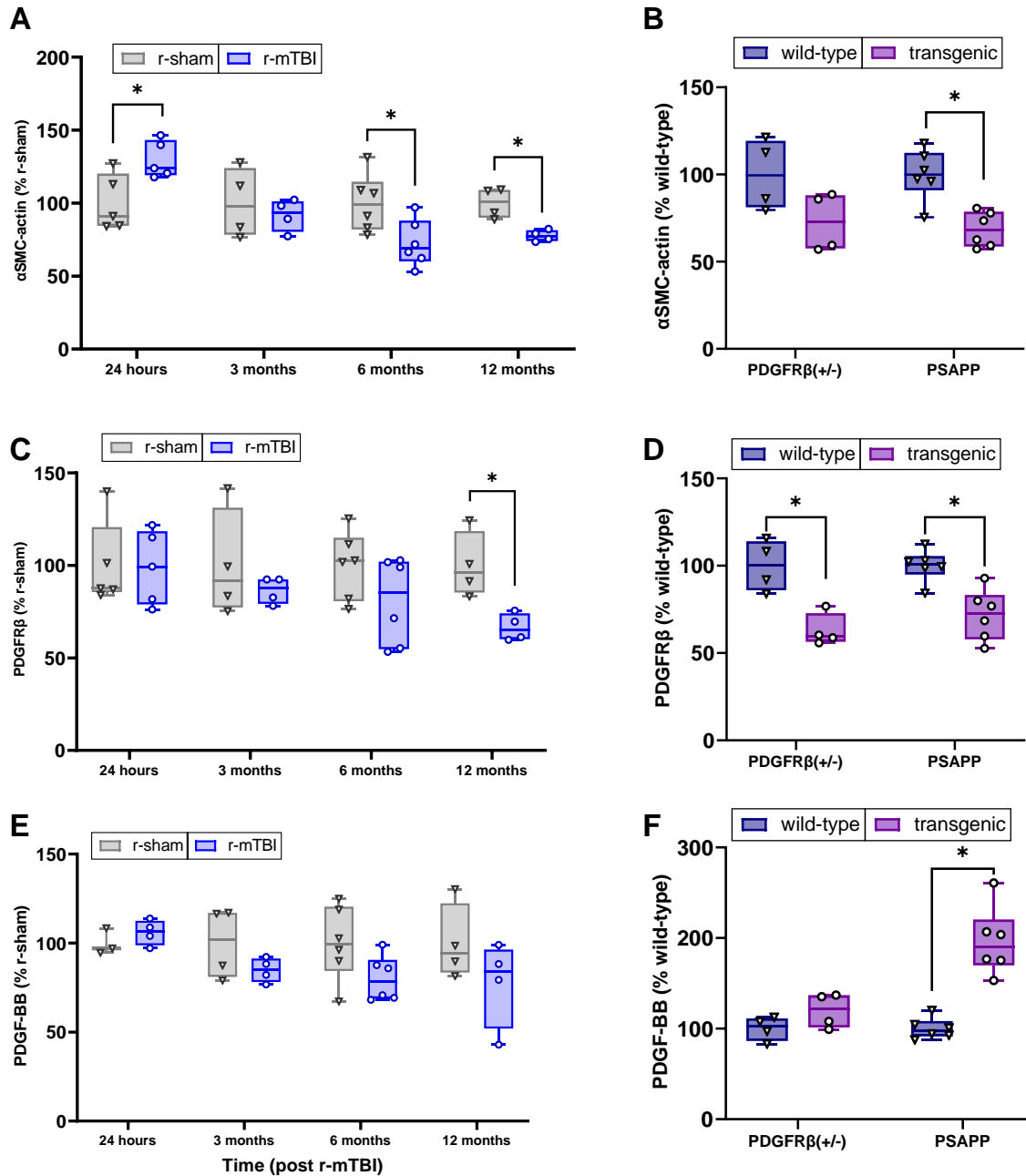


Figure 2.3 Expression of α SMA, PDGFR β and PDGFBB in r-mTBI and transgenic mice. Mural cell marker expression was evaluated in r-mTBI mice (24 h, 3 months, 6 months, and 12 months post-last injury), and PDGFR β (+/-) mice, PSAPP mice, and respective wild-type littermates. Freshly isolated cerebrovessels were probed for α SMA expression in (A) r-mTBI mice and (B) transgenic mice by ELISA and normalized to total protein using the BCA assay. Freshly isolated cerebrovessels were probed for PDGFR β expression in (C) r-mTBI mice and (D) transgenic mice by ELISA and normalized to total protein using the BCA assay. The sample sizes for the r-sham and r-mTBI groups were the same for each timepoint: 24 h (n = 5), 3 months (n = 4), 6 months (n = 6), and 12 months (n = 4). PDGFR β (+/-) and wildtype littermates (n = 4 each) and PSAPP and wild-type littermates (n = 6 each). Brain homogenate was probed for PDGFBB in (E) r-mTBI mice and (F) transgenic mice using ELISA and was normalized to total protein using the BCA assay. The sample sizes for the r-sham and r-mTBI groups were the same for each timepoint unless otherwise noted: 24 h (n = 3 r-mTBI, 4 r-sham), 3 months (n = 4), 6 months (n = 6), and 12 months (n = 4). PDGFR β (+/-) and wildtype littermates (n = 4 each) and PSAPP and wild-type littermates (n = 6 each). Values represent mean \pm SEM and are expressed as a percentage of each respective r-sham group or wild-type littermate. *P < 0.05 compared to each respective r-sham or wild-type littermate as determined by two-way ANOVA and Bonferroni's multiple comparisons test.

To examine whether head trauma resulted in altered mural cell expression in a mouse model of r-mTBI, the levels of the smooth muscle cell marker α SMA were evaluated in the isolated cerebrovessels from the *ex vivo* tau uptake study. In the r-mTBI mice, mural cell markers progressively diminished over time following injury. At both 6- and 12-months post injury, α SMA was significantly reduced (approximately 25%) compared to their respective r-sham (Figure 2.3A). To examine whether r-mTBI resulted in altered pericyte status, levels of the pericyte marker PDGFR β and its ligand PDGF-BB were investigated in the isolated cerebrovessels, and in the cortex homogenate from r-mTBI mice, respectively. At 12 months post injury, PDGFR β demonstrated a 30% decrease in r-mTBI mice compared to r-sham (Figure 2.3C). At 24 hours post injury, there was no difference observed in PDGF-BB levels between r-mTBI and r-sham. Though the results did not reach significance, there was a 15% decrease in PDGF-BB levels in r-mTBI at 3 months, a 20% decrease at 6 months and a 23% decrease at 12 months post injury in r-mTBI homogenate relative to r-sham (Figure 2.3E). Both α SMA (Figure 2.3B) and PDGFR β (Figure 2.3D) were significantly reduced (about 30% reduction) in the PSAPP mice compared to wild-type mice. The PDGFR β (+/-)mice demonstrated a 40% reduction in PDGFR β (Figure 2.3D) compared to wild-type littermates, and also showed a 30% reduction in α SMA (Figure 2.3B), though this did not reach statistical significance. There was no significant difference in PDGF-BB levels in homogenates from PDGFR β (+/-) mice compared to WT littermates. Interestingly, in PS1/APP homogenate, PDGF-BB levels were twice as high as their WT littermates (Figure 2.3F). There was a strong correlation between mural cell marker expression and tau uptake after r-mTBI (PDGFR β , $p = 0.0335$; α SMA, $p = 0.0195$) (Figure 2.4A, B). Of note, there was no significant change observed in mural cell markers between r-sham mouse groups with respect to age.

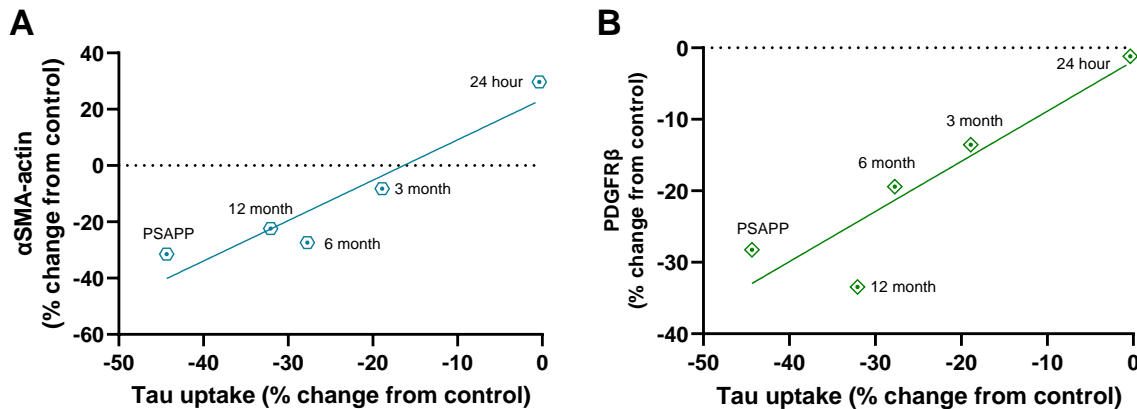


Figure 2.4 Correlation between mural cell expression and tau uptake in cerebrovessels isolated from r-mTBI (24 h, 3 months, 6 months, and 12 months post injury) and PSAPP mice. Cerebrovascular expression of (A) α SMC-actin, and (B) PDGFR β was plotted versus tau uptake (α SMC-actin, $r = 0.93$; PDGFR β , $r = 0.91$). Values represent the mean % change from each respective control (r-sham or wild-type littermates). α SMC-actin ($P = 0.0152$) and PDGFR β ($P = 0.0328$) as determined by a two-tailed Pearson correlation.

2.3.3 Flow cytometry and immunophenotypic analysis of cerebrovascular mural cells and endothelia

To determine whether r-mTBI resulted in overt mural cell loss or cellular dysfunction, the cerebrovasculature was isolated from mouse brains at 6 months post injury, when mural cell marker changes were first observed, and stained with antibodies against CD13 (mural cells) and CD31 (endothelial cells) before being evaluated using flow cytometry. The number of CD31+ endothelial cells and CD13+

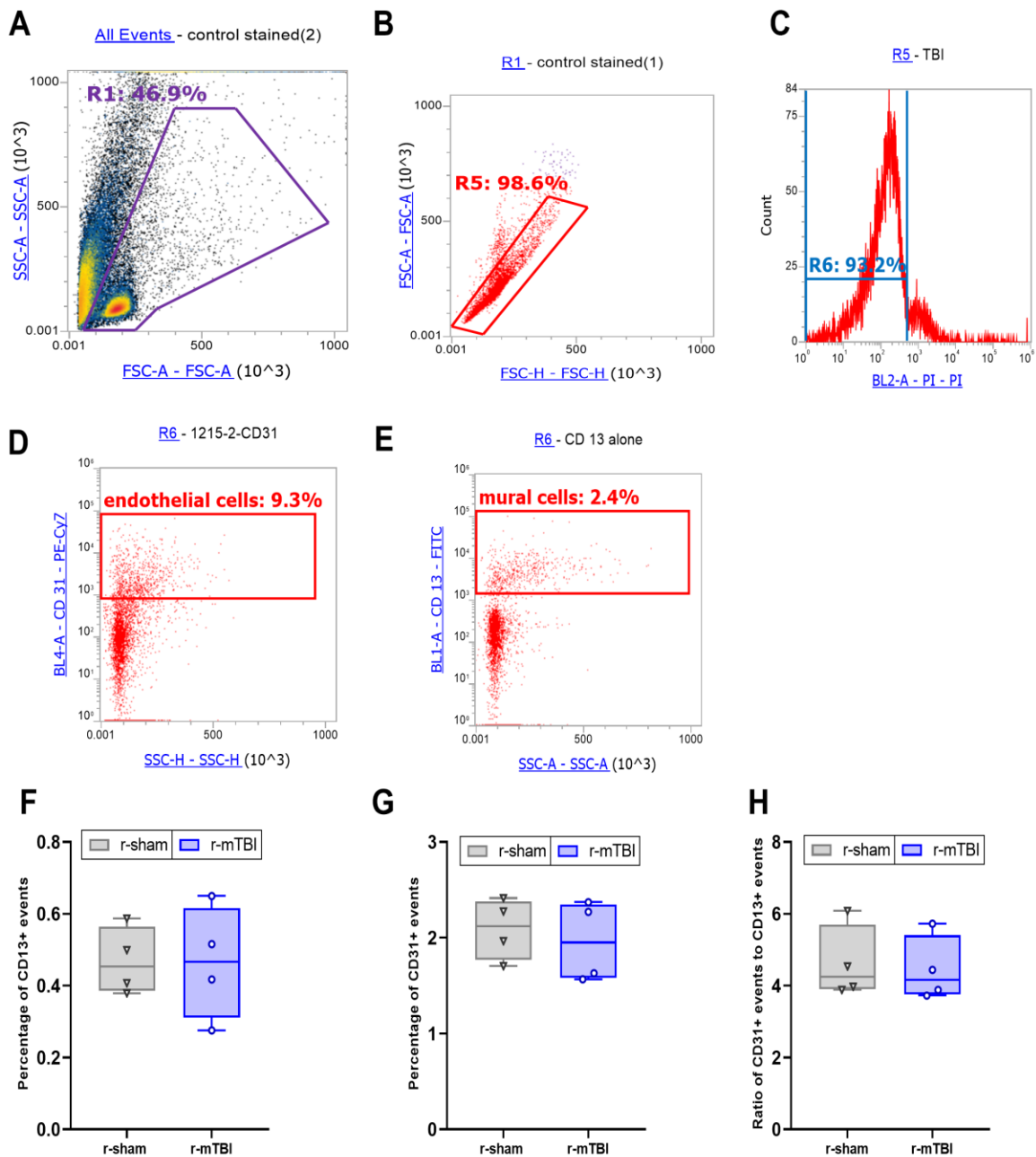


Figure 2.5 Flow cytometry analysis of brain endothelia and mural cells in freshly isolated cerebrovasculature from r-sham and r-mTBI mice at 6 months post injury. The scatter plot diagrams represent the gating strategy used during the identification and analysis of CD31 + ve endothelial cells and CD13 + ve mural cells. Cellular debris was excluded through gating based on (A) particle size (forward scatter area FSC-A) and particle complexity (side scatter area SSC-A). Gating based on (B) FSC-H (H- height) and FSC-A (A- area scaling) selected for single cell populations, while live cells were identified through (C) negative propidium iodide staining. The resulting sample populations were gated based on (D) positive expression of PE-Cy7 indicating CD31 + ve endothelial cells, and (E) positive expression of fluorescein isothiocyanate (FITC) indicating CD13 + ve mural cells. The percentages refer to the proportion of endothelia or mural cells in relation to the previous parent gate. Figs. F and G represent the percentage of CD31 + ve endothelial cells and CD13 + ve mural cells, respectively, in relation to the total number of gated events in r-sham and r-mTBI cerebrovessels. Figure H represents the ratio of CD31+ events to CD13+ events in r-sham and r-mTBI cerebrovessels. Values represent mean \pm SEM (n = 4). *P < 0.05 compared to r-sham as determined by a one-way ANOVA followed by post-hoc test.

mural cells was quantified and expressed as a percentage of the amount of gated cerebrovascular events (Figures 2.5A-E). There was no significant difference between the normalized percentage of CD13+ cells ($p = 0.9516$) (Figure 2.5F) or CD31+ cells ($p = 0.4147$) (Figure 2.5G). Additionally, the ratio of CD13:CD31 did not differ significantly between r-mTBI and r-sham cerebrovessels (Figure 2.5H).

2.3.4 Mural cell expression in cerebrovasculature isolated from human NDC, TBI, AD, and AD-TBI brain specimens.

To examine mural cell marker expression in humans, cerebrovessels were isolated from NDC, AD, TBI and AD-TBI human brain tissue (Table 2.1). α SMA expression in individuals with AD was diminished by 60% compared to NDC (Figure 2.6A). There was also a significant decrease in α SMA in the AD+TBI group compared to controls (50% reduction compared to NDC). For the TBI group, there was a trend towards a

Group	Sample size	Age \pm SEM (years)	Sex (M/F)	Years post injury \pm SEM	ApoE 2/3/4	Mean Braak Stage	Tangle Total \pm SEM
Control	15	79.4 \pm 1.9	8/7	n/a	9/4/2	1.5 (I-II)	1.8 \pm 0.6
TBI	12	85.2 \pm 1.9	6/6	38.1 \pm 9.2	3/8/1	3.5 (III-IV)	5.2 \pm 0.8
AD	15	82.5 \pm 1.7	7/8	n/a	3/5/7	5.1 (V-VI)	12.9 \pm 1.5
AD-TBI	14	79.6 \pm 2.3	7/7	41.5 \pm 7.9	0/4/10	5.3 (V-VI)	12.8 \pm 0.6

Table 2.1 Characteristics of human brain specimens. ApoE 2/3/4 refers to the presence of at least one copy of the allele.

reduction in α SMA expression, as their expression was diminished by 25% relative to NDC, however this effect did not reach statistical significance (Figure 2.6A). PDGFR β expression was reduced by 50% in AD cerebrovessels compared to NDC specimens (Figure 2.6B). Likewise, PDGFR β expression in AD-TBI was diminished by 40% relative to NDC (Figure 2.6B). There was no significant difference in PDGFR β expression in the TBI group compared to control samples, though a reduction was apparent (15% decrease compared to NDC). In human cortex homogenate, PDGF-BB levels were diminished approximately 15% in both the TBI and the AD-TBI, relative to NDC, whereas there was no difference observed in the AD compared to NDC (Figure 2.6C).

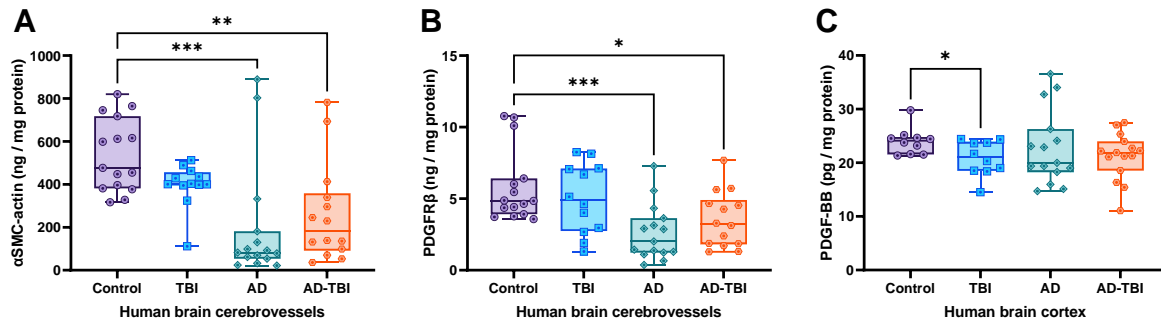


Figure 2.6 Mural cell marker expression in cerebrovasculature isolated from human brain cortex derived from, 1) non-demented control subjects (no history of TBI or AD diagnosis), 2) TBI, 3) AD, and 4) TBI and AD. Lysates were analyzed for (A) α SMC-actin, and (B) PDGFR β by ELISA and normalized to total protein using the BCA assay. Brain homogenates were analyzed for (C) PDGFBB and normalized to total protein using the BCA assay. Values represent mean \pm SEM and are expressed as ng per mg of total protein. Control (n = 15), TBI (n = 12), AD (n = 15), TBI-AD (n = 14). * $P < 0.05$ compared to control as determined by the one-way ANOVA followed by post-hoc test correction for false discovery rate using the Bonferroni correction.

2.3.5 Tau degradation in brain mural cells

To investigate the degradation processes by which mural cells metabolize extracellular tau, primary HBVP were exposed to inhibitors of proteasome activity (MG-132) and autophagy (3-MA and Spautin-1) *in vitro*. MG-132 significantly diminished pericyte proteasome activity (Figure 2.7A) and autophagy inhibitors significantly reduced pericyte autophagy (Figure 2.7B), suggesting that pericytes have the capacity for enzymatic degradation, and this machinery can be pharmacologically modulated. Next, to examine whether r-mTBI could impact mural cell degradation processes, isolated vessels from r-mTBI and r-sham WT mice were exposed to physiological levels of tau for 24 hours *ex vivo* and the lysates were analyzed for proteasome and autophagic activity. Cerebrovessels from r-mTBI mice, 6 months post injury,

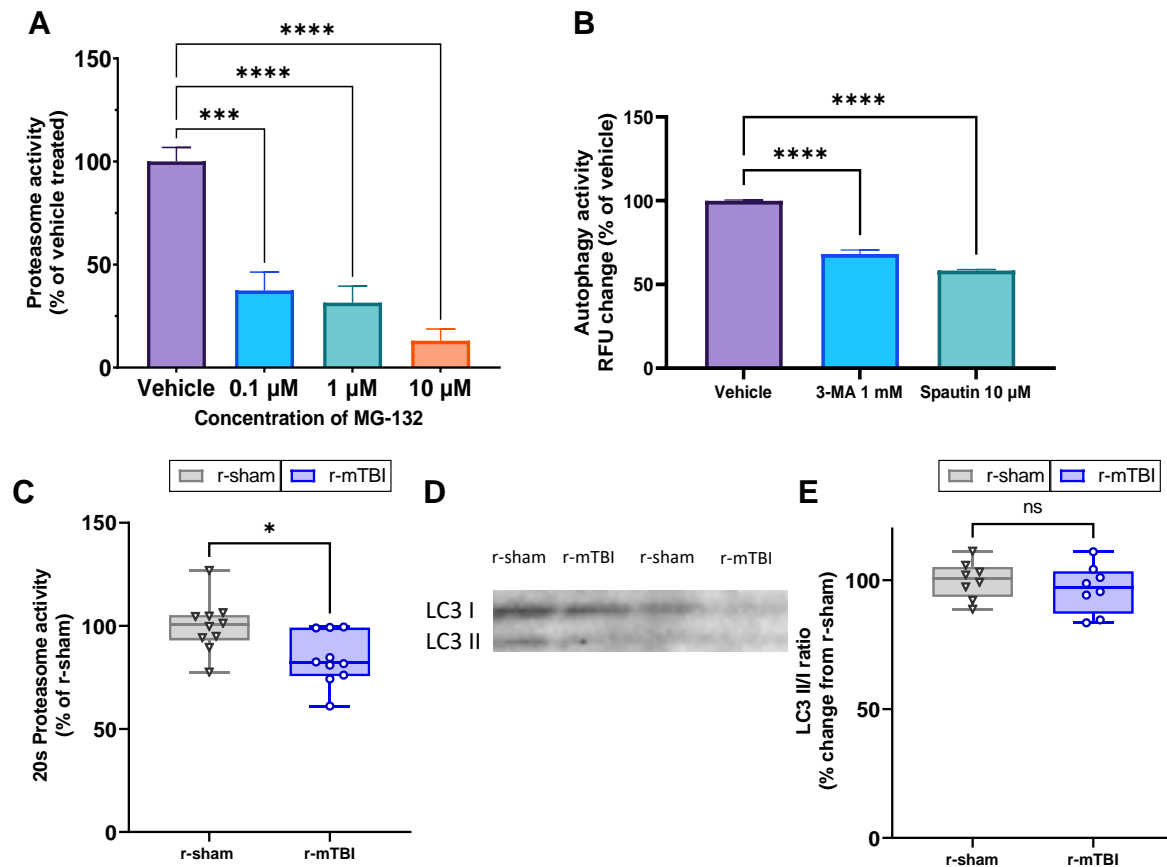


Figure 2.7 Cerebrovascular enzymatic degradation. (A) Pericytes were exposed to various concentrations of MG-132 or vehicle *in vitro* for 24 hours min at 37°C, then lysates were analyzed for proteasome activity using Suc-LLVY-AMC and normalized to total protein using the BCA assay. Values represent mean \pm SEM and are expressed as a percentage of the vehicle treated group (n = 4). (B) Pericytes were exposed to Rapamycin (500 nM) and Choloroquine (100 μ M) (vehicle treated) in the presence of absence of 3-MA (1 mM) or Spautin-1 (10 μ M) for 24 hours at 37°C. Cells were incubated with Cyto-ID and DAPI for 30 minutes at 37°C, then analyzed for autophagosome formation and normalized to total cell count. Values represent mean \pm SEM and are expressed as a percentage of the vehicle treated group (n = 2-3). (C) Lysates from isolated cerebrovasculature from r-mTBI and r-sham mice (6 months post injury) were analyzed for proteasome activity using Suc-LLVY-AMC and normalized to total protein using the BCA assay. Values represent mean \pm SD and are expressed as a percentage of r-sham (n = 10). (D, E) Lysates from isolated cerebrovasculature from r-mTBI and r-sham mice (6 months post injury) were analyzed by western blot for levels of LC3II/I. (D) Western blot showing bands of LC3 II/I in r-mTBI and r-sham cerebrovessels. (E) Quantification of western blot analysis. Autophagic activity values represent mean \pm SD and are expressed as a percentage of r-sham (n = 8). *P < 0.05 compared to each respective r-sham as determined by two-way ANOVA and Bonferroni's multiple comparisons test.

demonstrated a 15% decrease in 20S proteasome activity compared to r-sham (Figure 2.7C). There was no significant difference between r-mTBI and r-sham vessels with regards to the autophagic indicator LC3 II (Figure 2.7D, E).

2.3.6 Cerebrovascular proteasome elimination of extracellular tau

Cerebrovessels from r-mTBI and r-sham mice 6 months post injury were exposed to monomeric tau in the presence or absence of the proteasome inhibitor MG-132 for 24 hours, to investigate whether the cerebrovascular proteasome was involved in the degradation of extracellular tau, and whether r-mTBI

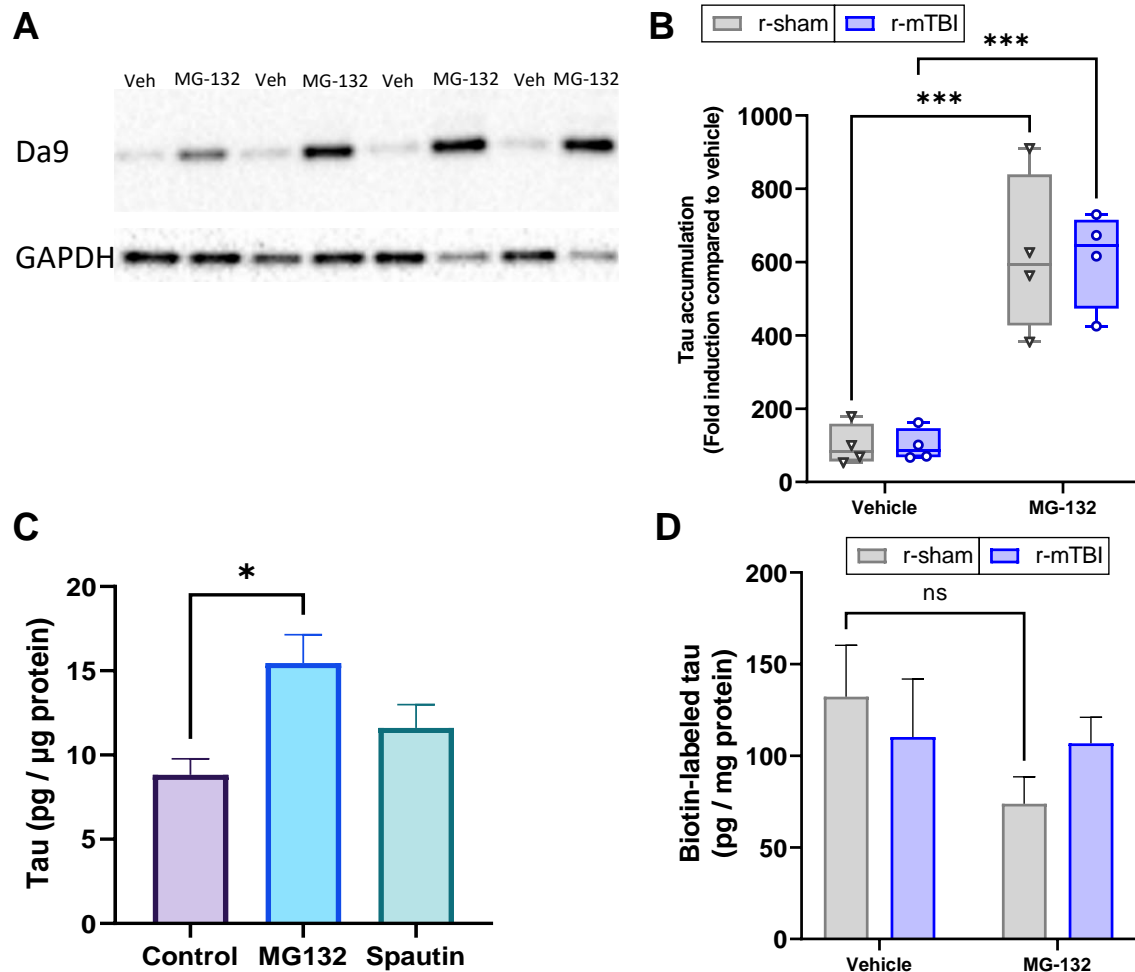


Figure 2.8 Influence of degradation inhibitors on cerebrovascular tau accumulation. (A, B) Isolated cerebrovessels from r-mTBI and r-sham mice (6 months post injury) were exposed to tau (50 ng/ml) in the presence or absence of MG-132 (10 μ M) for 24 hours at 37°C. (A) Western blot showing bands of cerebrovascular Da9 and GAPDH in the presence or absence of MG-132. (B) Quantification of western blot analysis. Values represent the mean \pm SD and are expressed as the tau fold induction normalized to vehicle treated cerebrovessels (n = 4). (C) Pericytes were exposed in vitro to either MG-132 (10 μ M), Spautin-1 (10 μ M), or a vehicle in media for 24 hours at 37°C, the lysates were analyzed for tau using ELISA and normalized to total protein using the BCA assay. Values represent the mean \pm SEM (n = 4). (D) Isolated cerebrovessels from r-mTBI and r-sham mice (6 months post injury) were exposed to biotin-labeled tau (50 ng/ml) in the presence or absence of MG-132 (10 μ M) for 24 hours at 37°C. Lysates were analyzed for tau accumulation using a modified biotin-labeled ELISA and normalized to total protein using the BCA assay. Values represent the mean \pm SEM (n = 3- 4). *P < 0.05 compared to each respective r-sham as determined by one-way or two-way ANOVA and Bonferroni's multiple comparisons test.

could influence tau processing. The presence of the proteasome inhibitor MG-132 and tau resulted in a ~6.5-fold increase in tau accumulation in the cerebrovessels compared to tau alone, when evaluated using western blot for total tau, but there was no significant difference in tau accumulation between r-mTBI and r-sham after 24 hours (Figure 2.8A, B).

2.3.7 Cerebrovascular endogenous expression of tau required biotin labeled tau.

To investigate whether the tau accumulation seen in these samples could be influenced by changes in endogenous tau processing within the mural cells, MG-132 was applied to pericytes *in vitro* in the absence of tau, and lysates were analyzed for tau accumulation via ELISA. In the presence of MG-132, levels of background signal in human pericytes increased by 1.6-fold over 24 hours, compared to vehicle (Figure 2.8C). Application of the autophagy inhibitor Spautin-1 did not significantly alter the background signal to the same extent as MG-132 over the same period, though there was trend suggesting an elevated signal. This elevated background signal necessitated the use of labeled tau to identify the mechanisms underlying the cerebrovascular processing of extracellular tau. For this purpose, I developed a biotin-labeled tau ELISA, and used biotin-labeled tau for subsequent studies, which did not detect any tau unless biotin-labeled tau was present. Biotin-labeled tau was applied to isolated cerebrovessels from 6-month post injury r-mTBI and r-sham mice for 24 hours in the presence or absence of MG-132. Contrary to the previous study using unlabeled tau, in this study there was no significant difference in the accumulation of biotin-labeled tau over 24 hours between r-mTBI and r-sham, nor was there any effect of MG-132. (Figure 2.8D). Altogether, these results indicated that while proteasome inhibition may alter endogenous tau dynamics within the cerebrovascular cells, the 20S proteasome was not involved in processing extracellular tau, which was the primary focus of our investigation.

2.4 DISCUSSION

Brain vascular mural cells are credited with a variety of roles in the CNS, including the maintenance of the BBB and the regulation of blood flow to neurons. Furthermore, they act as first responders to inflammation and can become scavengers if needed, removing neuronal waste from the ISF. The loss or dysfunction of mural cells has been associated with a variety of age-related neurodegenerative disorders, which can disrupt vascular resilience within the brain, resulting in the progression of cognitive decline (Eltanahy et al., 2021). To understand the influence of r-mTBI on mural cell status in comparison to other neurodegenerative diseases, I examined mural cell perturbations in mouse models of TBI or AD and human post-mortem tissue. Previously our group reported neurovascular disruption in the chronic stages of r-mTBI (Lynch et al., 2016), and preliminary evidence from our lab suggested that mural cells interact with tau, though the nature of that interaction was unclear. In this chapter, I looked at the influence of r-mTBI on mural cell status, and the potential impact on mural cell tau processing. The findings in this chapter suggest that r-mTBI leads to chronic mural cell dysfunction and diminished cerebrovascular tau uptake, potentially resulting in reduced perivascular tau elimination from the brain.

As described in Chapter 1, mural cells contribute to the elimination of solutes in the perivascular space, including A β (Ma et al., 2018). There has been very little investigation in the literature regarding an interaction between mural cells and tau. However, our lab found preliminary *in vitro* evidence that mural cells interact with tau to a greater extent than other cerebrovascular cells (Ojo et al., 2021), suggesting

they may play a similar role in tau elimination as has been reported for A β (Sagare et al., 2013). I exposed physiological levels of tau to isolated cerebrovasculature from r-mTBI and r-sham mice *ex vivo* for 1 hour and found progressively reduced tau uptake compared to r-sham mice at chronic time points (Figure 2.2). By 12 months post injury, the levels of tau uptake were diminished to a similar degree as age matched PSAPP mice. Furthermore, this diminished interaction with tau was observed in the isolated cerebrovessels of PDGFR β (+/-) relative to their WT littermates, suggesting a potential link between mural cell dysfunction and cerebrovascular tau uptake. This is consistent with previous reports of tau pathology and accumulation in PDGFR β (+/-) mice crossed with APP(sw/0), which are not observed in App(sw/0) alone (Sagare et al., 2013). Mural cells are known to interact with A β , likely through LRP1, and that interaction can be influenced by mural cell dysfunction or loss (Ma et al., 2018; Sagare et al., 2013). As cerebrovascular interactions with tau were diminished in neurodegenerative conditions, I investigated whether this diminished uptake of tau could be observed in the known mural cell ligand, A β (Ma et al., 2018). I found that A β uptake, but not uptake of the inert tracer dextran, was diminished in r-mTBI mice at 6 months post last injury. This suggested that the disrupted interactions with tau in the chronic period after r-mTBI may reflect disrupted mural cell status.

Mural cell marker degeneration or loss is commonly observed in AD (Nation et al., 2019) and has been observed in acute mouse models of mTBI (Bhowmick et al., 2019; Dore-Duffy et al., 2000). To understand whether r-mTBI can lead to alterations in mural cell markers at chronic time points, I probed the isolated cerebrovasculature from r-mTBI mice at 24 hours, 3-, 6-, and 12- months post injury using ELISA (Figure 2.3). In addition to a smooth muscle cell marker, I investigated changes to pericyte status, including the receptor and ligand changes to characterize the PDGF pathway. At 24 hours post injury, I found an upregulation in α SMA, and no significant change in PDGFR β , relative to age matched r-sham. This acute elevation in α SMA has been demonstrated previously in the literature and may represent a phenotypic change in mural cell status, potentially due to an increase in early endothelin-1 release from the vascular endothelium in response to injury (Dore-Duffy et al., 2011). This change in α SMA may reflect an acute shift from a synthetic to contractile mural cell phenotype, which could enable the vessels to modulate blood flow changes due to perfusion pressure fluctuations after TBI (Alford et al., 2011). In the initial 24 hours after a single TBI, a study found that over 40% of pericytes migrate into the parenchyma, which may leave their vacated vessels less resilient to an additional injury (Dore-Duffy et al., 2000). An acute study of the PDGFR β response after mTBI revealed that this receptor may be upregulated in pericytes in the first week after injury (Kyyriäinen et al., 2017), perhaps to promote pericyte migration or survival. This could provide infrastructural support to injured blood vessels in response to injury. However, at chronic time points post-injury, I observed diminished α SMA and PDGFR β relative to age matched r-sham mice, suggesting a progressive decline in mural cell homeostatic cell signaling and receptor expression.

It is important to note that the 24-hour time point post injury in the current study is in fact 3 months after the initial injury and may not be directly comparable to other studies investigating the effects of an impact 24 hours after a single injury or multiple injuries occurring over a much shorter period. There has been very little investigation into the chronic impact of r-mTBI on mural cell health, but mural cell differentiation markers can be influenced by a variety of molecular signaling ligands which may be present after TBI, potentially resulting in chronic alterations (Jullienne et al., 2016). I report that in the initial period after r-mTBI, there is not a substantial change in pericyte marker expression, but there is a progressive decline at chronic stages which may indicate a dysfunctional neurovascular environment that does not resolve, even at 1 year post injury. This mural cell degeneration may be associated with diminished

interaction with extracellular tau as there was a significant correlation between mural cell marker expression and cerebrovascular tau uptake, both in the context of r-mTBI and in the transgenic neurodegenerative mouse lines examined. Interestingly, the decline in mural cell marker expression after r-mTBI reflected a similarly diminished expression of those markers in the isolated cerebrovasculature of aged APP/PS1 mice, which may explain the apparent association between head trauma and the development of AD. The diminished expression of PDGFR β in APP/PS1 mice observed in this chapter is consistent with previous reports and is believed to precede vascular regression and other vascular abnormalities (Janota et al., 2015).

To further interrogate the vascular dysfunction associated with the observed decline in mural cell PDGFR β levels, I examined whether there was a corresponding change in levels of the ligand for this receptor, PDGF-BB. To maintain the BBB, pericyte expressed PDGFR β binds to its ligand PDGF-BB, which is released by endothelial cells into the surrounding extracellular matrix of the basement membrane. This binding stimulates downstream signaling cascades in the pericyte including proliferation, recruitment, survival, and migration (Sweeney et al., 2016). Pericytes require constant PDGF-BB stimulation from endothelial cells for their health and survival (Winkler et al., 2014), and it is critical to sustain normal pericyte numbers and phenotype over a chronic period (Vazquez-Liebanas et al., 2021). Perturbations in PDGF-BB/PDGFR β signaling are believed to result in vascular abnormalities (Vazquez-Liebanas et al., 2021), and while the mural cell receptor has been investigated to a limited degree in the context of AD (Sagare et al., 2013), there has been very little exploration into PDGF-BB fluctuations. At 6- and 12- months post injury, levels of PDGF-BB were diminished. This decrease in PDGF-BB has been observed previously after CCI in mice and persisted at 3 weeks post injury (Shang et al., 2014). Interestingly, the PS1/APP mice demonstrated a significant elevation in PDGF-BB levels compared to their WT littermates. This finding demonstrates the importance of uncovering the underlying mechanisms behind neurodegenerative disorders that may present with convergent pathologies, as it is likely that delivering PDGF-BB would be of limited efficacy in this disease model. The mechanism behind the elevated levels of PDGF-BB observed in PS1/APP mice is unclear, however A β is known to trigger neoangiogenesis and hypervascularity (Niwa et al., 2002), potentially leading to an increase in angiogenic endothelial sprouts. Those endothelial sprouts release elevated levels PDGF-BB, in order to recruit pericytes (Pan et al., 2021). In contrast to r-mTBI mice which have diminished PDGFR β expression as reported in Chapter 2, PS1/APP mice may experience a loss of mural cells as well (Janota et al., 2015). The uncovered endothelial sprouts in PS1/APP mice may continue to secrete PDGF-BB in the absence of mural cells, leading to a different, but still pathogenic, phenotype of cerebrovasculature than in r-mTBI. Collectively, these findings suggest mural cells play a role in the elimination of extracellular tau from the perivascular space in the brain, and mural cell disruption after r-mTBI may contribute to the accumulation of the perivascular tau pathology that has been observed following repetitive head trauma (McKee et al., 2013).

I next evaluated whether the diminished mural cell marker expression in r-mTBI was due to mural cell loss or cellular dysfunction. Previous reports have suggested that healthy aging in WT mice is associated with diminished PDGFR β levels and mural cell coverage, but not cellular loss, in contrast to APP/PS1 which experience both lower PDGFR β levels and mural cell loss (Janota et al., 2015). I used flow cytometry and stained for the canonical mural cell marker CD13, which is expressed by both pericytes and smooth muscle cells, as well as the endothelial marker CD31 (Figure 2.5). I evaluated the ratio of mural cells to endothelial cells, as well as the total number of mural cells in r-mTBI and r-sham isolated brain vasculature. At 6 months post injury, there was no significant change in the number of CD13 $^{+}$ cells or CD31 $^{+}$ cells between

r-mTBI and r-sham cerebrovessels, and no difference in the CD13+:CD31+ ratio, suggesting that mural cell marker differences were due to dysfunctional expression, instead of cellular loss. This was validated using confocal microscopy in r-sham and r-mTBI mice up to 9 months post-injury, which similarly saw no change in mural cell density using CD13+ (Lynch et al., 2021). Though mural cell loss may occur in first hours or days after injury (Bhowmick et al., 2019; Dore-Duffy et al., 2011), there appears to be a progressive decline in mural cell marker expression at more chronic time points post-injury, rather than sustained mural cell loss, at least in our brain injury model.

Current trends in the literature suggest that both AD and TBI have a degenerative cerebrovascular component. In this chapter, I report that individuals with AD had significantly lower expression of the pericyte and smooth muscle cell markers, PDGFR β and α SMA, than age matched controls, consistent with prior reporting in the literature (Winkler et al., 2014). When brain specimens from individuals that had suffered a TBI were compared with non-diseased control post-mortem tissue in this study, the TBI specimens showed a trend towards a decrease in the mural cell marker expression, though it did not reach significance (Figure 2.6). Though the individuals exposed to head trauma had higher tau tangle scores than NDCs, due to the tau tangle scoring system used, it was not possible to directly correlate tangle load to mural cell status. TBI specimens did demonstrate a diminished expression of the pericyte ligand, PDGF-BB relative to NDC. These individuals were exposed to a single injury of varied intensity, not to a chronic repetitive pattern of TBI which may occur in contact sports or the military. Additionally, the period between injury exposure and autopsy varied within our human population. It is possible that significant mural cell dysfunction could have been observed in the brain tissue of individuals with more frequent brain trauma, but this tissue was unavailable for comparison. Interestingly, while the AD and AD-TBI brains demonstrate mural cell marker changes, there did not appear to be a synergistic impact of brain trauma in the AD-TBI group, compared to AD alone. It is possible that the neurodegenerative conditions observed in AD have masked the contribution of exposure to earlier head trauma in such individuals. It is unclear from these results whether exposure to a single head injury early in life can accelerate or induce the pathogenic sequelae found in the cerebrovasculature in AD. Additionally, the neurodegenerative conditions found in AD differ from those found after a single head injury and likely influence differential cerebrovascular responses. It is possible that the altered endothelial response to TBI, represented by diminished PDGF-BB, which was not observed in the AD or AD-TBI group, may be masked under the phenotype found in AD. This is consistent with an altered response of PDGF-BB in transgenic AD mice, relative to r-mTBI, as discussed above. Finally, 70% of the individuals in the AD-TBI group were ApoE4 positive, compared to only 8% of the TBI group. As discussed in Chapter 1, ApoE4 may influence the progression of neurodegeneration in a variety of ways and may have contributed to the higher percentage of individuals with a TBI that eventually developed AD, compared to non ApoE4 carriers. The influence of ApoE isoform expression on extracellular tau elimination will be investigated in subsequent chapters.

Despite the observation that mural cells interact with extracellular tau, the purpose of this interaction remains unclear. There are several methods of solute elimination at the BBB, as discussed in Chapter 1, including enzymatic degradation. The ability of mural cells to enzymatically degrade internalized tau has not been examined in the literature, though they have previously demonstrated the ability to eliminate other extracellular solutes including A β (Ma et al., 2018). Moreover, if mural cells can eliminate extracellular tau at the BBB, their ability to do so may be impaired after r-mTBI, either through reduced interaction, or potentially due to dysfunctional elimination processes. First, I demonstrated that I could both detect and pharmacologically modulate the proteasomal and autophagic activity in pericytes *in vitro*.

Once I confirmed that mural cells are enzymatically active, I investigated whether r-mTBI could impact cerebrovascular degradation pathways (Figure 2.7). In isolated cerebrovessels from r-sham and r-mTBI mice 6 months post injury, I found that cerebrovessels from r-mTBI mice had diminished proteasome activity compared to r-sham. However, when I compared the LC3II changes to evaluate autophagy in isolated cerebrovessels, I did not see a significant difference in autophagic activity between r-mTBI and r-sham. While mural cell enzymatic processes appear to be influenced by TBI, it remains to be seen if these changes impact tau degradation.

These observations indicated that if mural cells have a role in tau degradation, tau elimination from the brain may be diminished following r-mTBI due to a confluence of reduced tau uptake and metabolism. To test this hypothesis, I investigated whether cerebrovascular interactions with extracellular tau resulted in the proteasomal degradation of tau, by exposing r-mTBI and r-sham cerebrovessels to monomeric tau for 24 hours in the presence of the proteasome inhibitor MG-132, as degradation process typically over a longer period of time (>12 hours) (David et al., 2002; Dolan & Johnson, 2010). The inhibition of proteasome activity led to significant increases in tau accumulation in both the r-mTBI and r-sham cerebrovessels, though there was no significant difference between them (Figure 2.8). However, these initial studies did suggest that mural cells use the proteasome to eliminate extracellular tau, which might indicate that the dysfunction of these processes could diminish their capability for tau elimination from the brain. Though there are no reports in the literature of endogenous tau expression in mural cells, it was important to ensure that the changes in tau accumulation were not influenced by the modulation of endogenous tau turnover in mural cells. I applied a proteasome inhibitor to human brain vascular pericytes *in vitro* in the absence of exogenous tau and found a significant accumulation in background signal over 24 hours, which may signify changes in endogenous tau, as measured by ELISA. When I applied an autophagy inhibitor, Spautin-1, under similar conditions it did not significantly influence the accumulation of tau. This change seen in the presence of MG-132 may have been driven by a non-specific signal due to our analytical approach, or potentially the modulation of mural cell derived tau.

To circumvent any background signals in our cerebrovascular preparations and more effectively study cerebrovascular tau degradation, I used biotin-labeled exogenous tau. To do this, I modified an Invitrogen hTau ELISA kit, which only detected the exogenously applied biotin-labeled tau, excluding potential confounding effects of endogenous tau accumulation. When I replicated the tau accumulation study in isolated cerebrovessels with or without MG-132 using biotin-labeled tau, the proteasome inhibitor did not significantly influence the cerebrovascular accumulation of exogenous biotin-labeled tau during the 24-hour period when differences in proteasome-mediated tau degradation would be expected to be observed. This indicated that the observed cerebrovascular tau accumulation over 24 hours in the presence of the proteasome inhibitor may have been due to an artefact of the inability to discriminate between extracellular tau, and tau that may have been expressed by cells within the cerebrovascular isolate. As the focus my investigations centered around the processing of specifically extracellular tau, subsequent studies examining the interactions and processing of extracellular tau used labeled forms of tau to avoid this potential confounding factor.

The observations in this chapter suggest that mural cells are capable of internalizing extracellular tau, and their ability to do so may be diminished after r-mTBI. After internalization, it is still unclear how the cerebrovasculature processes extracellular tau, but the results from this chapter suggest that enzymatic degradation does not play a significant role in cerebrovascular tau elimination. This reflects what has been observed previously in microglia, which have the capability to phagocytose monomeric and oligomeric

tau, and may degrade monomeric tau to some extent but appear to be inefficient at the degradation of oligomeric species of tau, particularly under pathogenic conditions (Majerova et al., 2014). In fact, there are conflicting reports regarding whether tau can be degraded via the proteasome (Y. Wang & Mandelkow, 2012), which may be cell type or cell line dependent. Differences in extracellular tau processing between neurons and glia are understudied and may shed light on whether glia contribute in a significant way to the tau clearance from the brain. Additionally, it is currently unclear how tau is internalized in non-neuronal cells, though recent evidence has demonstrated that neurons use LRP1 as a mechanism for tau uptake (Rauch et al., 2020), which may or may not be the method used by mural cells or other glia. Pericytes are capable of internalizing solutes through multiple mechanisms, including receptor mediated endocytosis or non-specific pinocytosis, as well as phagocytosis (Thomas, 1999). Solutes internalized by pericytes can be transported to lysosomes for degradation or transported to the blood (Z. Zhao et al., 2015). Though mural cells play a role in the clearance of monomeric and oligomeric A β at the capillary level (Ma et al., 2018), it is unclear whether that is through degradation or efflux to the

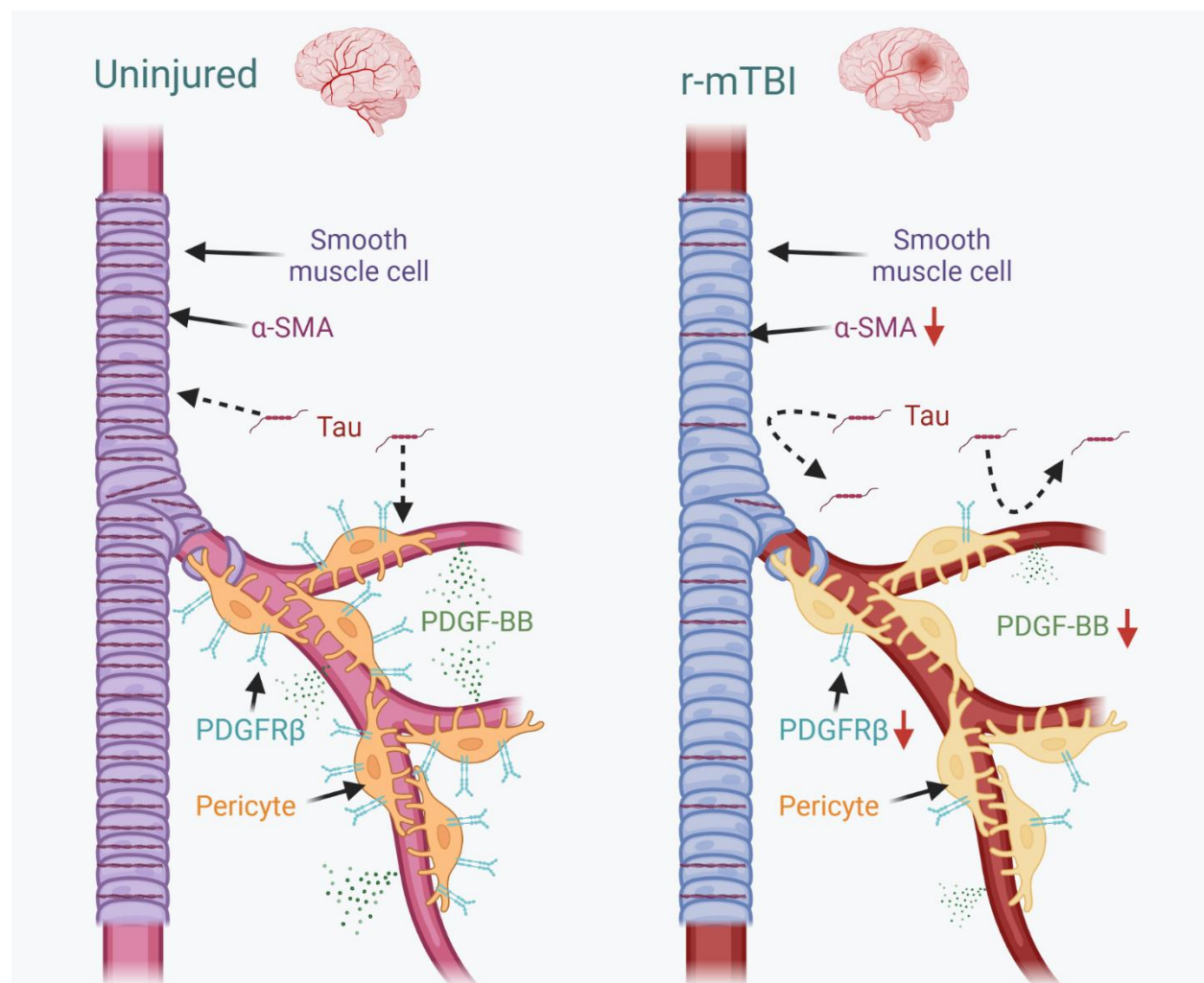


Figure 2.9 Proposed influence of r-mTBI on mural cells and tau uptake. Repetitive trauma to the brain results in altered mural cell status, correlating with diminished cerebrovascular interactions with tau. Following r-mTBI, there is a progressive decrease smooth muscle cell expression of α -SMA, and pericyte expression of PDGFR β , along with diminished levels of the endothelial cell-derived PDGFR β ligand, PDGF-BB. Disruptions to the PDGFR β pathway impair the cerebrovascular uptake of tau, contributing to tau pathology evident in human CTE brains.

periphery, either through their own transport mechanisms or via endothelial cells for transport through the BBB. In the case of extracellular tau, it appears that while the cerebrovessels may internalize the protein, they do not eliminate tau through degradation, indicating the existence of an alternative mechanism of tau clearance from the brain.

The results from this chapter suggest that the mural cells associated with the cerebrovasculature have an interaction with extracellular tau, which is impaired in the chronic stage of r-mTBI. This diminished interaction does not appear to be associated with a loss of mural cells, but rather dysfunction within the cell population. Though the mural cells appear to have the capacity to degrade extracellular solutes, the results from this chapter suggest these cells do not enzymatically degrade a significant amount of extracellular tau. An alternative elimination pathway mediated by mural cells may reflect what has been observed for A β . The half-life of A β in the extracellular space is short, and the protein is eliminated rapidly at the BBB, suggesting that a significant percentage of the elimination occurs through either bulk flow or transcytosis, rather than through degradation, which may occur more slowly. The half-life of tau in the extracellular space has not been thoroughly investigated, though an understanding of the time frame in which tau is eliminated from the extracellular space may shed light on the mechanism of clearance used to eliminate tau from the brain. This, as well as other potential mechanistic interactions between tau and brain endothelial cells in the context of r-mTBI will be discussed in detail in Chapter 3.

CHAPTER 3: INFLUENCE OF TBI ON EXTRACELLULAR TAU ELIMINATION AT THE BBB

3.1 INTRODUCTION

In Chapter 2, I showed that cerebrovessels interact with extracellular tau, and that interaction is attenuated in the context of chronic r-mTBI. In determining the nature of this interaction, I also showed that brain vessels do not process extracellular tau, to a large degree, through metabolic degradation. Another manner by which the cerebrovasculature could process tau is via elimination across the BBB. There is limited investigation into whether extracellular tau can transit across the BBB, though Banks and colleagues found rapid brain-to-blood tau efflux after intracerebroventricular injection of radioactively labeled tau (Banks et al., 2016), which may be facilitated to some degree by exosomes (M. Shi et al., 2016). Recent evidence has indicated a stronger link between ISF tau and blood-borne tau, than between ISF tau and CSF tau (J. Wang et al., 2018; Yamada et al., 2011) suggesting the potential existence of a route of BBB transit. While tau doesn't have transport systems as widely known as A β (Deane et al., 2009), recent studies have found that tau can interact with components of the BBB that facilitate A β transit (Z. Zhao et al., 2015) including LRP1 (Cooper et al., 2021; Rauch et al., 2020). In individuals with CTE, it is believed that trauma induced BBB dysfunction results in altered tau clearance and subsequent tau pathology, though the molecular mechanisms underlying this are unclear (Farrell et al., 2019).

Tau may share common elimination pathways as A β , which is predominantly cleared via BBB transit (Tarasoff-Conway et al., 2015), as the injection of tau into the cortex traced a similar clearance pattern as A β , quickly localizing to the BBB (Nimmo et al., 2020). The rapid appearance of brain delivered exogenous tau in the blood suggests that tau elimination from the brain could be driven largely by BBB transit, as opposed to tau degradation, for example (Banks et al., 2016). Prior reporting has indicated that the half-life of tau is 11 days in mice (Yamada et al., 2015), and 20 days in humans (Sato et al., 2018), but those studies tracked tau from expression to elimination. As tau largely exists as an intracellular protein, it may be cleared rapidly once it reaches the ISF, though the timeframe in which this occurs has not been established. To determine an optimal timepoint in which to interrogate the impact of head trauma on extracellular tau clearance from the CNS, I pursued a temporal elimination curve of exogenous tau.

There is no known tau-specific BBB transporter, but a common efflux mechanism involves endothelial caveolae-mediated transport. Caveolin-1 is an integral membrane component for the formation and function of caveolae-mediated cerebrovascular transcytosis. The levels of caveolin-1 are a fundamental differentiator between CNS and peripheral blood vessels, as those in the CNS that make up the BBB have significantly reduced caveolae-mediated transport to maintain a homeostatic environment. While the chronic effects of r-mTBI on cerebrovascular caveolin-1 expression are not known, there is evidence that in the acute phase after injury, there is a transient elevation of caveolin-1 at the BBB (Badaut et al., 2015), suggesting that head trauma can influence changes in caveolin-1 expression and BBB transit. In animal models with genetic (Head et al., 2010) or disease-associated (Bonds et al., 2019) diminished caveolin-1 expression however, there is an accumulation of pathogenic tau indicating that caveolin-1 may facilitate or modulate the movement of extracellular tau, however these mechanisms are not well understood.

Endothelial caveolae-mediated efflux of solutes is regulated by mural cells, but the nature of this interaction, particularly under conditions of trauma, is unclear. The caveolae-mediated transcytosis

pathway is regulated by the endothelial expression of the major facilitator superfamily domain-containing 2A (Mfsd2a), a lipid flippase which alters the plasma membrane such that caveolae cannot form (Andreone et al., 2017). Endothelial cells do not express Mfsd2a in the absence of pericytes (Ben-Zvi et al., 2014), suggesting direct pericyte regulation of caveolae-mediated transcytosis (Mäe et al., 2021). The mechanism underlying mural cell regulation of endothelial Mfsd2a expression has not been elucidated, but a potential signaling pathway could involve mural cell secreted angiopoietin-1 (Ang-1). Ang-1 binds to endothelial Tie2 receptors, triggering a signaling cascade, resulting in the nuclear translocation of β -catenin (Engelhardt & Liebner, 2014; J. Zhang et al., 2011), and subsequent translation of various proteins including Mfsd2a (Y. Wang et al., 2019). This β -catenin/Mfsd2a/caveolin-1 axis was recently demonstrated to be critical for the integrity of the blood-retinal barrier (Z. Wang et al., 2020) which resembles the BBB with respect to both structure and function. At this stage, the impact of chronic r-mTBI on this cerebrovascular signaling axis has not been investigated. A greater understanding of the mechanisms regulating tau elimination, particularly under pathogenic conditions where the elimination is impaired, could lead to the development of new therapeutic targets.

The results from Chapter 2 suggest that head trauma can impair the PDGFR β /PDGF-BB crosstalk between pericytes and endothelial cells, coinciding with attenuated cerebrovascular uptake of tau. The nature of this diminished interaction is unclear, but dysfunctional mural cell signaling could potentially lead to altered endothelial caveolae-mediated transcytosis, and by extension, impaired elimination of extracellular proteins like tau. Therefore, this chapter will investigate the role and mechanisms of endothelial BBB transit on tau elimination from the CNS, and how that process is impacted by head trauma.

I hypothesize that tau is eliminated from the brain through the BBB, and this is impaired after head trauma. The following sub-hypothesis will be interrogated:

- TBI results in diminished tau clearance from the brain
- Endothelial cells transport tau across the blood brain barrier
- The machinery used in endothelial tau transcytosis is impaired after r-mTBI

3.2 METHODS

3.2.1 Materials

Primary human brain vascular pericytes (HBVP) (cat#1200), primary human brain microvascular endothelial cells (HBMEC) (cat#1000), and associated culture reagents were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Fibronectin solution (cat#F1141), poly-L-lysine solution (cat#P4707), methyl- β -cyclodextrin (cat#C4555), heparin (cat#H3393-10KU) and Hanks' balanced salt solution (HBSS) (cat#H8264) were purchased from MilliporeSigma (St. Louis, MO, USA). Lucifer yellow dextran (10 kDa) and the human tau enzyme linked immunosorbent assay (ELISA) (cat#KHB0041) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Mammalian protein extraction reagent (M-PER) (cat#78505), Halt enzyme inhibitor cocktails (cat#78442), and the bicinchoninic acid (BCA) protein assay (cat#23225) were purchased from ThermoFisher Scientific (Waltham, MA, USA). The ELISA kits for mouse Major facilitator superfamily domain containing 2 (Mfsd2a) (cat#LS-F17827-1), and human Mfsd2a

(cat#LS-F17826-1) were purchased from LifeSpan BioSciences, Inc. (Seattle, WA, USA). The ELISA kit for mouse (cat#MBS721447) and human (cat#MBS727132) caveolin-1 were purchased from MyBioSource, Inc. (San Diego, CA, USA). The ELISA kit for mouse angiopoietin-1 (Ang-1) (cat#NBP2-62857) was purchased from Novus Biologicals (Littleton, CO, USA). The ELISA kit for mouse angiopoietin-2 (Ang-2) (cat#MANG20) was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human tau-441 (rhtau) was purchased from rPeptide (Watkinsville GA, USA) (cat#T-1001-2). Recombinant biotin-labeled human tau-441 (cat#T08-54BN) was purchased from SignalChem (Richmond, BC, Canada).

3.2.2 Animals

Both male and female mice [Human tau (hTau) (cat# 005491) and wild-type (C57BL/6) (cat# 000664)] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All studies used a mix of male and female mice. The hTau mice express six isoforms of human tau on a C57BL/6 background, but do not express murine tau, as previously described (Andorfer et al., 2003). The hTau genotype was confirmed after purchase using PCR from a tail snip via a third party (Transnetyx, Cordova, TN, USA). The mice were between 12 and 14 weeks old at the start of the study, housed in standard cages under 12-hour light/12-hour dark schedule at ambient temperature. Transgenic PS1/APPsw (PSAPP) mice overexpressing the “Swedish” mutation APP695 and mutant presenilin-1 (M146L) on a C57BL/6 background were bred in house and were used as a model of AD. The mural cell-depleted animals, PDGFR β (+/-), were kindly provided by Dr. Richard Daneman (University of California, San Diego, La Jolla, CA). Wild type mice littermates with a C57BL/6 background were used as controls and bred in house. All studies used mice housed 3 per cage under standard laboratory conditions (23 \pm 1°C, 50 \pm 5% humidity, and a 12-hour light/dark cycle) with free access to food and water throughout the study. All procedures involving mice were performed in accordance with Office of Laboratory Animal Welfare and National Institutes of Health guidelines under a protocol approved by the Roskamp Institute Institutional Animal Care and Use Committee.

3.2.3 Human brain tissue

Human brain specimens were acquired from Dr. Thomas Beach, Director of the Brain and Body Donation Program at the Sun Health Research Institute (Sun City, AZ, USA). Intact frozen human cortex samples from the inferior frontal gyrus (500 mg) were obtained from the autopsied brains of 1) control subjects (no history of brain injury or AD), 2) TBI subjects (established history of a single head injury), 3) AD subjects, and 4) TBI-AD subjects (history of a single head injury and AD), though the TBI brains did not have the pathognomonic lesion of CTE. Additional information on these samples can be found in Table 3.1. ApoE genotype status was designated as a carrier of at least one copy of either the ϵ 2, ϵ 3, or ϵ 4 allele. Where an individual had an ϵ 2 and an ϵ 3 allele, they were described as ApoE2 in Table 2.1, and a similar distinction was applied for the ϵ 4 allele.

3.2.4 Brain injury protocol

Repetitive mild traumatic brain injury (r-mTBI) was administered using a mouse model of closed head injury as previously characterized in Chapter 2. Mice (hTau) were euthanatized at 24 hours, 3 months, 6 months after the final brain injury or anesthesia exposure. The sample sizes for the r-sham and r-mTBI groups were the same for each post-injury time point: 24 hours (n = 4), 3 months (n = 7), and 6 months (n = 11). For the intracranial tau injection studies, mice (wild-type) were euthanatized at 12 months after the

final brain injury. For the intracranial tau injection studies, mice (wild-type) were euthanized at 12 months after the final brain injury. The sample sizes for the r-sham and r-mTBI groups in the intracranial tau injection study were $n = 4-8$ for each tau species.

3.2.5 Tau Aggregation

Enriched fractions of low molecular weight aggregated tau were generated as previously described (Mirbaha et al., 2017). Briefly, biotin-labeled tau (4.35 μM) was incubated with freshly prepared Heparin (1 μM) for 6 hours at 37°C. To monitor aggregation activity, monomeric biotin-labeled tau (4.35 μM) was incubated with or without heparin (1 μM) in the presence of the aggregation indicator dye, Thioflavin S (ThS) (20 μM). ThS fluorescence (excitation 450 nm, emission 510), was analyzed at baseline (0 hours) and at 6 hours for each group using a microplate spectrofluorometer (Biotek Cytation 3), to determine the change in relative fluorescence units. After confirmation of aggregation, the aggregate enriched solution of biotin-labeled tau was passed through a 100 kDa MWCO filter (Corning) and centrifuged at 14,000 $\times g$ for 25 min at 4°C. The concentrated, aggregate enriched fraction (MW > 100 kDa) was collected and stored at -80°C. The filtered fraction of seed competent monomers and dimers (MW < 100 kDa) were subsequently concentrated using a 30 kDa MWCO filter (Corning) and centrifuged at 14,000 $\times g$ for 25 min at 4°C. The final protein concentrations were determined using a biotin-labeled tau ELISA. Misfolding of the seed competent monomeric fraction was confirmed using dot blot. Concentration (50 ng/ml) matched aliquots (1 μl) of monomeric biotin-labeled tau, seed competent biotin-labeled tau, and aggregate enriched biotin-labeled tau, were placed on a nitrocellulose membrane for 30 minutes at room temperature before blocking in 10% BSA in tris buffered saline with 0.1% Tween-20 (TBS-T) overnight at 4°C. The membrane was incubated for 1 hour in MC1 (MC1, 1:1000 diluted in TBS-T with 5% BSA), vigorously washed with TBS-T, then incubated in horseradish peroxidase-conjugated anti-mouse secondary antibody (goat anti-mouse IgG 1:1000 in TBS-T with 5% BSA) for 1 hour at room temperature. After an additional wash in TBS-T, SuperSignal West Femto Maximum Sensitive Substrate (ThermoFisher) was used for chemiluminescence detection and signal intensity ratios were quantified with the ChemiDoc TM XRS (Bio-Rad). MC1 is a conformation-dependent tau antibody raised against paired helical filament tau from Alzheimer's disease brain homogenate, whose reactivity depends on the N terminus (amino acids 7-9) and the C-terminus (amino acids 313-322) (Jicha et al., 1997), and was generously provided by Dr. Peter Davies, The Feinstein Institute for Medical Research, Bronx, NY, USA.

3.2.6 Tau elimination

For the temporal tau elimination studies, 6 wild-type mice (9 months of age) were anesthetized via inhalation using a 3% isoflurane / oxygen mix and maintained at 37°C using a homeothermic blanket. mice were stereotactically injected into the brain with (50 $\mu\text{g}/\text{ml}$) human biotin-labeled tau in 3 μl of PBS (0.5 mm anterior to the bregma, 2 mm lateral to the midline, and 3 mm below the surface of the skull) as per our prior methods (Paris et al., 2011). In a separate cohort of mice, 10 kDa lucifer yellow dextran (LyD) (100 mg/ml) was stereotactically injected into the brain in the same manner as above, to provide context for the tau studies, as LyD does not readily cross the BBB (Natarajan et al., 2017). Mice were euthanized at 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, and 24 hours after the intracranial injection. The brain was harvested, and each hemisphere was homogenized with probe sonication in 500 μl of lysis

buffer. The half-life for both biotin-labeled tau and LyD was determined using nonlinear regression and a one phase decay fit (GraphPad Prism 8.0, GraphPad Software, Inc) using the following equation: $Y = (Y_0 - \text{Plateau}) * \exp(-K * X) + \text{Plateau}$. Y_0 is the Y value when X (time) is zero and the Plateau is the Y value at infinite times, both of which are expressed in the same units as Y. K is the rate constant, expressed in reciprocal of the X axis time units. If X is in minutes, then K is expressed in inverse minutes. Tau is the time constant, expressed in the same units as the X axis. It is computed as the reciprocal of K. The half-life is in the time units of the X axis. It is computed as $\ln(2)/K$. Lastly, the span is the difference between Y_0 and Plateau, expressed in the same units as the Y values (Motulsky, 2016). The value at time = 0 (y-intercept) was used as the theoretical initial concentration in the brain and the values at each time point were calculated as a percentage of this initial concentration.

3.2.7 Tau residence in the brain after r-mTBI

Biotin-labeled tau species and 10 kDa lucifer yellow dextran (LyD) were stereotactically injected into r-sham and r-mTBI wild-type mice (12 months post-injury), in the same manner as the temporal studies above, and euthanatized 2 hours after the intracranial injection. The brain homogenate was evaluated for biotin-labeled tau using a modified hTau ELISA (Invitrogen). The ELISA was performed as described in Chapter 2. Furthermore, each stereotaxic injection included LyD (80 mg/ml) to account for any nonspecific leakage out of the brain, as LyD typically demonstrates low BBB permeability (Natarajan et al., 2017). LyD fluorescence was analyzed using a microplate spectrofluorometer (Biotek Cytation 3). With respect to tau, all samples were evaluated for exogenous biotin-labeled tau remaining in the brain and normalized to LyD for each time point. To assess LyD alone, the amount of LyD at each time point was normalized to total protein content as determined using the BCA assay.

3.2.8 *In vitro* BBB assay

Using primary HBVP and HBMEC, a contact coculture version of our previously characterized *in vitro* BBB model (Bachmeier 2010) was used to evaluate tau transcytosis. Briefly, HBVP were seeded at 25,000 cells / cm^2 onto the exterior portion of poly-L-lysine coated 24-well 0.4 μm -pore membrane inserts. One hour after HBVP seeding, HBMEC were seeded at 50,000 cells / cm^2 onto the interior membrane of the fibronectin-coated insert to establish a polarized monolayer. The layer of cells separates this system into an apical ("blood" side) and basolateral ("brain" side) compartment. The basolateral compartment was exposed to monomeric or aggregated biotin-labeled tau (200 ng/ml) in the presence or absence of M β CD (10 mM), while fresh media was placed in the apical compartment. The inserts containing media were exposed to the wells containing biotin-labeled tau and incubated at 37 °C. The basolateral compartment was sampled at time 0 to establish the initial concentration of biotin-labeled tau. Samples were collected from the apical compartment at 0, 30, and 60 min to assess the rate of biotin-labeled tau transcytosis across the cell monolayer (basolateral-to-apical) and analyzed for biotin-labeled tau using a modified hTau ELISA. Furthermore, each basolateral compartment was exposed to a known paracellular marker, 10 kDa lucifer yellow dextran (LyD 10 μM), to monitor cellular integrity and/or nonspecific permeability, as we previously described (Bachmeier et al., 2010). The apparent permeability (Papp) was determined using the equation $P_{\text{app}} = 1 / A C_0 * (dQ / dt)$, where A represents the surface area of the membrane, C_0 is the

initial concentration of biotin-labeled tau in the basolateral compartment, and dQ / dt is the amount of biotin-labeled tau appearing in the apical compartment in the given time period (Artursson, 1990).

3.2.9 Tau uptake and caveolin inhibition *ex vivo*

Freshly isolated cerebrovessels (as described in detail in Chapter 2) from naïve wild-type mice (9 months of age) were pre-treated with methyl- β -cyclodextrin (0, 1, and 10 mM) for 30 minutes at 37°C followed by treatment with 5 ng/ml rhtau for 1 hour at 37°C. Following the treatment period, the extracellular media was removed, and the cerebrovessels were washed with ice-cold HBSS. Cell lysates were collected using lysis buffer (M-PER) supplemented with phenylmethanesulfonylfluoride (1 mM) and Halt protease and phosphatase inhibitor cocktail. The cell lysates were analyzed for total tau by ELISA and normalized to total protein content using the BCA protein assay.

3.2.10 Caveolin-1 expression in isolated vessels of animals and humans

Cerebrovessels from hTau r-sham and r-mTBI mice at 24 hours, 3 months and 6 months post-injury were isolated and collected using lysis buffer (M-PER) supplemented with phenylmethanesulfonylfluoride (1 mM) and Halt protease and phosphatase inhibitor cocktail, as described in Chapter 2. The cell lysates were analyzed for caveolin-1 by ELISA and normalized to total protein content using the BCA protein assay. Human cerebrovascular lysates were collected as described in Chapter 2, analyzed for caveolin-1 by ELISA and normalized to total protein content using the BCA protein assay.

3.2.11 Cerebrovascular angiopoietin 1/2 secretion *ex vivo*

Freshly isolated cerebrovessels from r-sham and r-mTBI mice 6 months post-injury were incubated in ECM for 72 hours at 37°C. Following the incubation, the extracellular media was collected, the cerebrovessels were washed in ice-cold HBSS, then collected in lysis buffer as described previously. The extracellular media was evaluated for secretion of Ang-1 and Ang-2 by ELISA and normalized to the corresponding cell lysate total protein content using the BCA protein assay.

3.2.12 Angiopoietin-1 modulates HBMEC expression of Mfsd2a *in vitro*

Fully confluent HBMECs were treated with 2.5 ng/ml of Ang-1 or a vehicle in ECM for 24 hours. Cells were washed with HBSS and cell lysates were collected using lysis buffer as described previously. The cell lysates were analyzed for Mfsd2a by ELISA and normalized to total protein content using the BCA assay.

3.2.13 Mfsd2a expression in animal cerebrovasculature after r-mTBI

Cerebrovessels from hTau r-sham and r-mTBI mice at 24 hours, 3 months and 6 months post-injury were collected using lysis buffer as described in Chapter 2. The lysates were analyzed for Mfsd2a by ELISA and normalized to total protein content using the BCA protein assay.

3.2.14 Statistical analysis

Randomization and blinding procedures were employed. Quantitative data were plotted as mean \pm standard deviation. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc).

The Shapiro-Wilk test was completed to assess normality. Tau uptake and human expression of caveolin-1 were evaluated for significance by ANOVA and the Bonferroni post hoc test with multiple comparisons. Mouse tau elimination, caveolin-1 and Mfsd2a expression were evaluated for significance as determined by two-way ANOVA Bonferroni post hoc test with multiple comparisons. For comparisons between two groups including tau transcytosis, angiopoietin secretion and stimulation studies, statistical significance was analyzed using a two-tailed unpaired Student's t-test or a Mann-Whitney U test. For all analyses, a p value of ≤ 0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 Tau residence time in the brain

To examine the elimination profile of exogenous tau from the brain, we evaluated the time course by which biotin-labeled tau is eliminated from the brain following intracranial injection. The half-life of exogenous biotin-labeled tau residing in the brain was 41 minutes (Figure 3.1A), while the half-life of 10 kDa LyD, which does not readily cross the BBB, was nearly 3-times greater (114 minutes) (Figure 3.1A). Next, the influence of r-mTBI on the elimination of exogenous tau species from the brain was evaluated after the intracranial injection of biotin-labeled tau (2 hours). Monomeric biotin-labeled tau residence was increased by 2.7-fold in the r-mTBI mice, compared to r-sham. While the aggregate enriched species showed a 2-fold elevation in biotin-labeled tau residence time in the brain post-injury compared to r-sham, this value did not reach statistical significance ($p = 0.20$) (Figure 3.1B). Furthermore, additional post hoc Bonferroni tests with multiple comparisons evaluated each of the biotin-labeled tau species under r-mTBI conditions. There was no significant difference between the amount of exogenous monomeric and aggregated enriched species residing in the brain in the r-sham mice. In the r-mTBI mice, there was a 4-fold decrease in the residence of exogenous seed competent biotin-labeled tau compared to the exogenous monomeric biotin-labeled tau, and similarly, a 2-fold decrease was observed in aggregate enriched biotin-labeled tau compared to monomeric biotin-labeled tau (Figure 3.1B). Of note, no statistically significant difference in the amount of dextran residing in the brain was observed between the r-mTBI and r-sham groups for any of the biotin-labeled tau species, indicating the effects of r-mTBI on tau elimination from the brain were not due to alterations in BBB integrity (Figure 3.1C). To characterize tau conformation status, MC1 immunoreactivity was elevated in seed competent and aggregate enriched biotin-labeled tau compared to monomeric biotin-labeled tau (Figure 3.1D, E). The heparin-induced aggregated tau demonstrated substantial activity in the ThS assay, while biotin-labeled monomeric tau showed minimal activity, near the assay limit of detection (Figure 3.1F).

3.3.2 Mechanisms of Tau transcytosis across an *in vitro* BBB model

To more specifically investigate the mechanism by which tau is eliminated from the brain, I examined tau transit across an *in vitro* model of the BBB. In comparing the basolateral-to-apical BBB transit of each biotin-labeled tau species, there were no differences in the apparent permeability of each biotin-labeled tau species across the BBB model under control conditions. Pretreatment with the caveolin-1 disruptor M β CD resulted in an even more pronounced decrease for both species. Monomeric tau transit diminished

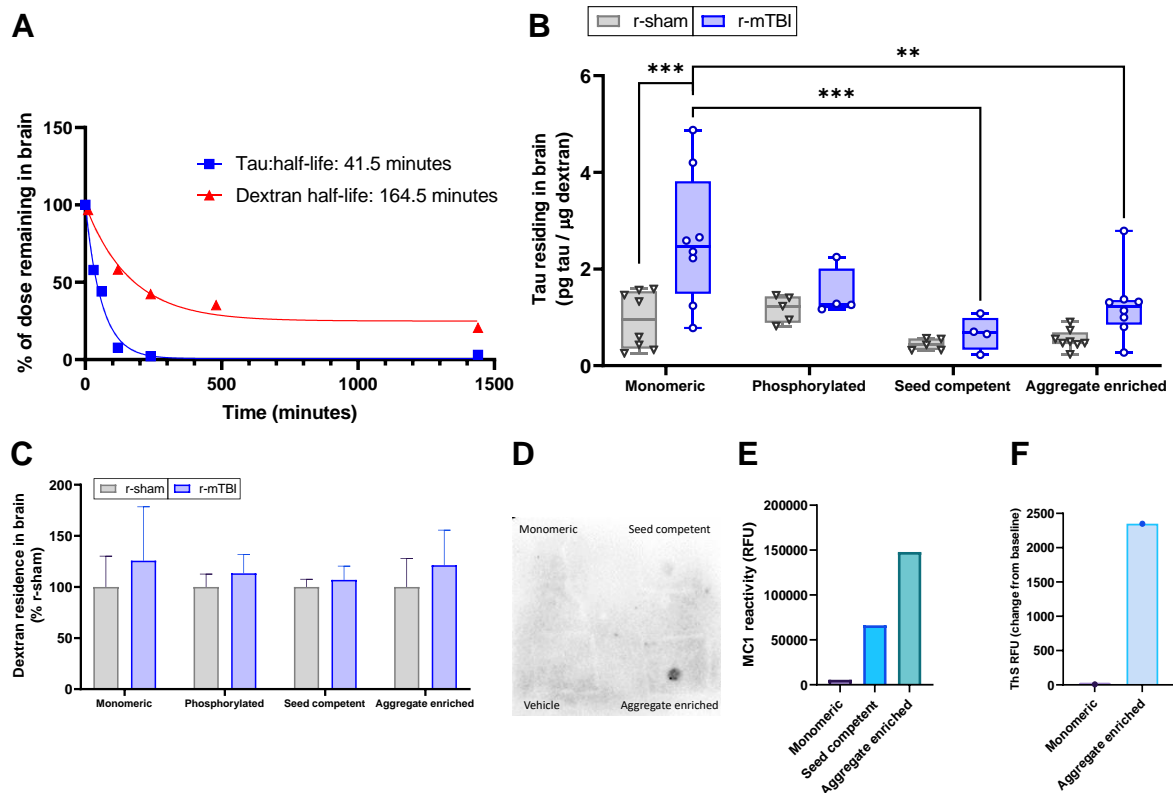


Figure 3.1 Effect of r-mTBI on the elimination of exogenous tau species from the brain. (A) The time course of tau elimination from the brain was established by examining biotin-labeled tau ($n = 6$) and 10 kDa LyD ($n = 5$) levels in the brain at various time points following intracranial injection into naïve wild-type mice (9 months of age). Biotin-labeled tau content was analyzed using an ELISA and LyD was analyzed via fluorescence. The half-life for both biotin-labeled tau and LyD were determined using nonlinear regression and a one phase decay fit. (B-C) Following intracortical injection in r-sham ($n = 5-8$) and r-mTBI mice ($n = 4-8$) (12 months post-injury), the amount of exogenous biotin-labeled tau species residing in the brain was determined at 2 hours post-injection. For each injection, biotin-labeled tau was co-injected with LyD. Biotin-labeled tau content was analyzed using an ELISA while LyD was analyzed via fluorescence and normalized to total protein used the BCA protein assay. Values represent mean \pm SD ($n = 4-8$) and are expressed as (B) pg of tau per μ g of LyD or (C) the percentage of dextran residence normalized to each respective r-sham. *** $P < 0.01$, **** $P < 0.001$ as compared to r-sham as determined by a two-way ANOVA and Bonferroni post-hoc test. (C-E) In characterizing each tau species, biotin-labeled tau (4.35μ M) was incubated with heparin (1μ M) for 6 hours at 37°C to induce aggregation. The aggregates were separated using a 100 kDa molecular weight cutoff filter into aggregate enriched (tau > 100 kDa) or seed competent (tau < 100 kDa) fractions. (D) The conformational status of the seed competent (50 ng/ml) and aggregate enriched (50 ng/ml) biotin-labeled tau fractions were compared to monomeric (50 ng/ml) biotin-labeled tau via dot blot using an MC1 antibody. (E) Quantitative analysis of MC1 immunoreactivity in the dot blot. (F) Heparin-induced in vitro aggregation of monomeric biotin-labeled tau (4.35μ M) was assessed using the indicator dye ThS. ThS fluorescence was measured in the presence of monomeric biotin-labeled tau (4.35μ M) immediately after the addition of heparin (1μ M) or vehicle (0 hours), and again after 6 hours. Values represent the change in ThS fluorescence (excitation 450 nm, emission 510 nm) over the 6 hour period.

by almost 6-fold compared to vehicle treated cells, and aggregated enriched tau transit diminished by 5.6-fold compared to the vehicle (Figure 3.2A). Notably, the individual biotin-labeled tau species did not appear to impact BBB integrity as dextran permeability across the BBB model was not different between the biotin-labeled tau species. The percentage of dextran crossing the BBB model over the 60 min period was $0.90 \pm 0.09\%$ for monomeric tau and $0.88 \pm 0.1\%$ for aggregate enriched tau. Likewise, treatment with M β CD had no effect on dextran BBB permeability compared to control conditions. The percentage

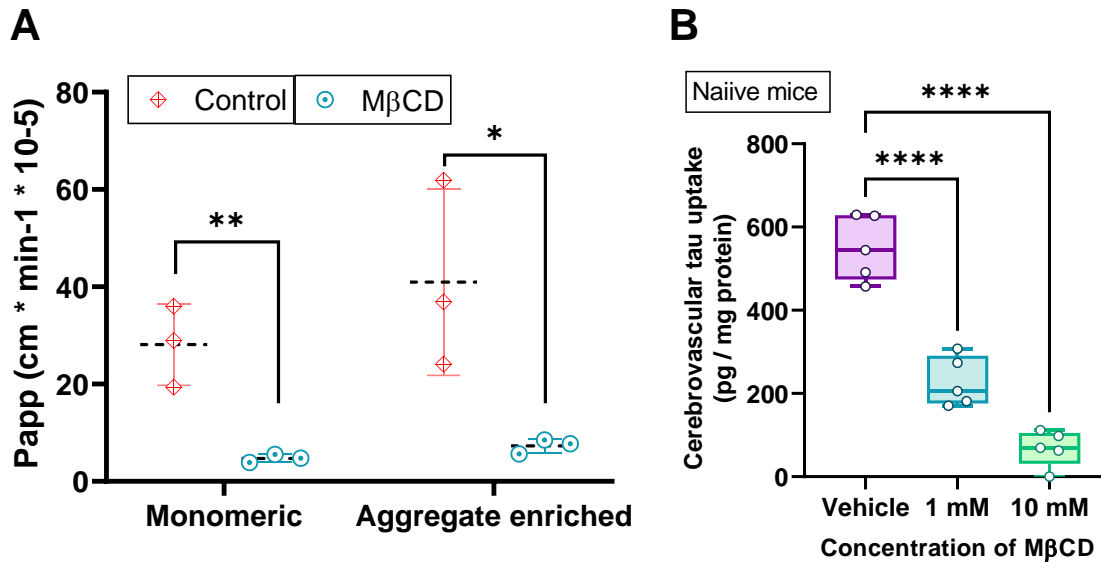


Figure 3.2 Effect of MβCD on tau transcytosis across an in vitro model of the BBB. (A) MβCD (10 mM) was exposed to the basolateral compartment of the in vitro BBB model for 30 minutes at 37 °C. Following the pretreatment with MβCD, biotin-labeled monomeric or aggregate enriched biotin-labeled tau was added alongside the known paracellular marker 10 kDa LyD to the basolateral compartment of the in vitro BBB model. Samples were collected from the apical compartment at 0, 30, and 60 minutes to determine the permeability of biotin-labeled tau and LyD across the BBB model. Values represent mean \pm SD ($n = 3$) and are expressed as the apparent permeability coefficient (Papp). * $P < 0.05$, ** $P < 0.01$ compared to each respective tau species with vehicle as determined by unpaired t-Test. (B) Freshly isolated cerebrovessels from naïve wild-type mice (9 months old) were pretreated with various concentrations of the caveolin inhibitor MβCD (0, 1 mM, 10 mM) for 30 minutes at 37 °C, before being exposed to recombinant human tau (5 ng/ml) for 1 hour at 37 °C. Lysates were analyzed for tau content using an ELISA and normalized total protein using the BCA assay. Values represent mean \pm SD ($n = 5$) and are expressed as pg of tau per mg of total protein. **** $P < 0.0001$ compared to vehicle as determined by one-way ANOVA and Bonferroni post-hoc test.

of dextran crossing the BBB model over the 60 min period was $0.89 \pm 0.04\%$ for control and $0.84 \pm 0.03\%$ for MβCD. To further investigate the interaction between cerebrovascular caveolin-1 and extracellular tau, freshly isolated cerebrovessels from naïve mice were treated with MβCD prior to exposure to unlabeled monomeric tau. 1 mM MβCD treatment resulted in a 2.4-fold decrease in cerebrovascular uptake compared to control, and an 8-fold decrease was observed in tau uptake when treated with 10 mM MβCD compared to control (Figure 3.2B).

3.3.3 Caveolin-1 expression in animal and human cerebrovasculature

Group	Sample size	Age \pm SEM (years)	Sex (M/F)	Years post injury \pm SEM	ApoE 2/3/4
Control	9	77.7 \pm 3.1	7/2		7/2/0
TBI	10	81.9 \pm 2.6	6/4	26.2 \pm 9.8	2/8/0
AD	8	85.9 \pm 2.2	4/4		1/4/3
AD-TBI	9	82.8 \pm 1.7	5/4	45 \pm 9.6	0/3/6

Table 3.1 Characteristics of human brain specimens. ApoE 2/3/4 refers to the presence of at least one copy of the allele.

As the results from the BBB transit study suggest that caveolin-1 may play a role in the cerebrovascular elimination of extracellular tau, I investigated whether caveolin-1 levels are impacted by r-mTBI. At 24

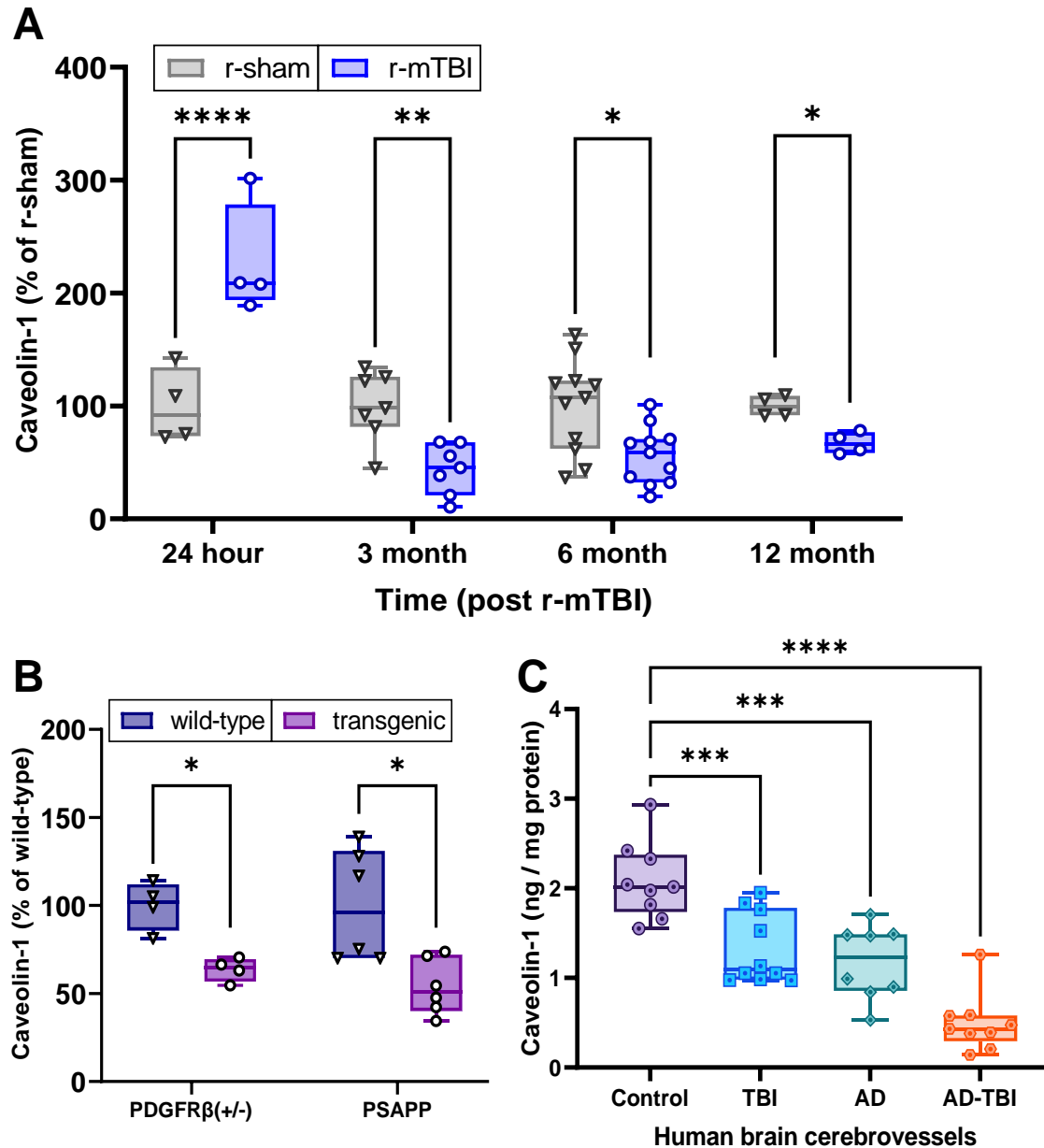


Figure 3.3 Effect of r-mTBI and AD on caveolin-1 in isolated cerebrovessels. Caveolin-1 expression was evaluated in lysates from (A) r-mTBI mice (24 h, 3 months, 6 months, and 12 months post injury), and (B) PDGFRβ(+/-) mice, PSAPP mice, and respective wild-type littermates using ELISA and normalized to total protein using the BCA assay. Values represent mean + SD (24 hour n = 4, 3 month n = 7, 6 month n = 11, 12 month (n = 4), PDGFRβ(+/-) and wildtype littermates (n = 4), and PSAPP and wild-type littermates (n = 6)) and are expressed as the percentage of each respective control. *P < 0.05, **P < 0.01, ****P < 0.0001 compared to each respective r-sham or control as determined by two-way ANOVA and Bonferroni's multiple comparisons test. (C) Caveolin-1 expression was evaluated in lysates from cerebrovasculature isolated from human brain cortex derived from, 1) non-demented control subjects (no history of TBI or AD diagnosis), 2) TBI, 3) AD, and 4) TBI and AD using ELISA and normalized to total protein using the BCA assay. Values represent mean ± SEM and are expressed as ng per mg of total protein. Control (n = 9), TBI (n = 8), AD (n = 10), TBI-AD (n = 9). *P < 0.05 compared to control as determined by the one-way ANOVA followed by post-hoc test correction for false discovery rate using the Bonferroni correction.

hours after r-mTBI, caveolin-1 levels were elevated by 2.3-fold relative to r-sham. Alternatively, at chronic timepoints post injury, caveolin-1 levels diminished by 65% at 3 months, 45% at 6 months and 35% at 12 months post injury, relative to age matched r-sham (Figure 3.3A). Similar reductions were observed in the cerebrovasculature of both the PS1/APP (35% decrease) and PDGFR β (+/-) (45% decrease) mice compared to their respective WT littermates (Figure 3.3A). With respect to human brain specimens (Table 3.1), isolated cerebrovasculature from TBI brain specimens had at 40% reduction in caveolin-1 levels compared to NDC (Figure 3.3B). AD cerebrovessels showed a 45% reduction, while caveolin-1 levels in AD-TBI brains were diminished by 4-fold compared to NDC cerebrovessels.

3.3.4 Altered Ang1/Tie2 signaling pathway after TBI

Prior reporting has indicated the growth factor Ang-1, which is primarily secreted by vascular mural cells, was elevated in the post-acute stages after TBI (Sabirzhanov et al., 2018) and associated with diminished caveolin-1 expression (Nag et al., 2017). To investigate whether cerebrovascular Ang-1 signaling was altered at chronic stages after r-mTBI, secreted Ang-1 and Ang-2 levels from r-mTBI and r-sham cerebrovessels 6 months post injury were evaluated. A 40% elevation in Ang-1 secretion was observed after r-mTBI, while there was no change in Ang-2 secretion between r-mTBI and r-sham (Figure 3.4A). Recent studies have indicated that Ang-1 signaling regulates Mfsd2a expression in endothelial cells. Ang-1 treatment to HBMECs *in vitro* resulted in a 30% increase in the expression of Mfsd2a (Figure 3.4B). As Mfsd2a has been shown to modulate endothelial caveolin-1 expression and function (Andreone et al., 2017), levels of cerebrovascular Mfsd2a were examined in isolated r-mTBI and r-sham cerebrovessels. At 24 hours post injury, levels of Mfsd2a in r-mTBI cerebrovessels were reduced by 55% relative to r-sham.

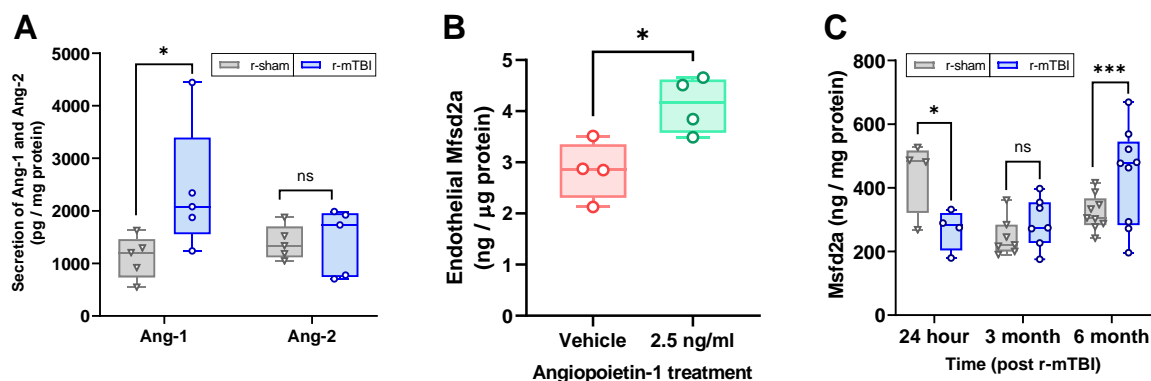


Figure 3.4 Influence of r-mTBI on cerebrovascular Ang-1/Tie2/Mfsd2a signaling pathway. (A) Secretion of Ang-1 and Ang-2 from fresh cerebrovessels isolated from r-sham and r-mTBI hTau mice. Following 72 hours of incubation at 37 °C, the cerebrovascular extracellular media was probed for Ang-1 and Ang-2 using an ELISA and normalized to total protein using the BCA assay. Values represent mean + SD (n = 5) and are expressed as pg of Ang-1 or Ang-2 per mg total protein. *P < 0.05 compared to each respective r-sham as determined by a Mann-Whitney U test. (B) Mfsd2a expression in HBMEC following treatment with Ang-1 (2.5 ng/ml) for 24 hours at 37 °C. Ang-1 expression was quantified by ELISA and normalized to total protein using the BCA assay. Values represent mean + SD (n = 4) and are expressed as ng Mfsd2a per μg of total protein. *P < 0.05 compared to vehicle as determined by unpaired t-Test. (C) Mfsd2a expression was evaluated in lysates from r-mTBI mice (24 h, 3 months and 6 months post injury) using ELISA and normalized to total protein using the BCA assay. Values represent mean + SD (24 h n = 4, 3 month n = 7, 6 month n = 11). *P < 0.05, ***P < 0.01 compared to each respective r-sham or control as determined by two-way ANOVA and Bonferroni's multiple comparisons test.

However, by 6 months post injury, Mfsd2a levels in r-mTBI cerebrovessels were 45% higher than age matched r-sham cerebrovessels (Figure 3.4C).

3.4 DISCUSSION

As pathological tau propagation has been observed in the chronic stages of TBI and other neurodegenerative diseases, I explored potential mechanisms responsible for the elimination of extracellular tau from the brain and the influence of head trauma on these processes. In Chapter 2, I demonstrated an interaction between extracellular tau and brain vascular mural cells (pericytes and smooth muscle cells) and showed a progressive decrease in cerebrovascular tau uptake up to 12 months post-injury in our mouse r-mTBI model (Ojo et al., 2021), though tau enzymatic processing by mural cells did not appear to play a significant role in extracellular tau degradation. However, extracellular tau interactions with the cerebrovasculature were significantly disrupted, and to this point, the mechanisms by which extracellular tau interacts with the endothelial cells that comprise the BBB, and the influence of mural cell signaling on those interactions are unclear. The present studies examined the role of the BBB in cerebrovascular tau elimination and the potential impact of head trauma on these processes. 1

The clearance of extracellular tau from the ISF has not been extensively characterized, though it appears that extracellular tau can readily enter the plasma as increases in ISF tau due to neuronal injury are reflected in the plasma shortly after injury (Yanamandra et al., 2017). After injection into the ISF, tau relocates the perivascular space within and around arteriole walls within minutes, and though it is not cleared as efficiently as A β , recent evidence suggests they share common routes of cerebrovascular elimination (Nimmo et al., 2020). Enlarged perivascular spaces, observed after TBI (Opel et al., 2019) and AD (Vilor-Tejedor et al., 2021), result in the impaired clearance of both metabolic waste, and solutes including tau, due to diminished fluid movement. In this chapter, I report that the half-life of exogenous tau was approximately 41 minutes in the brain following intracranial injection (Figure 3.1). For context, I also injected a 10 kDa dextran marker (LyD), that is not readily eliminated from the brain and does not cross the BBB (Natarajan et al., 2017), and found the half-life of LyD in the brain was 4-times the value we observed for exogenous tau (164 minutes vs. 41 minutes). Prior reporting has indicated a significantly longer half-life for tau in the brain, 11 days in mice (Yamada et al., 2015) and 20 days in humans (Sato et al., 2018). However, it is important to note these values encompassed the entire life cycle of tau, from neuronal synthesis and cellular secretion to elimination, whereas the half-life value for tau in the present study only reflects elimination from the brain. In line with the results from this chapter, a previous study determined that the half-life of tau injected into the cisterna magna was less than 2 hours and the exogenous tau was detectable in the plasma within minutes of the injection (Yanamandra et al., 2017). Thus, based on the elimination profile of tau from the brain in our studies, I used a 2-hour post-injection time-point to examine the influence of head trauma on tau residence in the brain.

The amount of exogenous monomeric tau residing in the brain was greater in the r-mTBI animals compared to r-sham, indicating tau elimination from the brain was reduced following head trauma. Interestingly, the monomeric tau residing in the r-mTBI brain was greater than that observed for the aggregate enriched tau species. While studies evaluating the interactions of various tau species with brain endothelia are lacking, earlier work suggested tau aggregates, but not monomeric tau, efficiently bind to

neurons and are internalized using bulk endocytosis (Wu et al., 2013). A more recent report found that both monomeric and aggregated tau can be internalized with similar efficiency in neurons, but follow distinct pathways (Evans et al., 2018). Solute internalization and trafficking at the BBB are both regulated by ligand binding avidity and particle size (Tian et al., 2020), characteristics which differ between tau aggregates, conformers, and monomers, and may describe any differences in endothelial internalizing and trafficking amongst various tau species. Clearly, further work is necessary to understand the regulatory mechanisms governing cerebrovascular tau elimination and the potential influence of head trauma on these processes.

There are several pathways through which tau can be eliminated from the brain including, but not limited to, 1) degradation, 2) perivascular drainage, and 3) BBB transcytosis. While tau has been shown to be degraded in the brain, these processes generally occur over a longer period of time (>12 hours) (David et al., 2002; Dolan & Johnson, 2010) than the 2-3 hour elimination time-frame in our experimental paradigm, so degradation does not appear to be a primary driver of extracellular tau elimination from the brain in our particular studies. Previous work found that paravascular tau clearance was reduced by approximately 60% following TBI and was associated with phospho-tau pathology and neurodegeneration (Iliff et al., 2014). The authors noted that if there were a tau efflux mechanism, at the BBB for example, their theory of convective bulk flow may contribute to tau ISF clearance by effectively distributing tau along the vascular bed for more efficient transcytosis (Iliff et al., 2014). That said, there has been little investigation into the transit of tau across the BBB. Banks and colleagues found that full-length tau and various truncated tau proteins readily crossed the BBB bidirectionally and entered the blood following injection into the brain (Banks et al., 2016), consistent with our intracranial tau injection studies.

To investigate potential mechanisms of tau transcytosis, I developed and employed an indirect co-culture BBB model, resembling one which has subsequently been validated (Kurmann et al., 2021), consisting of brain endothelia and pericytes. First, to understand whether the BBB could transport tau, I tested the BBB transit of several tau species along with LyD. In comparing the tau species, the aggregate enriched tau showed increased BBB transcytosis compared to monomeric tau, potentially explaining the enhanced elimination of these species in the tau residence studies above (Figure 3.2). To investigate potential tau transport mechanisms, tau transit was evaluated in the presence of a transcytosis inhibitor. To inhibit transcytosis, I used M β CD, which prevents the formation of caveolae and restricts caveolin-mediated endocytosis. The effect of caveolae modulation via M β CD demonstrated a dramatic reduction in BBB transit for both monomers and aggregate enriched species of tau. To further evaluate tau caveolin-1 interactions, I subsequently examined the influence of M β CD on *ex vivo* tau uptake in isolated cerebrovessels. These results supported the *in vitro* studies, demonstrating a significant reduction in cerebrovascular tau uptake that diminished as the dose of M β CD increased. Of note, while several studies have demonstrated M β CD to be an effective disruptor of caveolae and caveolin-1 (Jozic et al., 2019; Potje et al., 2019), I cannot rule out any nonspecific effects of M β CD (i.e., other than caveolin-1) on cerebrovascular tau transit. That said, it was previously reported that mice with reduced caveolin-1 brain expression showed elevated levels of total and phosphorylated tau in the hippocampus compared to wild-type animals (Bonds et al., 2019; Head et al., 2010), though perivascular tau was not examined in those studies. Future experiments will further investigate the effects of caveolin-1 on tau elimination *in vivo* using endothelial specific caveolin-1 knockout mice. Collectively these findings indicate tau proteins can be eliminated across the BBB, which may be mediated through caveolin-1.

To understand whether this potential mechanism of tau elimination could be impacted by r-mTBI, I probed the isolated cerebrovasculature for any potential alterations in caveolin-1 following head trauma (Figure 3.3). In the acute period after r-mTBI, the results demonstrate an upregulation of caveolin-1 expression at 24 hours post-injury, consistent with prior reporting of increased caveolin-1 expression within the neurovascular unit during the same 24-hour period following TBI in juvenile rats (Badaut et al., 2015). However, in this rat TBI model, the increased expression appeared to be transient, as caveolin-1 levels returned to baseline 3 days after the brain injury (Badaut et al., 2015). The acute upregulation of caveolin-1 post-injury could explain the transient increase in plasma tau levels observed immediately following sports-related concussive injury (Gill et al., 2017). In the more chronic phase post-injury, we observed decreased expression of caveolin-1 following r-mTBI at 3-, 6- and 12-months post-injury compared to r-sham. The decline in caveolin-1 expression at 12-months post injury reflected a similar diminished expression in both the cerebrovasculature of aged APP/PS1 mice and PDGFR β (+/-) mice relative to their age matched WT littermates. In fact, diminished caveolin-1 levels in both the APP/PS1 and chronic r-mTBI vasculature reflected what was observed in the human cerebrovasculature as there was a significant decrease in human cerebrovascular caveolin-1 in human TBI and AD cerebrovessels compared to NDC tissue. There is a dearth of research into the long-term effects of TBI on cerebrovascular caveolin-1, particularly in the context of repetitive injury, though recent examinations of CTE brain tissue found diminished caveolin-1 expression compared to control specimens (Cherry et al., 2021). It has been proposed that the upregulation of caveolin-1 acutely following cerebral insult is meant to facilitate vascular repair by promoting angiogenesis and stabilizing tight junction and efflux proteins (e.g., claudin-5 and P-glycoprotein) (Badaut et al., 2015). However, as observed in the present studies, the chronic downregulation of caveolin-1 after r-mTBI could hamper waste elimination from the brain, leading to the accumulation of extracellular solutes such as tau, as demonstrated in other animal models where caveolin-1 is chronically diminished in the brain (Bonds et al., 2019; Head et al., 2010).

To this point, the mechanism by which mural cells regulate the endothelial expression of caveolin-1 is unclear, though evidence suggests the signaling factors Ang-1 and Ang-2 may play a role via the Ang-1/Tie2 axis. Ang-1 is predominantly expressed and constitutively released by vascular mural cells (Gaengel et al., 2009; Sundberg et al., 2002) to facilitate vessel assembly and stability and is a critical survival factor in preventing endothelial cell death (Kim et al., 2000). In addition, Ang-1 is known to activate the signaling pathway that downregulates plasmalemma vesicle-associated protein expression, which is also responsible for caveolin formation (Laksitorini et al., 2019). Alternatively, Ang-2 is almost exclusively secreted by endothelial cells and functions as a negative regulator of the Ang-1 pathway in order to modulate vessel maturation (Felcht et al., 2012; Hansen et al., 2010). Proper ratios between Ang-1 and Ang-2 are necessary to ensure vessel integrity is maintained, and the disruption of the Ang-1: Ang-2 ratio has been observed in several vascular diseases (Pan et al., 2021) largely influenced by altered Ang-1 levels. The impact of Ang-1 on caveolin-1 expression was demonstrated recently in rats, where increased caveolin-1 levels following acute brain trauma were reversed with Ang-1 treatment (Nag et al., 2017).

Previous reports have shown vascular mural cells upregulate Ang-1 in response to insults such as hypoxic conditions, while Ang-2 levels were largely decreased or unchanged (Dore-Duffy et al., 2007; Park et al., 2016). With respect to TBI, prior studies have reported a progressive decrease in Ang-1 levels in the brain (Sabirzhanov et al., 2018) and capillaries (Dore-Duffy et al., 2007) over the first 48 hours following brain injury. At later stages of brain injury, pericytes become reactive and secrete angiogenic growth factors including Ang-1 to mediate endothelial cell activity and vascular integrity (Salehi et al., 2017). There has

been a lack of work examining Ang-2 levels following TBI, however Ang-2 levels were found to be increased acutely in the brain following subarachnoid hemorrhage (Gu et al., 2016) and cold-injury (Nourhaghi et al., 2003) but the chronic status of Ang-2 in the brain following cerebral insult is currently unknown. That said, as the time increases post-injury (days and weeks following head trauma), several studies have reported an increase in Ang-1 levels in the brain (Brickler et al., 2018; Sabirzhanov et al., 2018) and serum (Gong et al., 2011). The findings from the current study coincide with the literature as freshly isolated cerebrovessels from r-mTBI animals (6 months post-injury) secreted significantly higher levels of Ang-1 compared to r-sham animals, while cerebrovascular Ang-2 secretion was unchanged between the r-mTBI and r-sham animals (Figure 3.4). This suggests a persistent dysregulation of the Ang-1/Tie2 signaling axis within the cerebrovasculature in the chronic stages of r-mTBI.

A primary regulator of caveolae-mediated transcytosis in brain endothelia is the lipid transporter Mfsd2a, which inhibits caveolae vesicle formation by modulating the lipid composition of brain endothelial membranes to suppress transcytosis (Andreone et al., 2017) and regulate BBB integrity (Yang et al., 2017). As a result, transcytosis via caveolin-1 is inversely related to Mfsd2a expression in the BBB. Mfsd2a expression in brain endothelia has been shown to be directly regulated by cerebrovascular mural cells (Ben-Zvi et al., 2014). Dysfunctional endothelial transcytosis after injury appears to be primarily driven by altered pericyte crosstalk with endothelial cells (Sun et al., 2021). To investigate whether Ang-1 can modulate Mfsd2a in endothelial cells, I treated HBMECs with recombinant Ang-1 and found an increase in Mfsd2a expression. This signaling pathway has been recently validated by several studies (Mäe et al., 2021; Y. Wang et al., 2019) and implicates a potential link between mural cell dysfunction and endothelial transcytosis, through the modulation of the Mfsd2a/caveolin-1 pathway. To investigate whether endothelial Mfsd2a levels are impacted by r-mTBI, I probed the cerebrovascular lysates at 24 hours, 3- and 6-months post injury. At 24 hours post injury, Mfsd2a levels were significantly diminished in r-mTBI, corresponding with a significant elevation in caveolin-1 compared to r-sham animals during this time frame. Along these lines, prior reporting showed Mfsd2a levels were significantly decreased in the acute stage after surgical brain injury (Eser Ocak, Ocak, Sherchan, Gamdzyk, et al., 2020) and, interestingly, Mfsd2a upregulation was found to reverse BBB disruption by diminishing caveolin-1 levels, and altering caveolae-based transport, providing neuroprotection following injury-induced sub-arachnoid hemorrhage (C. Zhao et al., 2020). In terms of the chronic phase post-injury, there is a lack of evidence regarding the response of cerebrovascular Mfsd2a to head trauma. That said, while Mfsd2a levels are generally decreased in acute conditions such as intracerebral hemorrhage, brain Mfsd2a has been shown to be largely upregulated in more chronic disorders such as chronic liver injury and inflammatory bowel disease (Eser Ocak, Ocak, Sherchan, Zhang, et al., 2020). In line with these studies, I observed a progressive increase in cerebrovascular Mfsd2a levels in the chronic phase post-injury (6 months following r-mTBI), corresponding with elevated levels of Ang-1 and significantly diminished caveolin-1, though more work is necessary to understand the consequence of altered Mfsd2a expression in the brain following head trauma, especially in the more chronic stages post-injury. Altogether, alterations in the secretion of Ang-1 (from brain vascular mural cells) following brain injury could influence endothelial transcytosis by modulating the Mfsd2a/caveolin-1 pathway (Figure 3.5).

The observation that the Ang-1/Tie2 signaling pathway within the cerebrovasculature was altered at a chronic period post injury is consistent with the altered PDGFR β /PDGF-BB signaling pathway reported in Chapter 2. The sustained disruption to both the PDGFR β /PDGF-BB, and Ang-1/Mfsd2a/cav-1 pathways, support a new, pathogenic cerebrovascular microenvironment that persists and potentially contributes

to the development of neurodegenerative disease. The emergence of diminished caveolin-1 expression after r-mTBI coincided with the onset of mural cell dysfunction and reduced cerebrovascular tau uptake observed in Chapter 2. These studies indicate tau elimination from the brain is diminished following r-mTBI, which may be the result of decreased caveolin-1-mediated tau transit across the BBB. The current studies suggest the changes in caveolin-1 post-injury may be due to alterations in Mfsd2a expression in the BBB in response to increased Ang-1 secretion from dysfunctional mural cells in the aftermath of brain injury. Therapeutic modulation of the Ang-1/Tie2 signaling pathway has been extensively investigated in various preclinical studies and clinical studies (Saharinen et al., 2017), suggesting this may be a viable therapeutic option to restore vascular health after head trauma. Though this pathway was initially studied with respect to oncology, recent preclinical reports have pharmacologically modulated Ang-1/Tie2 to treat aberrant vascular pathology and have indicated beneficial effects on vascular stabilization (Saharinen et al., 2017). Future studies will fully characterize the Ang-1/Tie2/Mfsd2a signaling pathway and evaluate whether this therapeutic strategy can diminish trauma-induced vascular dysfunction. Furthermore, limited sample availability prevented the examination of Mfsd2a levels and Ang-1 secretion at 12 months post injury, though it is likely that the observed elevations at 6 months post injury may persist, as caveolin-1 was still diminished at 12 months post injury. Taken together, aberrant mural cell function following r-mTBI diminishes caveolae-mediated tau elimination across the BBB, which may describe the chronic accumulation of tau deposits typically observed in the brain following head trauma.

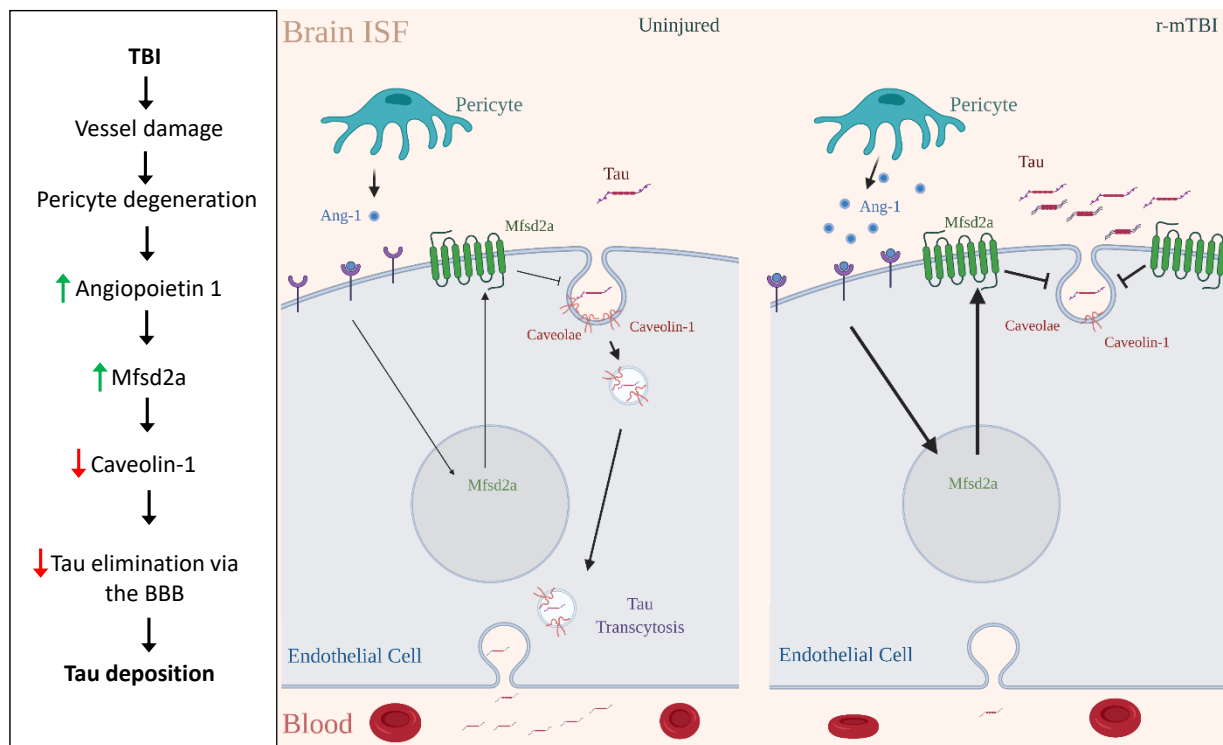


Figure 3.5 Proposed signaling cascade in brain endothelia following r-mTBI. In healthy aging, mural cells constitutively secrete Ang-1 which binds to endothelial Tie2 receptors and leads to the expression of Mfsd2a, which regulates the extent of caveolae-mediated transcytosis at the BBB. In the chronic phase following r-mTBI, Ang-1 secretion from reactive pericytes is elevated, which results in increased expression of Mfsd2a and reduced caveolae-mediated transcytosis across the BBB. The diminished caveolae activity and tau elimination at the BBB could lead to an accumulation of tau in the brain, which is a key pathological signature in the chronic phase following trauma to the brain.

As my investigation of cerebrovascular tau elimination following head trauma focused on mural cells (Chapter 2) and endothelial cells (Chapter 3), Chapter 4 will investigate the role of astrocytes in extracellular tau processing. The observations in this chapter suggest that after r-mTBI, tau may persist in the perivascular space, potentially enabling the uptake by perivascular astrocytes, though very little is known regarding how astrocytes internalize and process extracellular tau, particularly with respect to brain trauma. This, as well as other potential mechanistic interactions between tau and astrocytes in the context of r-mTBI, including ApoE isoforms, will be discussed in Chapter 4.

CHAPTER 4: ROLE OF ASTROCYTES AND APOE IN EXTRACELLULAR TAU ELIMINATION AFTER TBI

4.1 INTRODUCTION

In Chapters 2 and 3, I demonstrated that both mural and endothelial cells play a role in the elimination of extracellular tau, and these processes are impaired after r-mTBI. A third group of cells that comprise the BBB and could potentially influence the elimination of extracellular tau are astrocytes. As discussed in Chapter 1, CTE can be distinguished from other tauopathies by accumulation of perivascular astrocytic tau pathology in the sulci (Arena, Johnson, et al., 2020). Despite the prevalence of astrocytic tau pathology in some primary tauopathies and particularly after r-mTBI, the mechanisms by which perivascular astrocytes internalize, process, and eliminate extracellular tau species are not well understood. Furthermore, astrocytes are the predominant source of APOE in the CNS, which can exert isoform dependent influences on the cerebrovasculature (Main et al., 2018) and tau accumulation (Vasilevskaya et al., 2020) after TBI.

In the absence of injury, ApoE knockout mice appear normal (Fagan et al., 1998), but in response to injury, ApoE plays a critical role in the recovery of the BBB (Main et al., 2018). ApoE demonstrates a biphasic response to TBI, with an acute decrease after 24 hours, which resolves to elevated levels after 7 days (Main 2018) though this ApoE protein level restoration is attenuated with the ApoE4 isoform. The physiological upregulation of ApoE after injury likely facilitates membrane repair through the redistribution of lipids and cholesterol, though this recovery is isoform dependent (Slezak & Pfrieder, 2003). The expression of the ApoE4 isoform has been associated with higher levels of tau in the brain compared with ApoE2 or ApoE3, particularly under pathogenic conditions like AD and TBI, though the mechanism underlying this accumulation is unclear (Y. Shi et al., 2017). ApoE4 expression has been linked with dysfunctional endothelial cell regulation and basement membrane component secretion, which may be exacerbated and lead to BBB disruption under conditions of aging or r-mTBI (Yamazaki et al., 2020).

The results from Chapter 3 indicate that extracellular tau efflux at the BBB may be diminished after r-mTBI. Reduced BBB transit could facilitate local uptake of extracellular tau, potentially by astrocytes, whose end-feet encompass the perivascular space and form part of the BBB. As discussed in Chapter 1, the water channel aquaporin-4 (AQP4), polarized to the end-feet of astrocytes, may facilitate fluid movement and tau elimination via the glymphatic system, which becomes impaired after TBI (Iliff et al., 2014). However, a recent transcriptional network analysis of human astrocyte end-feet revealed an association between dementia status, localized tau pathology and dysfunctional elements of astrocyte perivascular end-feet other than AQP4 (Simon et al., 2018). The authors suggested that perivascular astrocyte end-feet play a previously unrecognized role in the dynamics of tau pathological progression, potentially through the uptake and intercellular spread of extracellular tau (Simon et al., 2018). The mechanisms by which astrocytes interact with tau are unclear, as HSPGs do not appear to mediate monomeric tau uptake in astrocytes (Perea et al., 2019), contrary to what has been observed in other cell types.

A potential mediator in the interaction between astrocytes and tau is the ApoE receptor LRP1, recently demonstrated to be a “master regulator of tau uptake” (Rauch et al., 2020). Astrocytes have been

observed to internalize other neurodegenerative disease associated ligands via LRP1, including ApoE and A β (Wong, 2020), indicating a potential mechanism for tau uptake. LRP1 is highly expressed in astrocytes, and typically internalizes ligands to the lysosome for degradation. Astrocyte derived ApoE has also demonstrated isoform specific interactions with both tau and LRP1, as discussed in Chapter 1 (Cooper et al., 2021). Astrocyte expression of ApoE may influence physiological and stimulated autophagy in an isoform-dependent manner, as ApoE4 expressing astrocytes were observed to demonstrate diminished autophagy and elevated accumulation of extracellular A β , relative to ApoE3 expressing astrocytes (Simonovitch et al., 2016). Additionally, disrupted autophagic flux has been observed in human TBI autopsy samples and in rodent models of TBI, though the impact on autophagy can vary depending on factors including injury severity and cell type (Cooper et al., 2021; J. Wu & Lipinski, 2019).

Though most of the ligands internalized by LRP1 are trafficked to the lysosome, it appears that certain species of tau in some cell types can avoid this fate at some point in the intracellular trafficking pathway (Cooper et al., 2021). The LRP1 mediated endosomal-lysosomal pathway is primarily responsible for the uptake, processing, and elimination of molecules (Wong, 2020), either through degradation or secretion. This system is commonly classified into early endosomes, late endosomes, recycling endosomes and lysosomes. Ligands internalized via LRP1-mediated endocytosis are contained within early endosomes, which rely on EEA1 and Rab5 for vesicle fusion and sorting. These vesicles exchange effector proteins like Rab5 within the endosomal membrane for proteins such as Rab7 when they mature into late endosomes (Winckler et al., 2018). From this point, they either fuse with Lamp1 containing lysosomes for degradation, or exchange Rab7 for Rab11 to transition into recycling endosomes as they return to the plasma membrane.

ApoE isoform expression can influence intracellular LRP1 trafficking through the endo-lysosomal pathway, and this effect appears to be independent of the role that ApoE isoforms play in the extracellular environment in binding to LRP1 on the cell surface (Cooper et al., 2021). Evidence suggests that astrocytes expressing ApoE4 have diminished early endosomal activity, without impacting later endosomal trafficking (Narayan et al., 2020), suggesting a toxic gain of function. It is possible that the ApoE isoforms may differentially regulate the ability of astrocytes to internalize and eliminate extracellular tau at the BBB both physiologically and after r-mTBI, potentially resulting in elevated levels of cerebrovascular tau, leading to the perivascular tau pathology seen following TBI. The studies in Chapter 4 will investigate the contribution of brain astrocytes to extracellular tau processing in the context of ApoE genotype, as a complement to my work evaluating the influence of vascular mural cells (Chapter 2) and brain endothelia (Chapter 3) on tau elimination from the brain following repetitive head trauma.

I hypothesize that astrocytes and ApoE isoforms are involved in the elimination of extracellular tau, which is impaired after r-mTBI. The following sub-hypothesis will be interrogated:

- ApoE4 impairs the elimination of tau after head trauma, relative to other ApoE isoforms
- ApoE4 and head trauma impair the tau degradative mechanisms in astrocytes resulting in impaired tau clearance.
- ApoE4 impairs the degradation of tau in astrocytes through the modulation of sub-cellular trafficking routes.

4.2 METHODS

4.2.1 Materials

C8D1A immortalized astrocytes (cat#CRL-2541) and associated culture reagents were purchased from the American Type Culture Collection (Manassas, VA, USA). Immunohistochemical studies were carried out with the following antibodies: monoclonal rabbit anti-mouse LRP1 antibody (cat#ab92544, Abcam, USA), monoclonal mouse anti-mouse EEA1 antibody (cat#sc-365652, Santa Cruz Biotechnology, USA), monoclonal mouse anti-mouse Rab7 antibody (cat#ab50533, Abcam, USA), monoclonal mouse anti-mouse Rab11A antibody (cat#sc-166912, Santa Cruz Biotechnology, USA), monoclonal mouse anti-mouse Lamp1 antibody (cat#MA1-164, ThermoFisher, USA), Donkey anti-Rabbit IgG Alexa Fluor 647 antibody (cat#A-31573, ThermoFisher, USA) and Donkey anti-Mouse IgG Alexa Fluor 568 antibody (cat#A100037, ThermoFisher, USA). Recombinant Fluorescein Tau-441 (mFtau) (cat#T-1113) was purchased from rPeptide (Watkinsville GA, USA). ATT 488 conjugated P301S tau pre-formed fibrils (aFtau) (cat#SPR-329B-A488) were purchased from StressMarq Biosciences (Victoria, BC, Canada). Lucifer yellow dextran (10 kDa) and the human tau enzyme linked immunosorbent assay (ELISA) (cat#KHB0041) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Mammalian protein extraction reagent (M-PER) (cat#78505), Halt enzyme inhibitor cocktails (cat#78442), and the bicinchoninic acid (BCA) protein assay (cat#23225) were purchased from ThermoFisher Scientific (Waltham, MA, USA). Recombinant biotin-labeled human tau-441 (cat#T08-54BN) was purchased from SignalChem (Richmond, BC, Canada). The proteasome inhibitor MG-132 (cat#ab141003) was purchased from Abcam (Cambridge, MA, USA). Suc-Leu-Leu-Val-Tyr-AMC (cat#10008119) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Fluoroshield mounting media with DAPI (Sigma Aldrich, USA). The adult brain dissociation kit, mouse and rat (cat#130-107-677), was purchased from Miltenyi Biotec (Waltham, MA, USA). The autophagy inhibitor Spautin-1 (cat#567569) and the antibody for LC3-II/I (cat#ABC929) were purchased from Millipore Sigma (St. Louis, MO, USA).

4.2.1 Animals

ApoE-targeted replacement (ApoE-TR) mice were purchased from Taconic Biosciences (Rensselaer, NY). All studies used a mix of male and female mice. The ApoE-TR mice were generated using a targeted replacement of the endogenous murine ApoE gene with human ApoE2, ApoE3 or ApoE4. The mice express, produce, lipidate and secrete human ApoE at physiological levels, (Verghese et al., 2013). EFAD mice were provided by Dr. Mary Jo LaDu (University of Illinois at Chicago). To generate EFAD mice, ApoE3 and ApoE4 mice were crossed with mice expressing 5 familial AD mutations (5XFAD, Tg6799 line), producing the E3FAD and E4FAD mouse models respectively, as previously described (Youmans et al., 2012). Mice were housed as previously described in Chapter 2. All procedures involving mice were performed in accordance with Office of Laboratory Animal Welfare and National Institutes of Health guidelines under a protocol approved by the Roskamp Institute Institutional Animal Care and Use Committee.

4.2.2 Brain injury protocol

Repetitive mild traumatic brain injury (r-mTBI) was administered using a mouse model of closed head injury as previously characterized in Chapter 2. Mice (ApoE2, ApoE3, ApoE4) were euthanized at 6 months after the final brain injury or anesthesia exposure.

4.2.3 Astrocyte isolation

Astrocytes were isolated from mice using the adult brain dissociation kit mouse and rat (Miltenyi Biotec), as per manufacturer instructions. Briefly, mice were perfused with PBS before freshly extracted mouse brains (minus the cerebellum) were collected under sterile conditions and then minced with a blade before enzymatic digestion. Digested cortex tissue was collected and passed through a 40 μ M cell strainer, before the addition Debris Removal solution. After mixing, PBS was gently overlaid, and the sample was centrifuged at 4°C and 3000xg for 10 minutes with full acceleration and full brake. The top two phases were aspirated, then the remaining sample was resuspended in PBS and centrifuged at 4°C and 3000xg for 10 minutes with full acceleration and full brake. Samples were resuspended in red blood cell removal solution for 10 minutes at 4°C, then cell pellets were obtained, resuspended in media and plated in T-25 flasks at 37°C until confluent.

4.2.4 Tau residence in the brain after r-mTBI

Biotin-labeled tau species and 10 kDa lucifer yellow dextran (LyD) were stereotactically injected into 12 month old r-sham and r-mTBI ApoE2, ApoE3 and ApoE4 mice (6 months post-injury) as well as E3FAD and E4FAD mice (12 months of age), in the same manner as described in Chapter 2, and euthanatized 2 hours after the intracranial injection. The brain homogenate was evaluated for biotin-labeled tau using a modified hTau ELISA (Invitrogen). The ELISA was performed as described in Chapter 2. Furthermore, each stereotaxic injection included LyD (80 mg/ml) to account for any nonspecific leakage out of the brain, as LyD typically demonstrates low BBB permeability (Natarajan et al., 2017). LyD fluorescence was analyzed using a microplate spectrofluorometer (Biotek Cytation 3). With respect to tau, all samples were evaluated for exogenous biotin-labeled tau remaining in the brain and normalized to LyD for each time point. To assess LyD alone, the amount of LyD at each time point was normalized to total protein content as determined using the BCA assay.

4.2.5 Proteasome activity assay

Primary astrocytes isolated from r-mTBI and r-sham ApoE2, ApoE3 and ApoE4 mice were seeded in a black clear bottom 96 well plate pretreated with poly-L-lysine in 100 μ L of culture medium at 10,000 cells per well and grown in Astrocyte Media for 48 hours until confluence. Cells were then treated with various doses of MG-132 (10 μ M, 1 μ M, 0.1 μ M) or vehicle in media for 24 hours. The media was aspirated, and cells were washed twice with ice cold PBS. Afterwards, 90 μ L of lysis buffer was added to each well, and cells were incubated for 30 minutes until lysis occurred. To evaluate 20S proteasome activity, 10 μ L of 1 mM Suc-LLVY-AMC, which fluoresces in response to 20S proteasome activity, was added to each well. The samples were analyzed for fluorescence (λ_{ex} = 380 nm, λ_{em} = 460 nm) every 5 minutes for 1 hour.

4.2.6 Astrocyte tau processing

Tau degradation was evaluated in primary astrocytes isolated from r-mTBI and r-sham ApoE2, ApoE3 and ApoE4 mice 6 months post injury, in a similar manner to what was described in Chapter 2. The experiments utilized 4R tau, not 3R tau, as astrocyte pathology after TBI is predominantly 4R (Cherry et al., 2020). Primary astrocytes were cultured in starvation media for 24 hours before exposure to 50 ng/ml of monomeric or aggregate enriched biotin-labeled tau for 24 hours at 37°C. Following the treatment period, the extracellular media was removed, and the astrocytes were washed with ice-cold PBS. Cell lysates were collected using lysis buffer and Western blotting analysis was used for the analysis of autophagic activity. Cell lysates were denatured at 95°C by boiling in Laemmli buffer (Bio-Rad). Samples were subsequently resolved on 4% to 12% gradient NuPage Novex Bis-Tris precast polyacrylamide gel. After electrotransferring, nitrocellulose membranes were blocked in 5% milk and then probed with primary antibodies (1:1000) overnight. After washing, HRP-conjugated secondaries were applied, and signal intensity ratios were quantified by chemiluminescence imaging with the ChemiDoc TM XRS as previously described (Ojo 2016). Autophagic flux was measured using an antibody for LC3 II/I and normalized to total protein using GAPDH. Biotin-labeled tau accumulation was analyzed for total tau content in cellular lysates by a modified biotin-labeled tau ELISA. The ELISA was performed according to the manufacturer's protocol, using stock biotin-labeled tau as the standard and excluding the 1-hour incubation step with a biotin-conjugated primary antibody. The samples were normalized to total protein content using the BCA assay.

4.2.7 Astrocyte clearance of tau

Immortalized C8-D1A astrocytes were seeded onto 24-well plates and grown to confluence in DMEM with 10% FBS, 1% penicillin/streptomycin. For tau clearance studies, cells were allowed to internalize monomeric or aggregate enriched biotin-labeled tau (50 ng/ml) for 1 hour at 37°C, in a similar manner as previously reported using A β (Liu 2017). Cultures were washed, then lysed and analyzed for tau using a modified biotin-labeled tau ELISA. Parallel cultures of astrocytes were washed, then incubated for an additional 4 hours in medium lacking tau, before being washed, lysed, and analyzed for tau content using ELISA and normalized to total protein using BCA. Clearance was measured as the percentage of tau remaining in the cells after 4 hours without tau exposure, compared to the initial 1 hour loading of tau.

4.2.8 Astrocyte clearance of tau with degradation inhibition

The influence of degradation on tau clearance was evaluated in a similar manner as described above, but with the presence or absence of proteasome and autophagic inhibition. Cells were loaded with 50 ng/ml of either monomeric or aggregated enriched biotin-labeled tau for 1 hour at 37°C in the presence or absence of the proteasome inhibitor MG-132 (10 μ M) and/or the autophagy inhibitor Spautin-1 (10 μ M). After 1 hour, the tau-laden media was removed, and cells were washed and then incubated for an additional 4 hours in fresh medium with or without degradation inhibitors, but lacking tau. Cells were then washed, lysed and analyzed for tau content using a modified biotin-labeled tau ELISA and normalized to total protein using BCA.

4.2.9 ApoE dependent colocalization of tau and LRP1 with endosomal and lysosomal compartments

Primary astrocytes (ApoE2, ApoE3 and ApoE4) were isolated from male mice (12 months of age) and grown to confluence as described. They were then seeded onto 8-well chamber slides and grown in DMEM with 10% FBS, 1% penicillin/streptomycin. When approximately 60% confluent cells were treated with 100 nM of either monomeric recombinant tau conjugated to FITC (mFtau) or briefly sonicated aggregated P301S tau conjugated to ATTO 488 (aFtau) for 30 minutes at 37°C. Following incubation, cells were washed with cold PBS and fixed in 4% PFA (2 hours) before permeabilization and blocking (10% normal donkey serum (NDS) in 0.2% Triton X-100) for 45 minutes. Afterwards, cells were incubated with primary antibodies in 2% NDS and 0.01% Triton X-100 overnight at 4°C. The primary antibody combinations used were LRP1 1:100, in addition to either EEA1 1:50, Rab7 1:100, Rab11 1:50, Lamp1 1:50, or ApoE 1:100. Cells were washed the times with PBS and incubated with the secondary antibodies in 2% NDS and 0.01% Triton X-100 (anti-rabbit 647 1:500, anti-mouse 568 1:500) for 1 hour at room temperature in the dark. All slides were washed three times with PBS before mounting with Fluoroshield mounting media with DAPI. All images were acquired using the confocal microscope LSM 800 (Carl Zeiss AG, German), the Zen Blue 2.3 (Carl Zeiss AG, Germany) software and a 63x objective. The acquisition settings were kept the same for all genotypes within the same experiment. For imaging and colocalization analysis, between 8-10 randomly selected images were used from each biological replicate. Colocalization coefficients were assessed using Zen blue edition 2.1 software (2011) (Zeiss, Germany), using thresholds set via Costes' automatic threshold (Costes et al., 2004).

4.2.10 Statistical analysis

Randomization and blinding procedures were employed. Quantitative data were plotted as mean \pm standard deviation. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc). The Shapiro-Wilk test was completed to assess normality. Statistical analysis was evaluated for significance as determined by one-way or two-way ANOVA and the Bonferroni's post hoc test with multiple comparisons as indicated in the figure legends. For all analyses, a p value of ≤ 0.05 was considered statistically significant.

4.3. RESULTS

4.3.1 Effect of ApoE, TBI and AD on tau residence time in the brain

To examine whether the expression of ApoE isoforms impacted the elimination of exogenous tau species after r-mTBI, the elimination of biotin-labeled tau was evaluated after intracortical injection of biotin-labeled tau (2 hours). Monomeric biotin-labeled tau residence was increased by 60% in the ApoE2 r-mTBI mice, compared to ApoE2 r-sham (Figure 4.1A). Alternatively, monomeric biotin-labeled tau residence decreased 2-fold in the ApoE4 r-mTBI mice, compared to ApoE4 r-sham (Figure 4.1A). Aggregate enriched tau residence in r-sham ApoE4 mice was 60% higher than r-sham ApoE3, and 2-fold higher than r-sham mice (Figure 4.1B). However, after r-mTBI, aggregate enriched tau residence diminished by 60% in ApoE4 mice, compared to ApoE4 r-sham (Figure 4.1B). In ApoE4 r-mTBI mice, dextran residence was diminished by 40% relative to ApoE4 r-sham, with no differences in dextran residence observed in other ApoE mice, regardless of TBI status (Figure 4.1C). A similar trend was observed in E4FAD mice compared to age matched naive ApoE4 mice, where monomeric biotin-labeled tau residence diminished by 55% in E4FAD

compared to ApoE4 (Figure 4.1D). Dextran residence also diminished by 40% in E4FAD compared to ApoE4 mice (Figure 4.1E). These trends were not observed when comparing E3FAD to ApoE3 mice (Figure 4.1D, E).

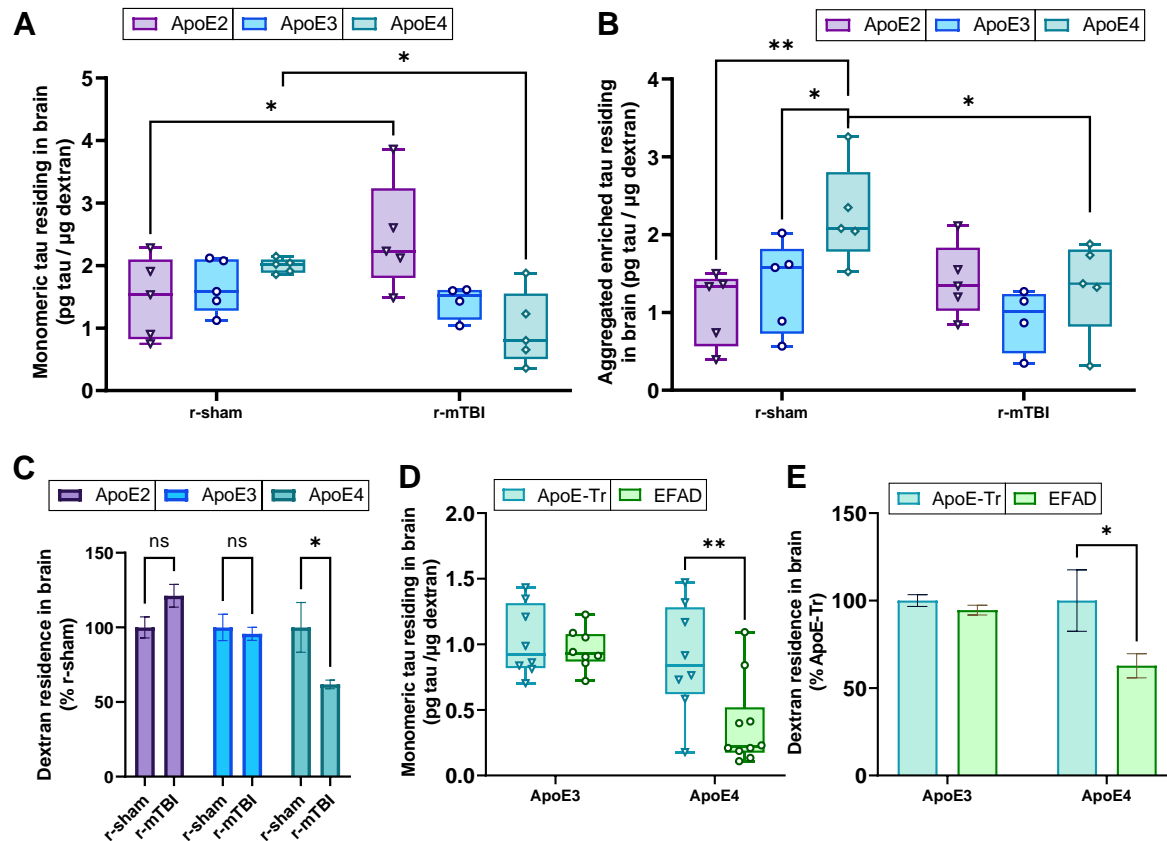


Figure 4.1 Effect of r-mTBI and ApoE isoform on the elimination of exogenous tau species from the brain. (A-C) Following intracortical injection in ApoE2, ApoE3 and ApoE4 r-sham (n = 5) and r-mTBI mice (n = 4-5) (6 months post-injury), the amount of exogenous biotin-labeled tau species residing in the brain was determined at 2 hours post-injection. For each injection, biotin-labeled tau was co-injected with LyD. Biotin-labeled tau content was analyzed using an ELISA while LyD was analyzed via fluorescence and normalized to total protein used the BCA protein assay. Values represent mean \pm SD (n = 4-5) and are expressed as pg of tau per μ g of LyD or (C) the percentage dextran residence normalized to each respective r-sham. *P < 0.05, **P < 0.01 as compared to r-sham as determined by a two-way ANOVA and Bonferroni post-hoc test. (D-E) Following intracortical injection in ApoE3 and ApoE4 Tr (n = 8) and FAD mice (n = 8) (12 months of age), the amount of exogenous biotin-labeled tau species residing in the brain was determined at 2 hours post-injection. For each injection, biotin-labeled tau was co-injected with LyD. Biotin-labeled tau content was analyzed using an ELISA while LyD was analyzed via fluorescence and normalized to total protein used the BCA protein assay. Values represent mean \pm SD (n = 8) and are expressed as (D) pg of tau per μ g of LyD or (E) the percentage dextran residence normalized to each respective ApoE-Tr. *P < 0.05, **P < 0.01 as compared to ApoE-Tr as determined by a two-way ANOVA and Bonferroni post-hoc test.

4.3.2 ApoE4 expression disrupts astrocyte tau processing after r-mTBI

To examine whether the expression of ApoE isoforms impacted how astrocytes process extracellular tau after r-mTBI, isolated astrocytes from ApoE2, ApoE3 and ApoE4 r-mTBI and r-sham mice (6 months post injury) were exposed to monomeric or aggregate enriched biotin-labeled tau for 24 hours. After exposure to r-mTBI, only ApoE4 demonstrated a difference in tau accumulation, with a 2-fold increase in monomeric tau accumulation compared to ApoE4 r-sham (Figure 4.2A), and a 2.3-fold increase in aggregate enriched

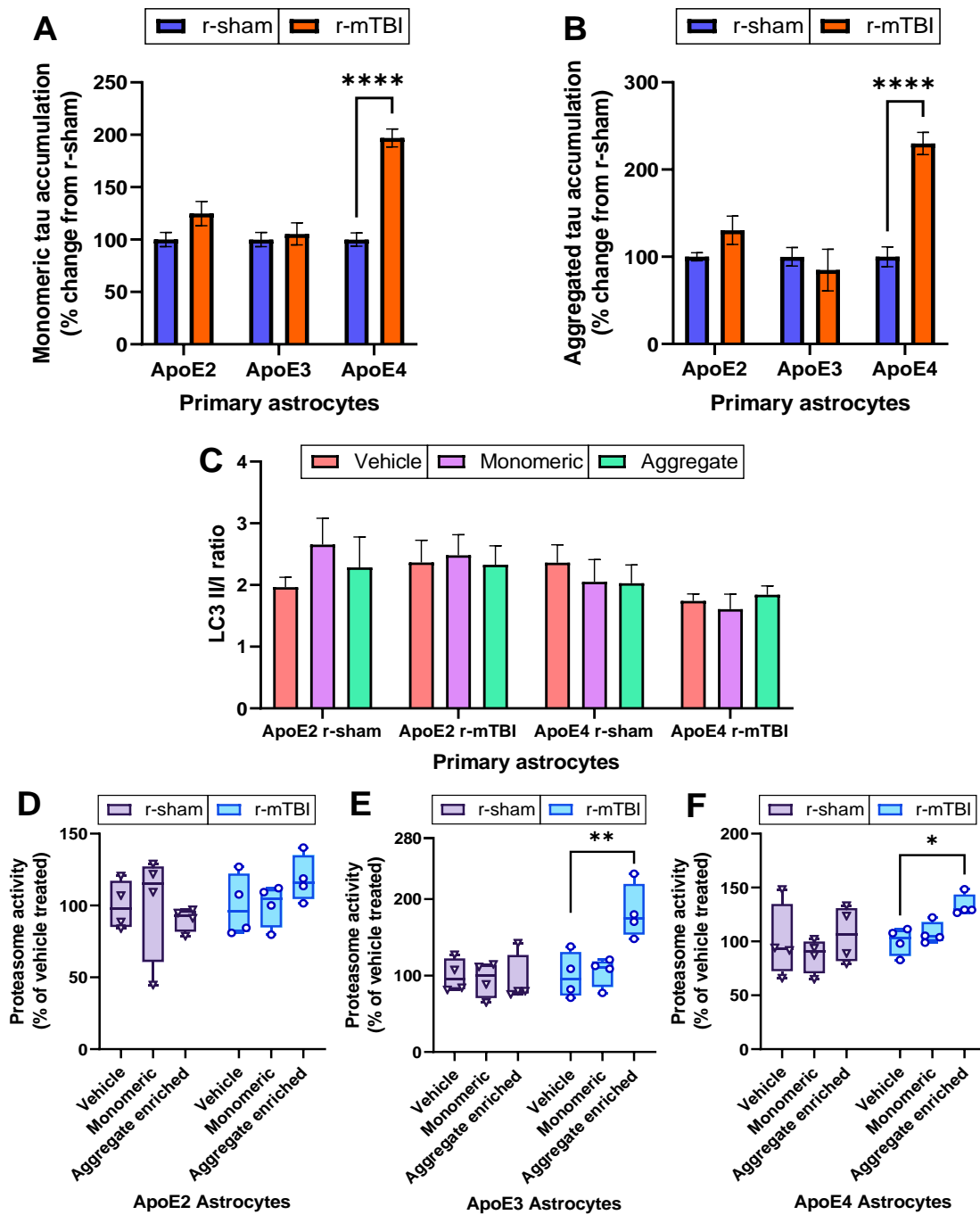


Figure 4.2 Influence of ApoE and r-mTBI on tau accumulation in primary astrocytes. (A, B) Isolated astrocytes from ApoE2, ApoE3 and ApoE4 r-sham and r-mTBI mice (6 months post injury) were grown to confluency and exposed to biotin-labeled (A) monomeric or (B) aggregate enriched tau (50 ng/ml) for 24 hours at 37°C. Accumulation of biotin-labeled tau was analyzed in astrocyte lysates using an ELISA and normalized to total protein using the BCA protein assay. Values represent mean \pm SD and are expressed as the percentage of tau accumulation normalized to each respective r-sham. **** P < 0.0001 as compared to r-sham as determined by a two-way ANOVA and Bonferroni post-hoc test. (C) Lysates from astrocytes exposed to monomeric or aggregate enriched biotin-labeled tau or a vehicle for 24 hours at 37°C were analyzed by western blot for levels of LC3II/I. Autophagic activity values represent mean \pm SD and are expressed as the ratio of LC3II/I (n = 4). (D-F) Astrocyte lysates from (C) were measured for proteasome activity using Suc-LLVY-AMC and normalized to total protein using the BCA assay. Proteasome activity values represent mean \pm SD and are expressed as a percentage of each respective vehicle (n = 4). * P < 0.05, ** P < 0.01 compared to each respective vehicle as determined by two-way ANOVA and Bonferroni's multiple comparisons test.

tau accumulation in the ApoE4 r-mTBI compared to ApoE4 r-sham (Figure 4.2B). There was no significant TBI effect in tau accumulation observed in the ApoE2 or ApoE3 astrocytes for either of the tau species.

4.3.3 Impact of ApoE and r-mTBI on astrocyte degradation processes

To investigate whether the disrupted tau processing in ApoE4 r-mTBI astrocytes was due to dysregulated degradation processes, autophagic flux was quantified in isolated r-mTBI and r-sham ApoE2 and ApoE4 astrocytes exposed to either a vehicle or different species of tau over 24 hours. There was no significant ApoE or r-mTBI effect change observed in autophagic activity in isolated astrocytes (Figure 4.2C). Additionally, tau exposure did not significantly impact autophagic flux. Proteasome activity was evaluated in r-mTBI and r-sham ApoE2, ApoE3 and ApoE4 primary astrocytes exposed to either a vehicle or different species of tau over 24 hours. Astrocytes isolated from ApoE3 and ApoE4 mice exposed to r-mTBI, but not sham, demonstrated an 80% and 35% increase in activity, respectively, when exposed to aggregate enriched tau compared to a vehicle (Figure 4.2E-F). Other isolated astrocytes did not demonstrate a change in proteasome activity in response to tau treatment (Figure 4.2D-F).

4.3.4 Mechanisms and efficiency of astrocyte elimination of internalized tau

To investigate the efficiency of astrocyte elimination of extracellular tau, immortalized astrocytes were exposed to either monomeric or aggregate enriched tau for 1 hour, followed by the removal the extracellular media, which was exchanged for fresh media that did not contain any tau. The amount of intracellular tau residing within the astrocyte lysates after 4 hours was compared to a parallel group of astrocytes that were lysed immediately following the 1-hour tau exposure. Astrocytes retained less than 2% of their initial monomeric tau uptake after 4 hours, compared to 15% of their initial aggregate enriched

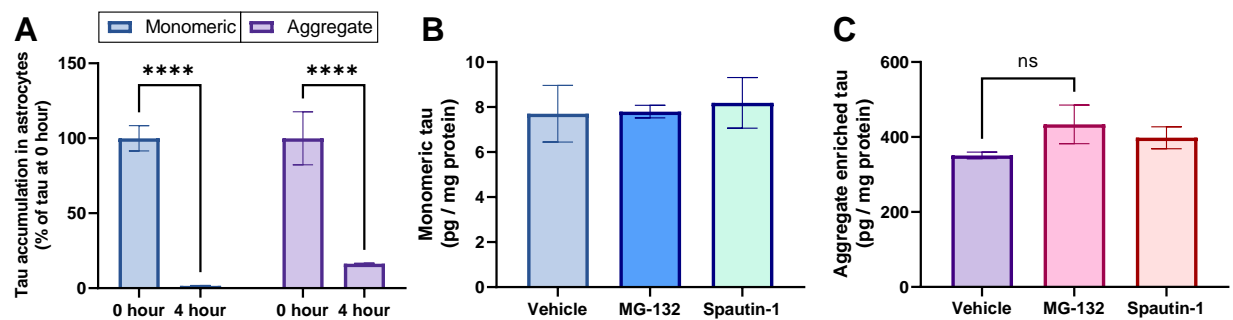


Figure 4.3 Tau clearance from immortalized astrocytes. (A) Immortalized mouse C8D1-A astrocytes were allowed to internalize monomeric or aggregate enriched biotin-labeled tau (50 ng/ml) for 1 hour at 37°C. Parallel cultures were washed and incubated an additional 4 hours in media lacking biotin-labeled tau at 37°C. Lysates were analyzed for biotin-labeled tau by ELISA and normalized to total protein using the BCA protein assay. Values represent mean + SD and are expressed as a percentage of the tau accumulated in astrocytes prior to media change (0 hour). ****P < 0.0001 compared to each respective 0 hour accumulated tau as determined by two-way ANOVA and Bonferroni's multiple comparisons test. (B, C) Immortalized mouse C8D1-A astrocytes were allowed to internalize (B) monomeric or (C) aggregate enriched biotin-labeled tau (50 ng/ml) for 1 hour at 37°C in the presence or absence of a MG-132 (10 µM), Spautin-1 (10 µM), or a vehicle. Cells were washed and incubated an additional 4 hours at 37°C in media lacking tau but containing the respective degradation inhibitors or vehicle. Lysates were analyzed for biotin-labeled tau by ELISA and normalized to total protein content using the BCA protein assay. Values represent mean + SD and are expressed as pg tau per mg protein. *P < 0.05 compared to each respective vehicle as determined by ANOVA and Bonferroni's multiple comparisons test.

tau uptake after 4 hours (Figure 4.3A). Moreover, the study was replicated in the presence of proteasome and autophagy inhibitor astrocytes to determine if the decrease in intracellular tau levels over time was due to degradation. The presence of degradation inhibitors had no significant effect on intracellular tau retention after 4 hours, suggesting the changes in intracellular tau were not predominantly due to cellular metabolism, but potentially excretion back into the extracellular space (Figure 4.3B-C).

4.3.5 ApoE isoform impact on endosome-lysosome pathway and tau trafficking

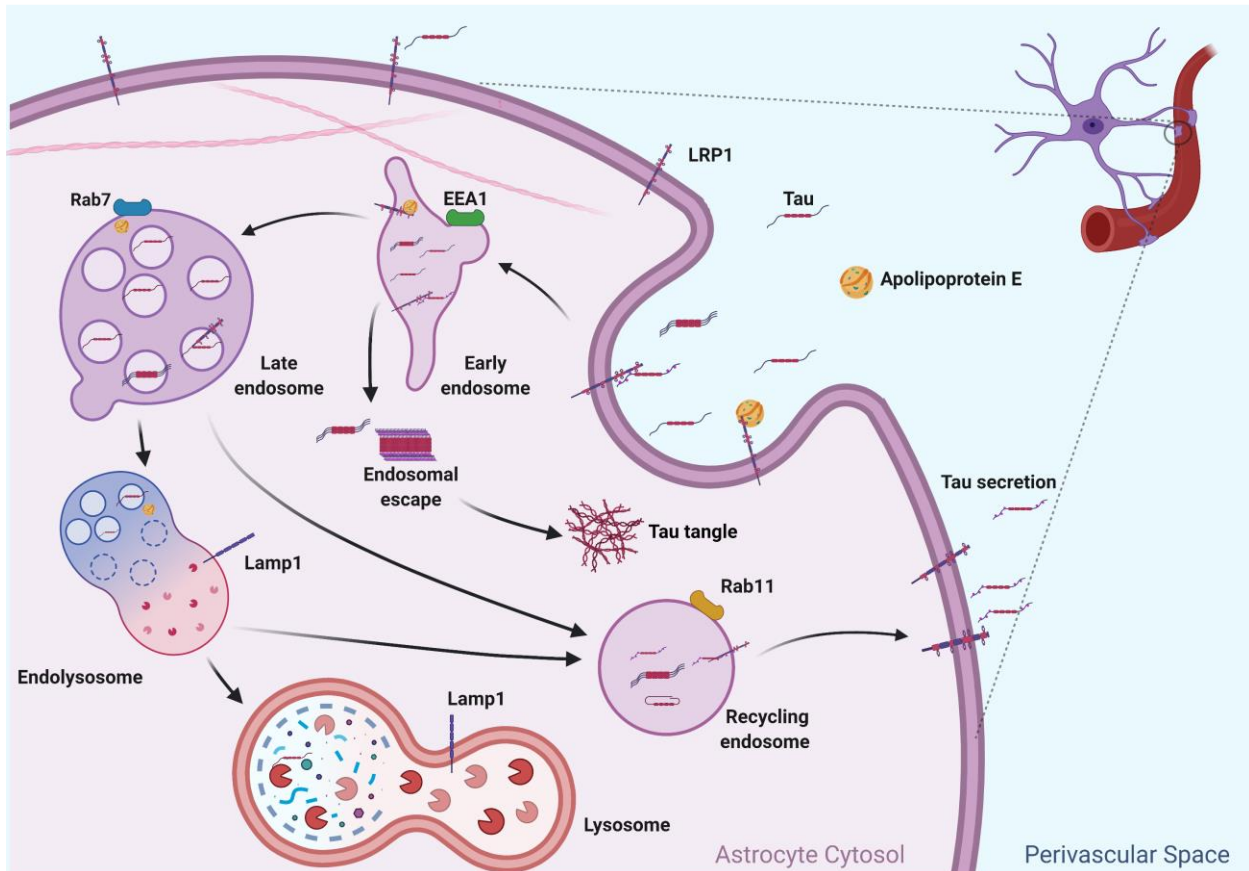


Figure 4.4 Schematic of tau sub-cellular trafficking steps probed in ApoE2, ApoE3 and ApoE4 astrocytes. Astrocytes were isolated from ApoE2, ApoE3 and ApoE4 male mice (12 months of age) and exposed to with 100 nM of either monomeric recombinant tau conjugated to FITC (mFtau) or aggregated tau conjugated to ATTO 488 (aFtau) for 30 minutes at 37°C. Cells were stained with antibodies for the receptor LRP1, the early endosome marker EEA1, the late endosome marker Rab7, the recycling endosome marker Rab11, or the lysosomal marker lamp1, along with a DAPI nuclear stain and imaged using confocal microscopy.

To visualize the effects of ApoE isoforms on tau localization and movement through the endo-lysosomal pathway, astrocytes were isolated from ApoE2, ApoE3 and ApoE4 mice and exposed to fluorescently tagged tau for 30 minutes. Astrocytes were stained with antibodies for the receptor LRP1, indicators of several endocytic compartments including EEA1, Rab7, and Rab11, as well as the lysosomal marker lamp1 (Figure 4.4). Tau colocalization with the cell surface receptor LRP1 was examined in astrocytes, and approximately 30% greater colocalization was seen between monomeric tau and LRP1 in ApoE2 astrocytes compared with ApoE3 and ApoE4 (Figure 4.5A, B), though this difference was not observed for aggregated tau (Figure 4.5C, D). Next, tau colocalization was investigated with the early endosome marker EEA1. For

both monomeric and aggregated tau, ApoE4 astrocytes demonstrate enhanced early endosome colocalization with tau, compared to ApoE3 astrocytes (Figure 4.6). In late endosomes, however, this trend was reversed as ApoE4 astrocytes showed a 30% reduction in Rab7 colocalization with monomeric tau (Figure 4.7A, B), and a 35% reduction in rab7 colocalization with aggregated tau (Figure 4.7C, D), relative to ApoE2 and ApoE3 astrocytes. A similar trend was observed with respect to recycling endosomes, as the colocalization between monomeric tau and Rab11 was diminished by 50% in ApoE4 astrocytes compared to ApoE2 astrocytes (Figure 4.8 A, B). Similarly, the colocalization between Rab11 and aggregated tau was diminished by 30% in ApoE4 compared to ApoE2 astrocytes. (Figure 4.8 C, D) There was no significant difference seen amongst ApoE isoforms when examining tau colocalization with the lysosome marker lamp1 (Figure 4.9).

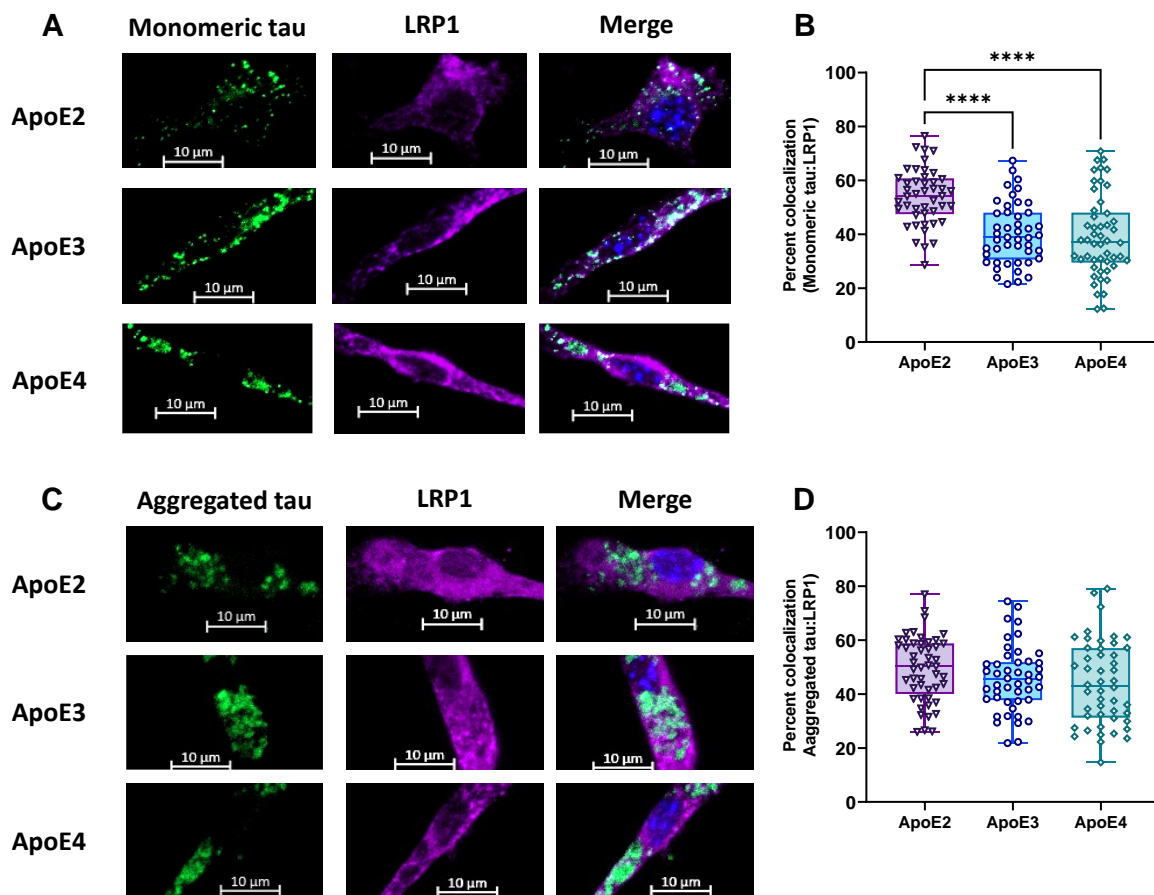


Figure 4.5 The colocalization percentage between tau and LRP1 in ApoE2, ApoE3 and ApoE4 astrocytes. Scale bar, 10 μ m. (A) Representative images of mFtau (green) colocalization with LRP1 (purple). (B) The colocalization of mFtau with respect to LRP1 was quantified and expressed as a percentage. Values represent mean \pm SD and are expressed as a percentage of mFtau colocalized to LRP1 (n = 45-50). (C) Representative images of aFtau (green) colocalization with LRP1 (purple). (D) The colocalization of aFtau with respect to LRP1 was measured as in (B). ****P < 0.0001 as determined by ANOVA and Bonferroni's multiple comparisons test.

4.4 DISCUSSION

In this chapter I investigated the role of astrocytes in extracellular tau elimination after r-mTBI. The accumulation of perivascular astrocytic tau pathology has been suggested to be the distinguishing pathognomonic difference between CTE and AD (Arena, Johnson, et al., 2020), though the mechanisms by which astrocytes interact with and eliminate extracellular tau, particularly after TBI, are unclear. Additionally, several groups have presented conflicting reports on the influence of ApoE isoforms on astrocytic tau pathology (Y. Shi et al., 2017; N. Zhao et al., 2018). Confounding factors from these reports include both the use of mutated tau and the employment of different promoters for tau expression, limiting the extrapolation of such studies into the pathophysiology underlying r-mTBI in the context of ApoE genotype (Y. Shi et al., 2017; N. Zhao et al., 2018). However, as several studies (Y. Shi et al., 2017; N. Zhao et al., 2018) have observed an influence of ApoE genotype on tau pathology, and the various ApoE

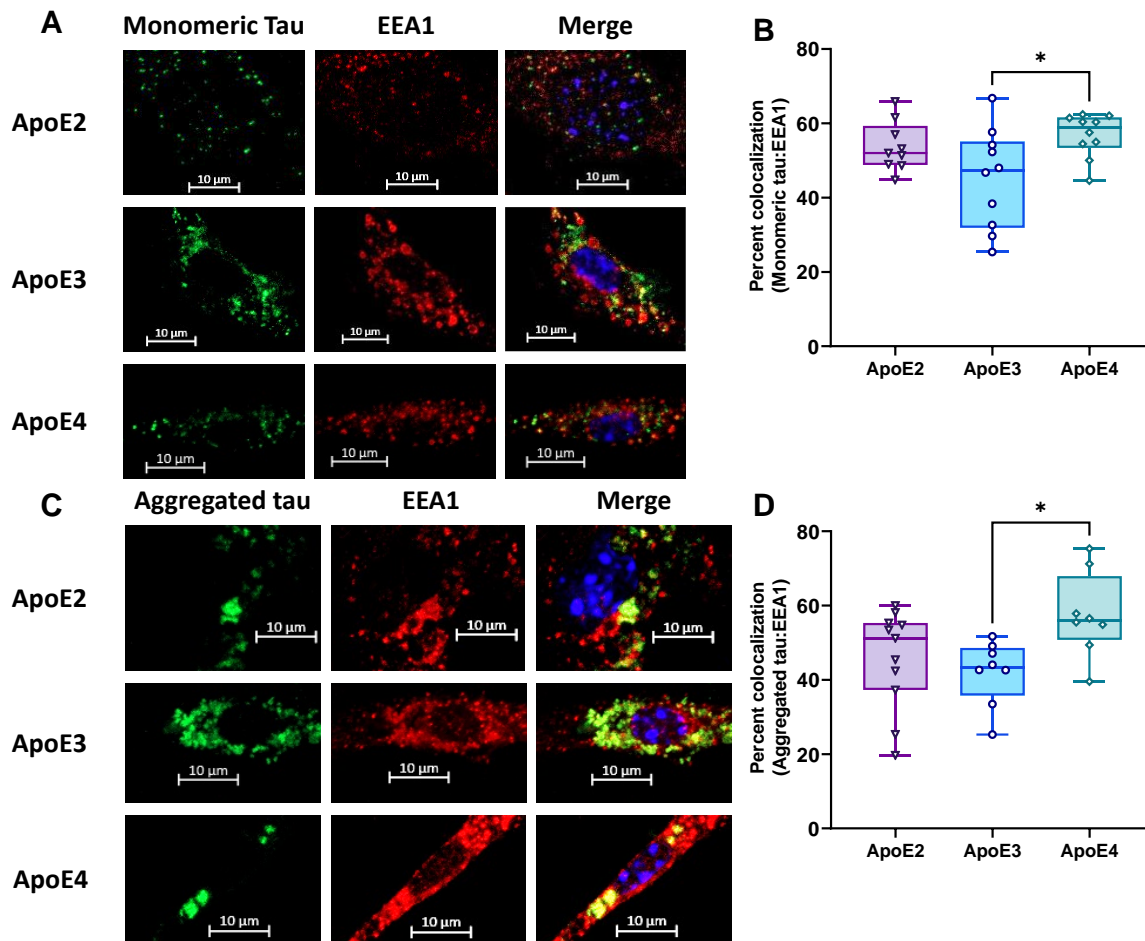


Figure 4.6 The colocalization percentage between tau and the early endosome marker EEA1 in ApoE2, ApoE3 and ApoE4 astrocytes. Scale bar, 10 μ m. (A) Representative images of mTau (green) colocalization with EEA1 (red). (B) The colocalization of mTau with respect to EEA1 was quantified and expressed as a percentage. Values represent mean \pm SD and are expressed as a percentage of mTau colocalized to EEA1 (n = 8-10). (C and D) Representative images and quantification of aTau colocalization with EEA1 was measured as in (A and B). *P < 0.05 as determined by ANOVA and Bonferroni's multiple comparisons test.

isoforms may contribute to chronic r-mTBI pathophysiology and outcome following TBI, we investigated the role of ApoE on tau processing. In Chapter 3, I demonstrated that extracellular tau can be eliminated through the BBB, and this elimination impacted by r-mTBI. However, it is unclear whether ApoE isoform status can influence the movement or processing of extracellular tau, particularly after r-mTBI.

To investigate whether ApoE isoforms affect the elimination of extracellular tau, different species of biotin-labeled tau were injected into age matched r-sham ApoE2, ApoE3 and ApoE4 mice, and tau residence in the brain was evaluated in a similar fashion to the method used in Chapter 3. I observed that ApoE influenced the physiological elimination of extracellular tau in an isoform-dependent manner (ApoE2>ApoE3>ApoE4), with a particularly pronounced difference seen with aggregate-enriched tau (Figure 4.1). This is consistent with what has been observed for extracellular A β , where the presence of ApoE4 lead to diminished elimination of A β from the brain compared to ApoE2 (Verghese et al., 2013).

Under physiological conditions, the levels of extracellular tau may be at a manageable level, where the brain can efficiently process and eliminate tau. To investigate the effect of r-mTBI on extracellular tau

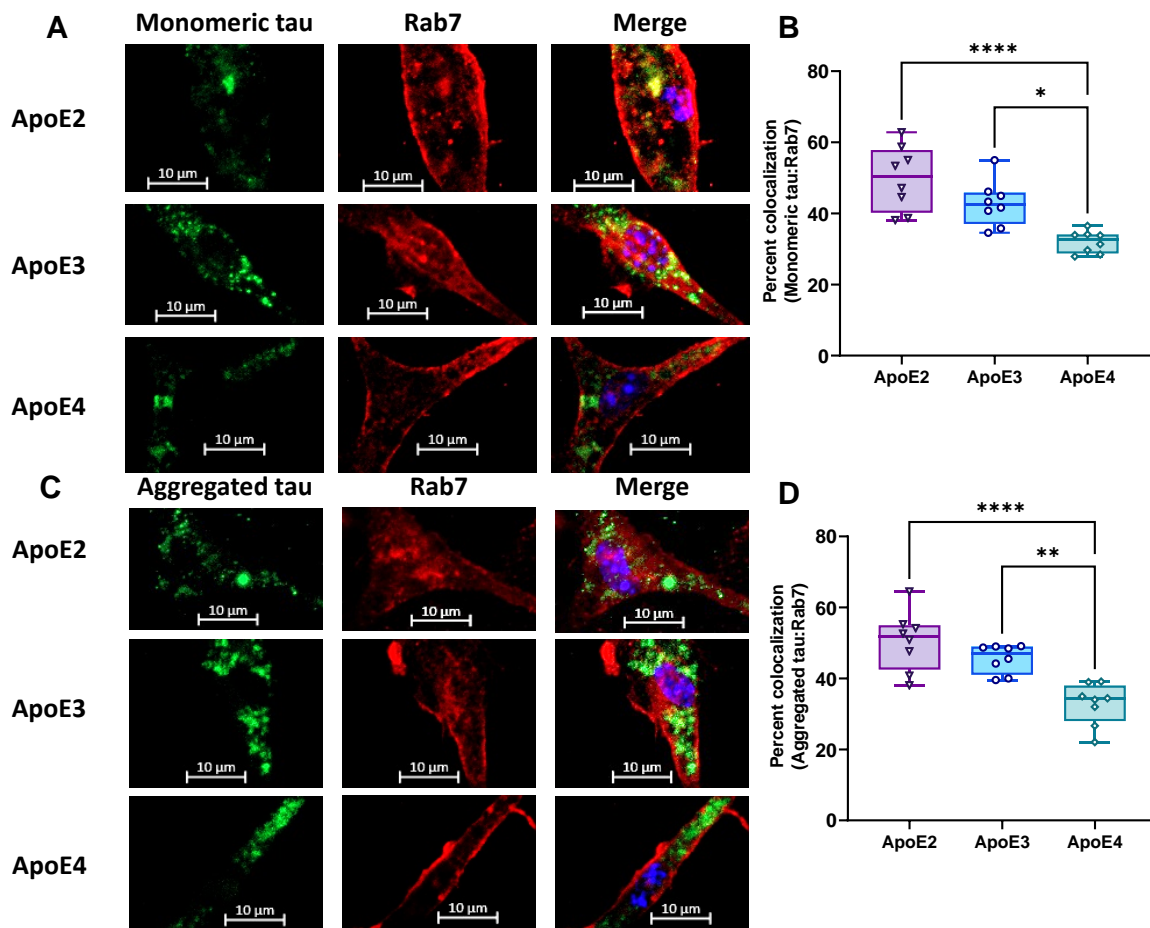


Figure 4.7 The colocalization percentage between tau and the late endosome marker Rab7 in ApoE2, ApoE3 and ApoE4 astrocytes. Scale bar, 10 μ m. (A) Representative images of mFtau (green) colocalization with Rab7 (red). (B) The colocalization of mFtau with respect to Rab7 was quantified and expressed as a percentage. Values represent mean \pm SD and are expressed as a percentage of mFtau colocalized to Rab7 (n = 8-10). (C and D) Representative images and quantification of aFtau colocalization with Rab7 was measured as in (A and B). *P < 0.05 as determined by ANOVA and Bonferroni's multiple comparisons test.

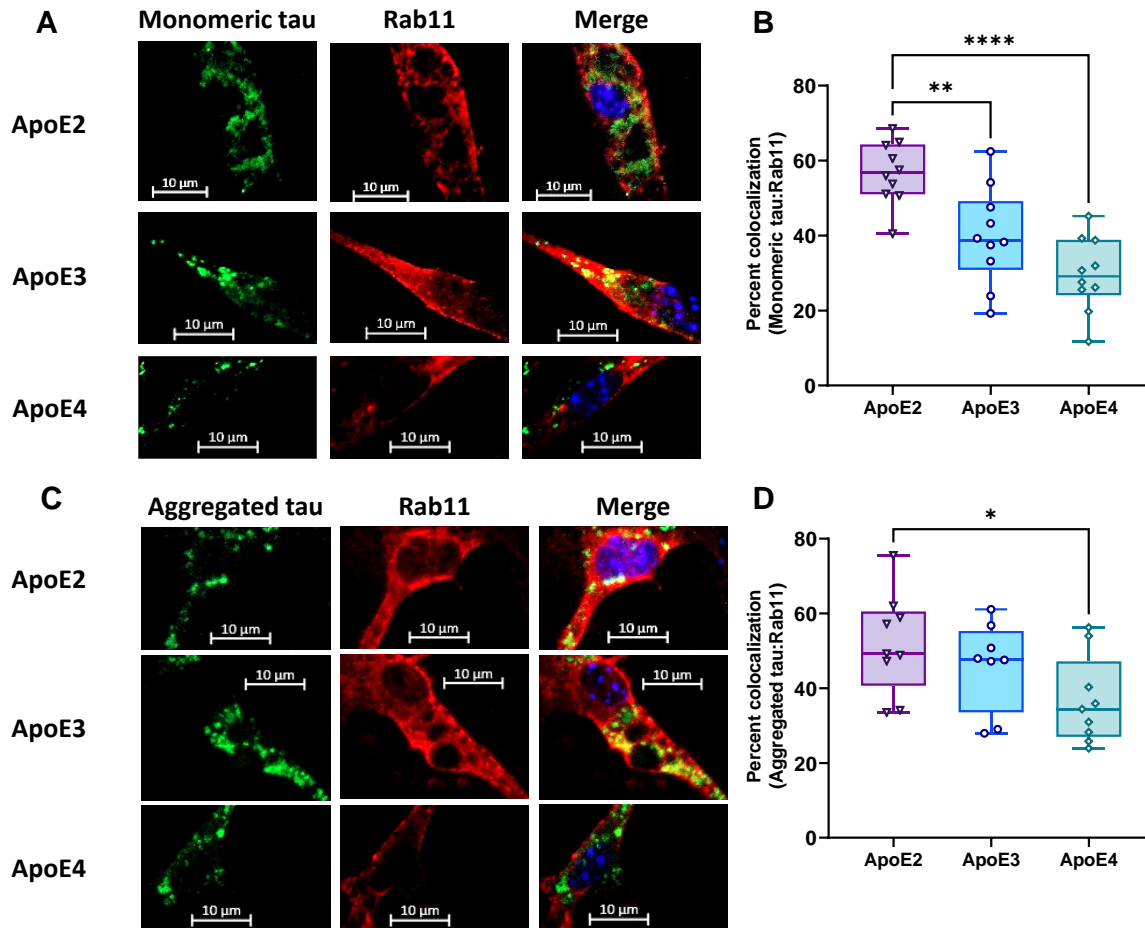


Figure 4.8 The colocalization percentage between tau and the recycling endosome marker Rab11 in ApoE2, ApoE3 and ApoE4 astrocytes. Scale bar, 10 μ m. (A) Representative images of mFtau (green) colocalization with Rab11 (red). (B) The colocalization of mFtau with respect to Rab11 was quantified and expressed as a percentage. Values represent mean \pm SD and are expressed as a percentage of mFtau colocalized to Rab11 (n = 8). (C and D) Representative images and quantification of aFtau colocalization with Rab11 was measured as in (A and B). *P < 0.05, **P < 0.01, ****P < 0.0001 as determined by ANOVA and Bonferroni's multiple comparisons test.

elimination from the brain in relation to ApoE genotype, I examined tau residence in the r-mTBI brain following intracranial injection of exogenous tau in ApoE mice. Consistent with what was observed in WT mice in Chapter 3, r-mTBI lead to a diminished elimination of extracellular monomeric tau in ApoE2 mice compared to r-sham ApoE2 mice, though this effect was not observed in ApoE3 mice. Interestingly, this effect was seemingly reversed in ApoE4 mice, as tau residence in the brain was reduced following r-mTBI compared to r-sham animals. However, the co-injected dextran tracer also had lower residence in the brains of the r-mTBI animals, suggesting possible disruptions to the BBB in these mice, which could also describe the diminished tau levels remaining in the brain in the ApoE4 r-mTBI mice. The increased BBB permeability after r-mTBI in the ApoE4 genotype, but not other ApoE isoforms, is consistent with previous reports. In a mouse model of TBI, the ApoE4 genotype resulted in delayed pericyte repopulation and impaired closure of the BBB after TBI, relative to ApoE3 (Main et al., 2018), with elevated MMP9 expression and sustained BBB disruption, though these measures were only examined out to 10 days after a single mild injury (Main et al., 2018). It is possible that under conditions of repetitive mild TBI, the ApoE4-associated impairment in BBB closure persists in the chronic phase post-injury. The diminished residence

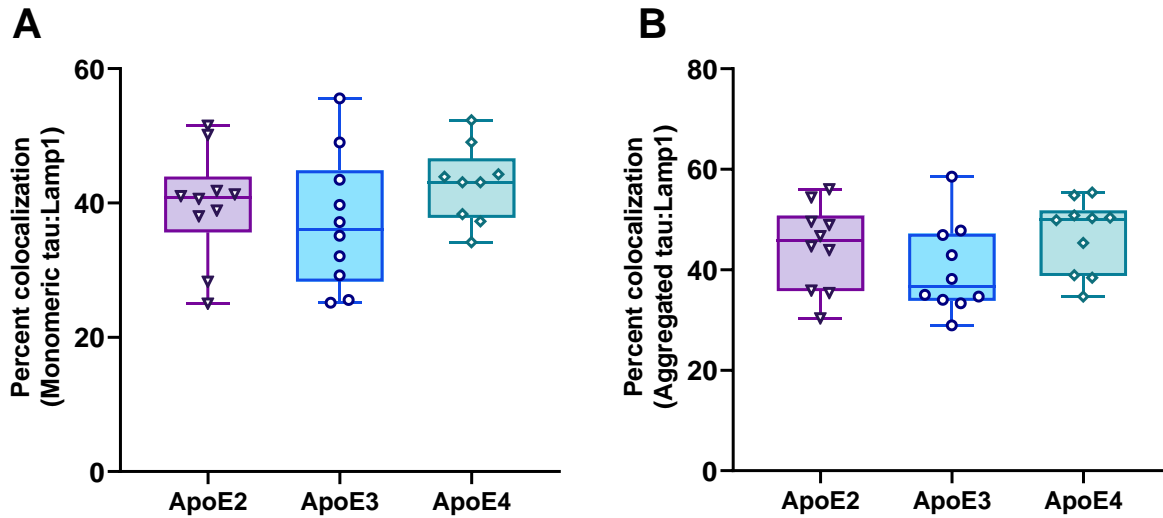


Figure 4.9 The colocalization percentage between tau and the lysosome marker lamp1 in ApoE2, ApoE3 and ApoE4 astrocytes. Scale bar, 10 μ m. (A) The colocalization of mFtau with respect to lamp1 was quantified and expressed as a percentage. Values represent mean \pm SD and are expressed as a percentage of mFtau colocalized to lamp1 (n = 8-10). (B) The colocalization of aFtau with respect to lamp1 was quantified and expressed as a percentage. Values represent mean \pm SD and are expressed as a percentage of aFtau colocalized to lamp1 (n = 8-10).

of dextran in the brain of the ApoE4 r-mTBI mice supports this hypothesis. That said, I cannot rule out the possibility that a confluence of ApoE4 and r-mTBI resulted in altered glymphatic influx, which is also responsible for the elimination of dextran from the brain and will be investigated in future studies. Nevertheless, the results from these studies suggest that the ApoE4 isoform impairs extracellular tau elimination from the CNS, particularly with respect to pathological tau species. We believe the prominent ApoE genotype effect on BBB leakiness masked the injury effect on tau elimination that we would typically see, particularly as our group has previously observed pathological tau accumulation in ApoE4 mice following r-mTBI (Muza et al., 2019). As such, the ApoE4 associated BBB alterations after insult prevented the current experimental setup from revealing such impairments.

In a parallel set of studies, I examined the influence of ApoE genotype on tau elimination from the brain in the context of another neurodegenerative condition, AD. In the same manner as the studies above, I examined tau elimination in EFAD mice compared to age matched ApoE mice (no transgenic AD mutations). In these studies, I observed that E4FAD mice exhibited enhanced elimination of tau from the brain relative to the ApoE4 animals, however as observed in the r-mTBI studies above, I also found the brain residence of the dextran marker was significantly lower in the E4FAD animals compared to the ApoE4 mice, again indicating BBB breakdown with the combination of ApoE4 and AD. This contrasts with ApoE3 where no difference in dextran brain residence was observed between the E3FAD and ApoE3 animals. Previous studies have demonstrated that ApoE4 is less effective at enabling LRP1 to inhibit a signaling cascade in pericytes, which ultimately results in the breakdown of the BBB (Bell et al., 2012). It is unclear whether this is due to the binding affinity of ApoE isoforms to LRP1 (Bell et al., 2012), the effect of ApoE4 expression in pericytes on endosomal LRP1/PDGFR β signaling cascades (Muratoglu et al., 2010), or some other variable. Altogether, our studies showed that ApoE isoforms influence the elimination of extracellular tau with (ApoE2>ApoE3>ApoE4), and after insult, the detrimental impact of the ApoE4 isoform leads to BBB breakdown not seen with other ApoE isoforms. The ApoE4-associated deleterious

effect on BBB health could impair physiological mechanisms of tau efflux at the BBB, resulting in the previously reported accumulation of pathogenic tau species after TBI (Muza et al., 2019).

In the intracranial tau studies above, I clearly show an effect of ApoE genotype on tau elimination from the brain. As astrocytic tau pathology is commonly observed in the post-mortem examination of CTE tissue (Arena, Johnson, et al., 2020) and prior reporting shows an association between neuronal tau pathology and the ApoE4 isoform (Y. Shi et al., 2017), particularly after TBI (Vasilevskaya et al., 2020), I investigated tau accumulation in r-mTBI astrocytes in the context of ApoE genotype (Figure 4.2). While tau accumulated in the astrocytes from each ApoE group, ApoE4 expressing astrocytes isolated from r-mTBI brains had significantly higher accumulation of extracellular tau than ApoE4 astrocytes isolated from r-sham brains. This r-mTBI effect was not observed in astrocytes expressing ApoE2 or ApoE3, suggesting that the expression of ApoE4 may play a deleterious role after brain trauma in astrocytes with respect to tau elimination. Interestingly, the aberrant influence of ApoE4 was only apparent when combined with an insult (in this case TBI), consistent with the tau residence studies above, where either TBI or AD was necessary to induce the aberrant effect of ApoE4.

The physiological role of astrocytic tau internalization is unclear, though experimental models have demonstrated an inverse relationship between astroglial and neuronal tau pathology, suggesting that astrocytes may remove extracellular tau to prevent neurotoxicity to neurons (Narasimhan et al., 2017), which could be neuroprotective (Hampton et al., 2010). Astrocytes may express subtle levels of endogenous tau, as reports have found astrocyte expression of *MAPT* mRNA (Y. Zhang et al., 2014) *in vivo*, though they do not develop tau aggregates *in vitro* (Narasimhan et al., 2020). Furthermore, pathogenic tau is unable to seed astrocytic tau pathology *in vitro* or astrocytic tau propagation *in vivo* (Narasimhan et al., 2020), in contrast to neurons (Sanders et al., 2014) or oligodendrocytes (Narasimhan et al., 2020) which express higher levels of tau (Y. Zhang et al., 2014). It is likely that tau pathology observed in astrocytes primarily originated from neurons or oligodendrocytes. More research is required to determine if astrocytes express physiologically relevant levels of endogenous tau in a manner that contributes to pathology. However, prolonged accumulation of tau in astrocytes may contribute to functional deficiencies (Reid et al., 2020) that are detrimental to surrounding neurons, potentially due to altered calcium signaling (Piacentini et al., 2017) and glutamate transport (Dabir et al., 2006). Reports have demonstrated that extracellular oligomeric tau can induce astrocytic senescence (Gaikwad et al., 2021), and the release of inflammatory and neurotoxic factors (P. Wang & Ye, 2021), that diminishes their capacity to support neurons (Sidoryk-Wegrzynowicz et al., 2017), contributing to clinical and neurodegenerative (Maté De Gérand et al., 2021) decline. Taken together, these results suggest that after r-mTBI, astrocytes may facilitate the clearance of extracellular tau, but the expression of ApoE4 impairs this process in astrocytes which could potentially contribute to localized perivascular neuronal toxicity. Previous studies have suggested that astrocytes internalize solutes like A β through a lysosomal pathway (Prasad & Rao, 2018), but there has been limited investigation into whether tau follows a similar route. Furthermore, it has been reported that the ApoE4 isoform can impair autophagy in astrocytes (Simonovitch et al., 2016), though the influence of brain trauma on this pathway is unclear, particularly with respect to tau.

To examine whether brain trauma could influence astroglial metabolic tau processing, I investigated the impact of r-mTBI on lysosomal activity in isolated ApoE2 and ApoE4 astrocytes. In this set of studies, there was not enough cellular lysate from isolated ApoE3 astrocytes to examine lysosomal activity. Exposure to extracellular tau did not induce autophagic flux, compared to a vehicle, suggesting that astrocytes do not

use the lysosome as a primary means to eliminate tau. Furthermore, there wasn't a significant impact of r-mTBI nor ApoE genotype on autophagic flux, as measured by the ratio of LC3-II:LC3-I, indicating the accumulation of extracellular tau in the r-mTBI ApoE4 astrocytes was not driven by autophagic dysfunction. In the same manner, I investigated the astrocyte proteasome and did not find significant differences in proteasome activity upon tau exposure, nor across ApoE genotypes. Interestingly, I did observe an increase in proteasome activity in ApoE3 and ApoE4 astrocytes from r-mTBI brains that were exposed to aggregate enriched tau, relative to vehicle (i.e., no tau exposure). This suggested that the physiological mechanisms used to process aggregate enriched tau are dysfunctional in astrocytes exposed to r-mTBI, prompting a response in activity by the proteasome in the presence the accumulation of aggregated tau. It should be noted that the increase in proteasome activity does not necessarily mean there is increased tau degradation. Internalized tau may be predominantly encapsulated in endosomal vesicles, preventing interactions with the 20S proteasome in astrocytes under physiological conditions, though if tau escapes into the cytosol, there may be a corresponding increase in proteasome activity. Higher order oligomeric species of tau tend to be inaccessible to the narrow opening of the proteasome, suggesting the elevated proteasome activity observed in these studies may be the result of cellular stress due to the r-mTBI exposure and accumulation of aggregated tau (Lee et al., 2013). Furthermore, the elevated proteasome activity did not result in a decrease in tau accumulation, nor was there a change in autophagic flux with tau exposure. Taken together, these results suggest that astrocytes do not rely on enzymatic digestion to process internalized tau.

As discussed, alterations in metabolic processes do not always correlate with tau degradation. A more direct way to interrogate this pathway can be achieved using degradation inhibitors (Figure 4.3). I exposed tau to astrocytes for 1 hour in the presence or absence of MG-132, Spautin-1 or a vehicle, then replaced the tau-laden extracellular media with fresh media containing inhibitors, and evaluated the tau remaining the lysates after 4 hours. The presence of the degradation inhibitors did not have a significant impact on the accumulation of either monomeric or pathogenic tau in the astrocytes. Based on these observations, degradation does not appear to be a prominent mechanism in the elimination of extracellular tau from brain astrocytes.

In continuing my examination of the interaction between astrocytes and extracellular tau, I monitored tau exchange between the intracellular and extracellular compartments in astrocyte cultures. I exposed astrocytes to monomeric or aggregate enriched tau for one hour. Next, I replaced the tau containing media with fresh media, and lysed the cells after 4 hours of incubation. In parallel, I performed the experiment on a second set of astrocytes exposed to monomeric or aggregate enriched tau and lysed the cells immediately after the 1-hour incubation period, to establish a baseline of initial tau uptake. The overarching purpose was to evaluate the proportion of intracellular tau that is simply effluxed to the extracellular environment in a given time frame. I evaluated the percentage of intracellular tau remaining in the lysates after 4 hours, compared to the initial intracellular tau levels, and found that almost 99% of the intracellular monomeric tau had been eliminated after 4 hours. While the aggregate enriched tau was not eliminated to the same degree as the monomeric tau, 85% of the aggregate enriched tau was eliminated after 4 hours. Previously, a study using a similar experimental design to evaluate astrocyte clearance of A β 42 observed approximately 60% of A β 42 was eliminated 6 hours after initial uptake (Liu et al., 2017). Astrocyte elimination of A β is believed to be carried out through enzymatic degradation (Liu et al., 2017), in contrast to what was observed in the current studies, where tau degradation does not appear to play a significant role in astrocytic tau clearance. The rapid and efficient elimination of tau observed in

these studies, compared to what was previously observed with A β 42 (Liu et al., 2017), indicates that the more likely mechanism for tau clearance involves cellular efflux, rather than degradation. As with mural cells, astrocytes may not play a direct role in tau elimination, though under neurodegenerative conditions, astrocytic interactions with extracellular tau may result in tau sequestration, resulting in diminished cerebrovascular elimination of tau.

Expression of the ApoE4 isoform has been linked to impaired endosomal (Nuriel et al., 2017), exosomal (K. Y. Peng et al., 2019) and lysosomal (Li et al., 2012) processes, likely predisposing ApoE4 carriers to develop neurodegenerative diseases associated with protein accumulation, though the impact of this dysfunction on tau trafficking within astrocytes has not been examined. As my studies suggested that astrocytes internalize tau, and subsequently appear to release it back to the extracellular environment, I investigated the subcellular trafficking of tau in astrocytes, and in particular, the effect of ApoE isoforms on these processes (Figure 4.5 – 4.9). Astrocytes likely use several different mechanisms to internalize tau, though unpublished observations in our lab have suggested that tau exposure induces LRP1 internalization in ApoE astrocytes in an ApoE isoform dependent manner with ApoE2>ApoE3>ApoE4. As previous studies have declared that LRP1 internalizes tau (Rauch et al., 2020) and LRP1 has been shown to regulate both A β 42 internalization and processing in astrocytes (Liu et al., 2017), the current subcellular trafficking studies monitored LRP1 as well. Recent investigations have suggested an unusually strong affinity between LRP1 and tau, due to ability of the microtubule binding region of tau to bind tightly to several ligand binding domains of LRP1 located on the cell surface, indicating that tau may bind to several LRP1 clusters at a time to facilitate internalization (Cooper et al., 2021). In my studies, I observed that astrocytes expressing ApoE2, 3, or 4 all demonstrated significant colocalization with LRP1 and tau, though the astrocytes expressing ApoE2 demonstrated a 30% greater interaction between LRP1 and monomeric tau than ApoE3 or ApoE4. This difference in LRP1 colocalization between ApoE isoforms was not observed in aggregated tau.

As ApoE2 is believed to bind less efficiently to LRP1 than ApoE3 or ApoE4 (Cooper et al., 2021), ApoE2 would indirectly facilitate tau binding to LRP1 to a greater extent than other isoforms, through diminished competition for the receptor. This might lead to differences in tau internalization via LRP1 in ApoE2 expressing cells, compared to ApoE3 or ApoE4. While tau pathology has been shown to be ApoE isoform-dependent, both ApoE2 (N. Zhao et al., 2018) and ApoE4 (Y. Shi et al., 2017) were found to exacerbate tau pathology, demonstrating the contribution of the individual ApoE isoforms to tau pathology is not entirely understood. Notably, many primary tauopathies are characterized by astrocytic tau pathology (Kovacs, 2020). Astrocytes may use several routes to internalize tau, including either HSPG-dependent and independent mechanisms (Martini-Stoica et al., 2018; Perea et al., 2019), or an integrin receptor complex (P. Wang & Ye, 2021). The mechanism of uptake likely varies between tau species, but others have observed that LRP1 may contribute significantly to tau uptake (Cooper et al., 2021). Recent results suggest that monomeric tau is efficiently internalized and processed via LRP1, though pathogenic tau may escape the processing pathway and deposit into the cytosol (Cooper et al., 2021). Further investigation into the interactions between LRP1 and tau are required, as their unusual binding affinity (Cooper et al., 2021), may differentiate LRP1 mediated tau processing from other ligands like A β or ApoE.

Once internalized via LRP1, ligands enter the cell within early endosomes, and are subsequently sorted as the intracellular vesicles mature to late endosomes and either fuse with lysosomes or return to release their cargo at the plasma membrane. The expression of ApoE4 has previously been associated with an impaired maturation from early endosomes to other phases of the endocytic pathway, though this

impairment is cargo-specific (Prasad & Rao, 2018). In this study, I observed an increase in the colocalization between tau and the early endosome marker EEA1 in ApoE4 astrocytes compared to ApoE2 or ApoE3 astrocytes. In contrast, there was decreased colocalization between tau and the late endosome marker Rab7 in ApoE4 astrocytes compared to ApoE2 or ApoE3 astrocytes. These results suggest that the time spent in the early endosome of ApoE4 astrocytes is prolonged, whereas tau internalized into ApoE2 and ApoE3 astrocytes demonstrate efficient tau sub-cellular processing throughout the early stages of the endosomal pathway.

The effects of ApoE isoform expression on astrocyte endocytic pathways, both generally and in ligand-specific circumstances are not fully understood at this time (Schmukler et al., 2018), though recent research has indicated that the biochemical properties of ApoE4 may be responsible for the observed defective early endosomes (Xian et al., 2018). More specifically, due to residue differences between ApoE isoforms, ApoE4 has an isoelectric point (IEP) of 5.9, which is close to the pH found in early endosomes, and different from the IEP of ApoE2 or ApoE3 (Xian et al., 2018). This means that once ApoE4 reaches the acidic conditions within the endosome, ApoE4 partially unfolds to form a protein folding intermediate, termed a 'molten-globule' (Morrow et al., 2002), where its hydrophobic core is exposed. This results in a condition where ApoE4 aggregates form and prevent early endosomes from maturing into late endosomes and/or recycling endosomes and proceeding along the endosome-lysosome pathway. Early endosomes subsequently are trapped in a nonproductive intracellular pool, along with their cargo (Prasad & Rao, 2018). This would likely impair the processing of internalized extracellular tau, as ApoE4 induced alterations in endolysosomal pH increase the likelihood that pathogenic tau will escape the endosomal-lysosomal pathway into the cytosol (Cooper et al., 2021), leading to intracellular tau accumulation. The aberrant influence of ApoE4 on this pathway could explain the increased tau levels in the ApoE4 astrocytes following r-mTBI compared to the other ApoE genotypes, suggesting that perivascular ApoE4 astrocytes may predispose an individual to pathological tau accumulation in astrocytes after injury due to aberrant endosomal trafficking of extracellular tau.

In continuing our examination of the subcellular trafficking of extracellular tau by astrocytes, I interrogated the recycling endosomal pathway, which enables the constitutive return of LRP1 to the plasma membrane surface after internalization allowing the cell to deliver and release surplus proteins and debris into the extracellular space. ApoE4 is associated with diminished exosome production and secretion at later ages (K. Y. Peng et al., 2019), which may result in pathogenic protein accumulation. In this study I investigated whether internalized tau was trafficked back to the cell surface in recycling endosomes. I found that in ApoE2 astrocytes, there was increased colocalization between Rab11 labeled recycling endosomes and tau compared to ApoE4 astrocytes, consistent with the dysfunctional ApoE4 early endosomes observed in this study and reported by other groups (Rawat et al., 2019). The colocalization between Rab11 and tau supports my *in vitro* studies which found that almost 99% of internalized tau was released by astrocytes into the extracellular environment in a span of 4 hours. Taken together, these results suggest that astrocytes internalize extracellular tau but do not seemingly utilize or degrade it, but rather return it to the cell surface for release, a process which is impaired in astrocytes expressing ApoE4. Other studies have indicated that diminished ApoE4 recycling of tau extends to other cargo and receptors like LRP1 (Prasad & Rao, 2018). In these prior studies, 50% less LRP1 was observed at the cell surface of ApoE4 astrocytes relative to ApoE2 astrocytes (Prasad & Rao, 2018) due to impaired endosomal recycling. As such, the diminished surface expression of LRP1 in ApoE4 astrocytes, in conjunction with a greater degree of LRP1 binding interference between ApoE4 and tau, would result in

a reduced capacity for ApoE4 astrocytes to release extracellular tau, akin to what has been previously observed for another LRP1 ligand, A β (Prasad & Rao, 2018; Verghese et al., 2013). My *in vitro* findings indicate that exposure to r-mTBI results in an accumulation of tau in ApoE4 astrocytes. Though the impact of r-mTBI on sub-cellular trafficking machinery in astrocytes is not well understood, particularly in the context of ApoE isoforms, future investigations could investigate whether dysfunctional endosomal machinery contributes to the observed accumulation of tau in ApoE4 astrocytes after r-mTBI.

Lastly, I did not observe significant differences amongst ApoE isoforms in regard to tau colocalization with the lysosomal marker, lamp1. This coincides with my *in vitro* results that did not demonstrate a significant role for the lysosome in tau processing. I did observe tau colocalization with lamp1 in the astrocytes, though this may be due to the non-specificity of lamp1. Lamp1 resides on the surface of lysosomes and has been used historically as a marker for lysosomal distribution and colocalization. However, some lamp1-labeled organelles may not contain lysosomal hydrolases, and may represent other multivesicular bodies, as lamp1 has been observed on endosomes as well. Though there may be potential limitations with the lysosome-label used in this study, there is support in the literature for the observation that there may be reduced LRP1 mediated lysosomal trafficking and autophagic flux in ApoE4 astrocytes (Simonovitch et al., 2016). Most LRP1 ligands are degraded via LRP1 mediated delivery to the lysosome (Cooper et al., 2021). This indicates that ApoE4 expressing astrocytes have diminished capacity to eliminate other extracellular LRP1 ligands through the lysosome, which is consistent with previous studies (Prasad & Rao, 2018). As the impaired endosomal maturation in ApoE4 also leads to elevated pH in the lysosomes of astrocytes (Prasad & Rao, 2018), this suggests that the expression of ApoE4 results in a greater propensity for the accumulation of proteins and other extracellular waste.

The elevation of tau in the extracellular fluids of the brain following head trauma, due to axonal injury or diminished removal of extracellular tau at the BBB as observed in Chapter 3, would increase the exposure of tau to perivascular astrocytes, and provoke astrocytic tau internalization. Based on my findings, it appears astrocytes do not utilize nor degrade extracellular tau but release it back to the extracellular environment. Astrocytes may be ineffective at eliminating excessive pathogenic species of tau, particularly under neurodegenerative conditions, resulting in the astrocytic tau accumulation observed in tauopathies. This dysfunction is exacerbated by the ApoE4 genotype, which both facilitates insult-induced BBB breakdown, and impairs intracellular trafficking efficiency. Taken together, an accumulation of extracellular tau after r-mTBI, combined with mural and endothelial cell dysfunction, may overwhelm the tau processing pathways in perivascular astrocytes associated with the BBB, particularly in individuals that carry the APOE4 genotype, resulting in pathogenic tau accumulation within astrocytes, that may directly lead, both directly and indirectly, to neurodegeneration.

CHAPTER 5: CONCLUSIONS AND FINAL REMARKS

5.1 RATIONALE

Traumatic brain injuries are common in the military and contact sports (Omalu et al., 2005), though they have received increased attention in recent years, particularly due to the chronic brain damage observed in high-profile American (Mez et al., 2017) and European (Lee et al., 2019) football players. This chronic brain damage is likely the result of repetitive brain trauma, and is associated with a wide variety of neurological, cognitive, behavioral, and psychiatric disturbances (Blennow et al., 2012), which typically emerge years or decades after exposure (McKee et al., 2009). A prominent feature of brain trauma is the accumulation of hyperphosphorylated and aggregated tau, which is chemically and structurally akin to the tau tangles observed in AD. Emerging evidence has indicated that tau accumulation in the brain may be due to diminished tau elimination, rather than increased tau production. The mechanisms responsible for tau elimination from the CNS are unclear, and the destination of tau after it is released from neurons is not fully characterized. Furthermore, there is very little understanding with respect to the interactions between extracellular tau and the non-neuronal cells that comprise the CNS, particularly cerebrovascular cells. Interestingly, TBI-associated tau pathology has been observed in neurons and astrocytes surrounding small blood vessels, and while several studies have demonstrated a link between tau and the cerebrovasculature (Banks et al., 2016; Merlini et al., 2016), the underlying mechanisms driving perivascular tau accumulation are not well understood.

Cerebrovascular degeneration is commonly observed in neurodegenerative diseases like AD and ALS (Winkler et al., 2014) and has been associated with the diminished capacity to eliminate pathogenic proteins including A β (Ma et al., 2018). Recent studies have linked brain trauma with cerebrovascular dysfunction, though the impact of r-mTBI on the cerebrovasculature, particularly at a chronic stage after injury, has not been examined. While preclinical models of r-mTBI are limited in their ability to replicate several of the mechanical forces and pathological outcomes observed in humans, they are quite effective at capturing the trauma-induced cerebrovascular consequences that have been observed in humans and provide an excellent platform for examining the chronic outcomes after injury (Smith et al., 2021). The purpose of this study was to interrogate the interactions between cerebrovascular cells and extracellular tau, and how such interactions are influenced by r-mTBI. Understanding mechanisms by which the cerebrovasculature contributes to tau elimination from the brain and the influence of r-mTBI on these processes could reveal new therapeutic strategies to promote tau elimination from the brain and delay the progression of neurodegenerative tauopathy following brain trauma.

5.2 SUMMARY OF FINDINGS

In this thesis, I found that the isolated cerebrovessels interact with extracellular tau, and this interaction is diminished at chronic stages after r-mTBI, in line with what I observed in a mouse model of AD. Preliminary findings from our lab indicated that brain mural cells interact with extracellular tau to a greater extent than other cerebrovascular cells. After r-mTBI, I observed a progressive degeneration in the smooth muscle cell marker α SMA, and both the pericyte-derived receptor, PDGFR β , and its endothelial cell-derived ligand, PDGF-BB. This mural cell degeneration, which was not due to cellular loss, correlated with progressively diminished cerebrovascular tau uptake post-injury. In addition, I identified

similar decreases in these mural cell markers in cerebrovessels isolated from human TBI and AD brain tissue compared to non-demented controls. Collectively these results indicated that after r-mTBI, the ability of the cerebrovasculature to interact with extracellular tau was diminished, which may be attributed to mural cell dysfunction post-injury. Interestingly, while mural cells demonstrated the capacity to internalize tau, this interaction did not result in the enzymatic digestion of tau. These results indicated that while the brain vasculature interacts with tau, and this interaction is reduced after r-mTBI, the cerebrovasculature does not play a significant role in the degradation of extracellular tau.

An alternative mechanism of solute elimination at the BBB involves cellular transcytosis. To determine the half-life of tau in the brain, exogenous tau was injected into the cortex and the elimination profile was examined over time. Interestingly, tau demonstrated rapid elimination compared to a smaller co-injected tracer (10 kDa dextran) that is not known to cross the BBB. As degradation is believed to take place over a much longer period of time than our intracranial tau elimination studies (i.e., 2 hours) these results indicated that extracellular tau may be largely eliminated from the brain via the BBB. My investigation of disease associated influences on tau elimination found that exposure to r-mTBI delayed tau elimination from the brain. In addition to these studies, previous neuroimaging reports have found a correlation between the ApoE4 genotype and tau burden in the brain, as determined by tau PET in former professional athletes that participated in contact sports (Vasilevskaya et al., 2020). In my studies, I observed that ApoE genotype influenced tau residence within the brain (ApoE2<ApoE3<ApoE4). Moreover, ApoE4 mice exposed to neurodegenerative insult (r-mTBI, AD mutations) resulted in tracer leakage indicative of chronic BBB breakdown, which was not observed in other ApoE genotypes. Of note, future studies will incorporate E2FAD mice, which were not available during the current experiments. In total, while several studies have shown an association between ApoE4 and tau accumulation in the brain, I was unable to fully evaluate the influence of ApoE4 on tau elimination from the brain using our methodological approach, due to the inherent breakdown of the BBB following brain injury in the ApoE4 animals.

The elimination of solutes across the BBB can be achieved through a variety of mechanisms. One of these, caveolin-1-mediated transcytosis, is a non-specific mechanism which has previously been linked to mural cell status (Ben-Zvi et al., 2014) and head trauma (Badaut et al., 2015). Though previous reports have demonstrated that reduced caveolin-1 expression is associated with tau accumulation (Bonds et al., 2019; Head et al., 2010), the interaction between tau and the BBB has not been studied. Therefore, I investigated the role of endothelial caveolin-1 in cerebrovascular tau uptake and transit across the BBB. I observed that the inhibition of caveolin-1 impairs tau transit across the BBB *in vitro*, and diminishes tau uptake in isolated cerebrovessels *ex vivo*. The direct role of caveolin-1 expression on cerebrovascular tau transit will be conducted in future studies using inducible endothelial caveolin-1 knockout mice, to further validate these observations. In our model of r-mTBI, there was a transient increase in cerebrovascular caveolin-1 in the acute stage after injury, consistent with previous reports (Badaut et al., 2015). Interestingly, in the more chronic phase post-injury, I observed persistent downregulation in caveolin-1, which was also evident in the cerebrovasculature isolated from human TBI samples when compared to non-demented human control brains. The diminished cerebrovascular caveolin-1 was also observed in a mouse model of AD and in human AD cerebrovessels, suggesting a potential neurodegenerative convergence between chronic TBI and AD. These findings demonstrate that tau can transcytose the BBB, and does so via caveolin-1, which is downregulated in chronic disease. Taken together, these results demonstrate that

the accumulation of tau pathology after TBI may be influenced by cerebrovascular dysfunction and a diminished capacity to eliminate tau across the BBB.

The regulation of brain endothelial caveolin-1 expression and caveolae-mediated transcytosis has been characterized to some extent, with most reports linking caveolin-1 directly to mural cell signaling (Andreone et al., 2017; Ben-Zvi et al., 2014). In particular, the Ang/Tie2 signaling axis between mural cells and endothelia, known to regulate BBB permeability, is transiently disrupted after TBI (Sabirzhanov et al., 2018), though there have not been investigations into the chronic outcomes of Ang signaling after injury. I hypothesized that mural cells contribute to endothelial caveolin-1 mediated tau transit through altered Ang-1 signaling after r-mTBI. In this thesis, I showed that mural cells release elevated levels of Ang-1 at a chronic stage after injury, which leads to increased endothelial expression of the caveolin-1 inhibitor Mfsd2a *in vitro*. Furthermore, I demonstrated an inverse relationship between endothelial Mfsd2a and caveolin-1 levels after injury, with elevated expression of Mfsd2a at a chronic period after r-mTBI.

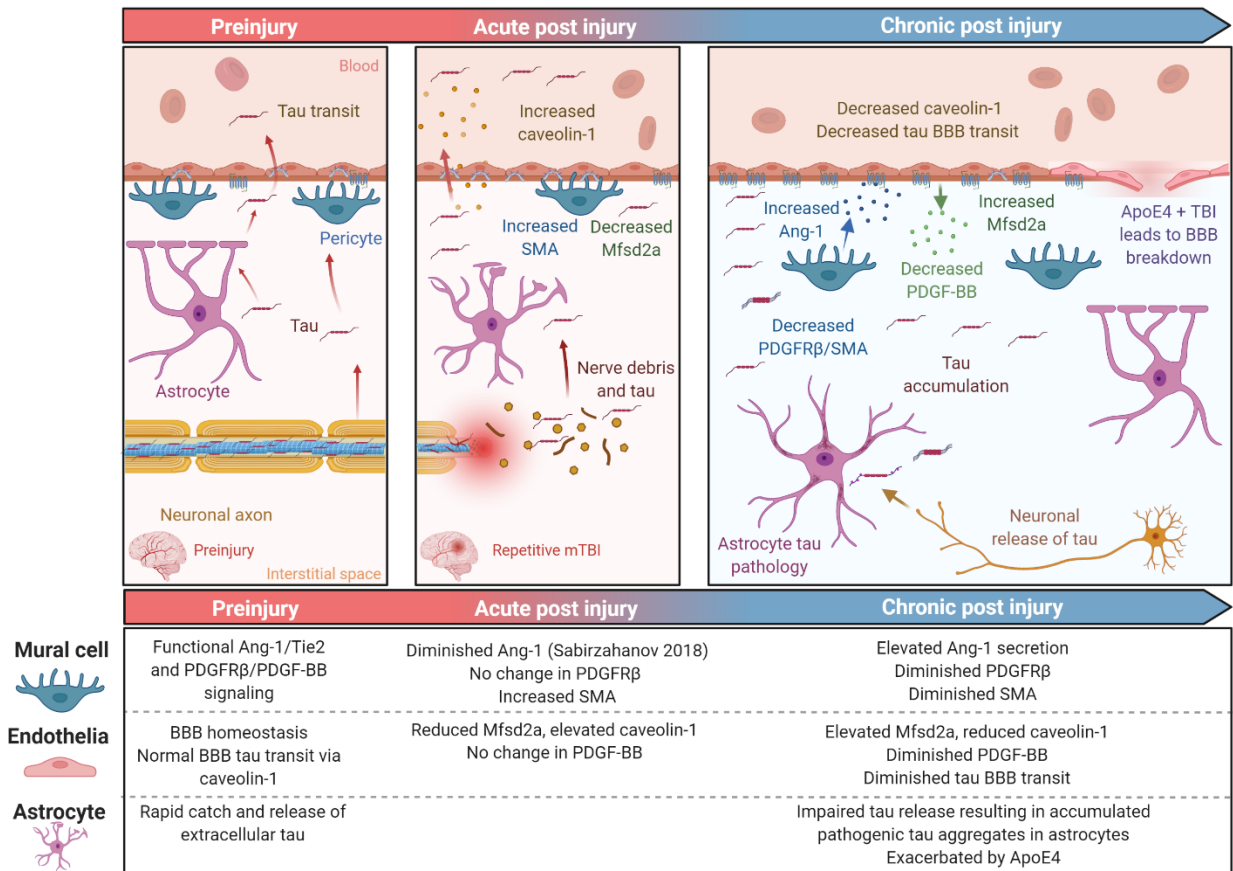


Figure 5.1 Proposed impact of r-mTBI on cerebrovascular elimination of extracellular tau. Low levels of tau released physiologically by neurons can transit through the BBB via caveolae-mediated transcytosis. In the acute phase post injury, endothelial cells express elevated levels of caveolin-1, and diminished levels of the caveolin-1 inhibitor, Mfsd2a. This facilitates the elimination of trauma induced cellular debris, enables angiogenesis, and facilitates vascular repair. In the chronic period after repetitive head trauma, PDGFRβ and SMA levels are diminished in brain mural cells, while endothelial cells secrete reduced levels of PDGF-BB. The dysfunctional pericytes secrete elevated levels of Ang-1, resulting in elevated endothelial expression of Mfsd2a, and diminished caveolin-1. As tau elimination via endothelial BBB transit is impaired, extracellular tau accumulates and initiates/exacerbates perivascular tau pathology in neurons and astrocytes. Furthermore, under r-mTBI conditions, the ApoE4 isoform contributes to basement membrane degeneration and BBB breakdown, leading to vascular leakiness.

Altogether, these findings indicate that dysfunctional mural cell signaling at a chronic stage after r-mTBI leads to reduced endothelial tau transit at the BBB, resulting in tau accumulation in the brain post-injury, as described by Figure 5.1. Therefore, we propose that modulating mural cell Ang-1 signaling after injury could be a potential therapeutic strategy to facilitate tau elimination from the brain following head trauma, though further studies are required to fully validate each step of the proposed Ang-1/Tie-2/Mfsd2a signaling cascade. Alternatively, endothelial cells could be targeted therapeutically via the PDGFR β /PDGFBB pathway as a more direct approach to restore cerebrovascular health after brain injury.

The findings from Chapters 2 and 3 suggest that r-mTBI leads to altered interactions between extracellular tau and several types of cerebrovascular cells, potentially contributing to elevated extracellular tau in the perivascular space. As perivascular astrocytic tau pathology is strongly associated with TBI (Arena, Johnson, et al., 2020), we examined the interactions between these cerebrovascular cells and extracellular tau. There is uncertainty with respect to the mechanisms by which astrocytes process tau, and whether that is influenced by TBI. Furthermore, astrocytes are the predominant source of ApoE, and differential ApoE isoform expression has been linked to progressive tau pathology (Y. Shi et al., 2017) and tau burden after injury (Vasilevskaya et al., 2020). In my studies, I investigated whether astrocytes internalize different tau species, the sub-cellular mechanisms by which astrocytes process tau, and whether these mechanisms are impaired after r-mTBI. My findings suggest that astrocytes internalize both monomeric and aggregated forms of tau, and after exposure to r-mTBI, ApoE4 astrocytes exhibit impaired tau processing. Though exogenous tau accumulation was observed in isolated ApoE4 astrocytes exposed to r-mTBI, this was not strongly influenced by impaired degradation mechanisms. There were no significant changes in either autophagic or proteasomal activity in astrocytes exposed to tau, and inhibition of the degradation pathways did not result in an accumulation of tau. Interestingly, tau that was preloaded into astrocytes *in vitro* was rapidly and efficiently cleared from the cells within hours, through a cellular mechanism that was not influenced by pharmacological inhibition of the proteasome or autophagy. Therefore, I hypothesized that under normal conditions astrocytes internalize tau, and rapidly release it back to the extracellular space, as opposed to degrading the tau internally.

To investigate the influence of ApoE genotype on tau subcellular trafficking in astrocytes, I followed the movement of tau through multiple stages of the endocytic pathway in astrocytes expressing ApoE2, ApoE3, or ApoE4. Confocal microscopic analysis showed that astrocytes demonstrated rapid (< 30 minutes) endosomal internalization and processing of tau for release at the plasma membrane. This observed mechanism for rapid astrocytic tau processing and efflux is consistent with our previous studies. Notably, I also detected ApoE isoform-specific differences in the subcellular trafficking of tau in astrocytes. Specifically, ApoE4 demonstrated delayed endosomal processing compared to the other isoforms, as evidenced by the increased percentage of tau located in early endosomes, and diminished tau located in late and recycling endosomes, suggesting that ApoE4 may impair tau release from astrocytes. This is consistent with previous reports, which demonstrated a deleterious influence of ApoE4 on astrocytic subcellular trafficking (Xian et al., 2018), potentially contributing to the leakage of endosomal pathogenic tau into the cytosol (Cooper et al., 2021) where it may escape endocytic processing and accumulate (Figure 5.2). Furthermore, I observed significant colocalization between tau and LRP1 in astrocytes, indicating a potential mechanism to facilitate tau internalization (Cooper et al., 2021). The results of these studies suggest that astrocytes rapidly internalize and release tau, but this process may be impaired following r-mTBI or the presence of ApoE4. The observations in Chapter 4 suggest that astrocytes are vulnerable to elevated extracellular tau concentrations, and as the tau levels rise in the brain post-injury,

due in part to reduced BBB elimination (as observed in Chapters 2 and 3), tau may accumulate in perivascular astrocytes over time.

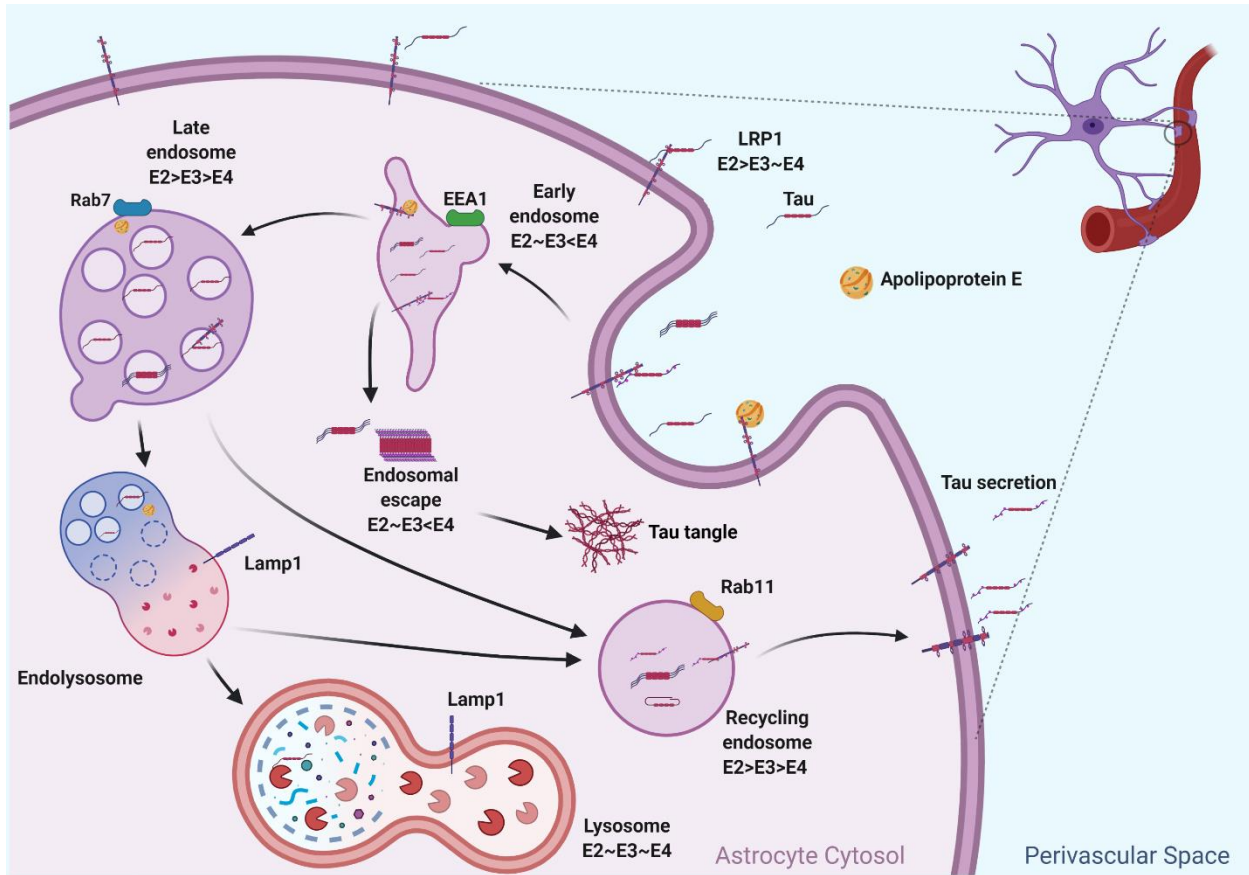


Figure 5.2 Proposed effect of ApoE genotype on astrocyte interactions with extracellular tau. Excess extracellular tau in the perivascular space exacerbated by the diminished tau BBB transit is internalized by astrocytes through an endosomal pathway, which is mediated by LRP1. Tau rapidly progresses through the endosomal pathway from the early endosomes and late endosomes to the recycling endosome where it is returned to the plasma membrane and efficiently released back to the extracellular environment. The ApoE4 isoform impairs the transition of early endosomes to late or recycling endosomes compared to ApoE2 or ApoE3, resulting in prolonged early endosomal tau residence. The ApoE4-associated delay in early endosomal maturation to recycling endosomes can increase the incidence of endosomal tau leakage into the cytosol, contributing to astrocytic tau pathology. Furthermore, aggregated tau species are more likely to leak out of the endosomal pathway and deposit in the cytosol, where they accumulate into astrocytic tau tangles.

5.3 CONCLUSIONS

Despite the increasing awareness of TBI-related neurodegeneration in recent years, and the significant advancements in our understanding of TBI pathophysiology, efforts to translate this progress into therapeutic success in the clinic have fallen short. My work identified a novel deleterious cerebrovascular response to head trauma and characterized the consequences of that dysfunction on the elimination of extracellular tau from the brain. I also investigated the mechanistic link between ApoE genotype and the cerebrovascular elimination of extracellular tau.

We identified several neurodegenerative cerebrovascular aberrations that were influenced by chronic r-mTBI, including altered mural-endothelial cell crosstalk, which contributes to reduced extracellular tau elimination across the BBB. We believe that diminished tau transit at the BBB in the chronic stage after r-mTBI represents a driver of perivascular tau pathology and may be an effective therapeutic target to enhance tau elimination from the brain following head trauma. In addition, the ApoE4 genotype was found to increase extracellular tau residence in the brain, potentiate BBB breakdown after injury, and impair astrocyte processing of extracellular tau. These novel associations could represent further targets for therapeutic intervention after head trauma and provide valuable insight into the mechanistic progression of TBI-induced tau pathology.

To our knowledge, this is the first demonstration that extracellular tau can be eliminated across the BBB via caveolae-mediated transcytosis, a mechanism which is impaired after r-mTBI. Consequentially, this may enhance the exposure of perivascular neurons and astrocytes to elevated levels of pathogenic tau and could potentiate the pathognomonic perivascular tau pathology observed after TBI. Importantly, these findings may provide therapeutic opportunities for preventing/reducing tau pathology in the brain following head trauma.

5.4 FUTURE DIRECTIONS

In this thesis I mentioned several mechanisms for tau elimination from the brain, including degradation and BBB transit, which I investigated in the current studies. In future studies, we will investigate another mechanism, perivascular drainage, and its contribution to tau elimination following head trauma. Mural cells contribute to the development (Munk et al., 2019) and maintenance of proper perivascular drainage (Carare et al., 2008), and previous reports suggest r-mTBI may result in diminished tau elimination via the perivascular drainage channels (Iliff et al., 2014). To interrogate these interactions, we will first examine whether tau is eliminated via these channels, and then characterize potential disease associated influences on this clearance route. Intracortical and intracisternal injections of fluorescent tau and other tracers into PDGFR β (+/-) mice, r-mTBI mice, and ApoE r-mTBI mice, may reveal possible alterations in CSF influx, ISF tau movement, and perivascular tau drainage which could contribute to the diminished tau elimination post-injury that was reported in prior Chapters. Findings from these studies will provide further clarity into potential therapeutic interventions to restore cerebrovascular health and delay neurodegeneration after head trauma.

We found that pericytes are dysfunctional in the chronic period after r-mTBI, which correlated with altered cerebrovascular interactions with tau, and impaired cerebrovascular status. One approach that could be employed to mitigate these abnormalities is stimulation of the pericyte population, which may rejuvenate endothelial tau transit after injury and promote tau elimination from the brain. Future studies will further interrogate the influence of the PDGFR β /PDGF-BB pathway on tau elimination after injury. Specifically, we will investigate whether therapeutic interventions that enhance PDGF-BB after r-mTBI can upregulate pericyte PDGFR β expression, promote cerebrovascular recovery, restore cerebrovascular interactions with tau, facilitate cerebrovascular elimination of tau, and improve behavioral outcomes after head trauma.

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