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Delineating the Contribution of Tau Astrogliopathy to the Neurodegenerative Sequelae of Repetitive Mild Traumatic Brain Injury.

Camila Renata Ortiz Torres

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

Supervisors

Dr. Joseph Ojo
Dr. Fiona Crawford

Date of Submission: January 2024
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.

Camila Ortiz
PUBLICATIONS


ACKNOWLEDGEMENTS

I would like to thank the people that have been supporting me throughout this journey.

First, I would like to thank my supervisors Dr. Joseph Ojo and Dr. Fiona Crawford for their useful guidance and support during the four years of education.

Second, I would like to thank other principal investigators at Roskamp Institute, Dr. Daniel Paris, Dr. Corbin Bachmeier, Dr. Benoit Mouzon for sharing with me their vast knowledge on techniques used in this thesis.

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Finally, I would like to send a massive GRACIAS to my parents, brothers, close family members and friends in Ecuador for always believing in me and providing their support when I needed the most.
ABSTRACT

Epidemiological studies have unveiled a robust link between exposure to repetitive mild traumatic brain injury (r-mTBI) and the elevated susceptibility for developing neurodegenerative diseases, notably chronic traumatic encephalopathy (CTE). A distinguishing pathological hallmark identified in postmortem CTE-affected brains is the presence of pathogenic tau within astrocytes, located predominantly at the depths of sulci, a phenomenon referred to as tau astrogliopathy. However, whether tau astrogliopathy is an active contributor to the pathology of r-mTBI/CTE or merely a consequence of aging is still extensively debated in the scientific community. Furthermore, the functional consequences of tau in astrocytes and how this may affect the pathophysiological response to r-mTBI are still unclear in the literature. This thesis addresses these pivotal questions by utilizing novel mouse models harboring tau-bearing astrocytes, subjected to our r-mTBI paradigm. Our investigations yield compelling insights into the intricate relationship between pathogenic astroglial tau and the chronic negative consequences of r-mTBI. Through gene ontology analysis, we observed profound alterations in key biological processes such as immune response and mitochondrial bioenergetics of tau-bearing astrocytes following r-mTBI. Additionally, ex-vivo analysis of primary astrocytes indicates that tau-bearing astrocytes after r-mTBI exhibit a more pronounced state of reactivity after encountering second inflammatory stimuli. Furthermore, in-vitro experiments focusing on the astroglial secretome identified that tau-bearing astrocytes after rmTBI induce increases in microglial-derived cytokines, which can be correlated with changes observed in the astroglial secretome. Moreover, our gene array analysis in CTE astrocytes revealed an immunosuppressed astroglial phenotype similar to tau-bearing astrocytes in our model. Collectively, the findings of this thesis underscore the significance of unraveling the enigma surrounding tau astrogliopathy and illustrate its role in propagating the chronic astroglial and microglial-mediated neuroinflammation observed after r-mTBI. The studies performed in this thesis represent the establishment of a valid platform for future research focused on identifying astrocyte-specific molecular mechanisms. The identification of these mechanisms holds the potential for targeted interventions and the development of effective disease-modifying therapeutics for r-mTBI.
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>ACM</td>
<td>astrocyte-conditioned media</td>
</tr>
<tr>
<td>ACSA</td>
<td>astrocyte cell surface antigen-2</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APOE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>AQP1/4/9</td>
<td>aquaporin 1/4/9</td>
</tr>
<tr>
<td>ARTAG</td>
<td>Age-related Tau Astroglioniopathy</td>
</tr>
<tr>
<td>Atg3/Atg4a</td>
<td>autophagy-related 3/4a</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BCL6</td>
<td>B-cell lymphoma 6</td>
</tr>
<tr>
<td>CamkIIa</td>
<td>calcium-calmodulin-dependent protein kinase II alpha</td>
</tr>
<tr>
<td>c-AMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CC</td>
<td>corpus callosum</td>
</tr>
<tr>
<td>CD44</td>
<td>cluster of differentiation 44</td>
</tr>
<tr>
<td>CDB</td>
<td>Corticobasal Degeneration</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebral spinal fluid</td>
</tr>
<tr>
<td>CTE</td>
<td>Chronic Traumatic Encephalopathy</td>
</tr>
<tr>
<td>CTX</td>
<td>cortex</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
</tr>
<tr>
<td>DAI</td>
<td>diffuse axonal injury</td>
</tr>
<tr>
<td>DEG</td>
<td>Differentially expressed genes</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK5</td>
<td>extracellular-signal-regulated kinase 5</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>FPI</td>
<td>fluid percussion injury</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GCS</td>
<td>Glasgow Coma Score</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GGT</td>
<td>Globular Glial Tauopathies</td>
</tr>
<tr>
<td>GLAST</td>
<td>glutamate transporter</td>
</tr>
<tr>
<td>GLT1</td>
<td>glutamate transporter 1</td>
</tr>
<tr>
<td>GS</td>
<td>glutamine synthetase</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HIPPO</td>
<td>hippocampus</td>
</tr>
<tr>
<td>IBA1</td>
<td>ionized calcium-binding adaptor molecule 1</td>
</tr>
<tr>
<td>IL-1α/β</td>
<td>Interleukin 1 alpha/beta</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin 12</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>Interleukin 12 subunit p70</td>
</tr>
</tbody>
</table>
CHAPTER 1 - INTRODUCTION

1.1 Traumatic Brain Injury epidemiology

Traumatic brain injury (TBI) results from a violent insult to the head that distorts brain function and damages cerebral tissue.

TBI is a leading cause of death and life-long disability worldwide and represents a significant public health problem. It is estimated that 69 million individuals worldwide suffer from TBI every year (Dewan et al., 2019). In the United States, between 2010 and 2013, the Centers for Disease Control and Prevention recorded 2.5-2.8 million cases of TBI, where 50,000 cases had a deadly outcome and 80,000-90,000 experienced the onset of long-term disability (CDC, 2003, 2015; Taylor et al., 2017).

The causes of TBI are diverse; however, three main causes have been identified in the US population by Taylor and colleagues (2017): 1) falls (47.2%); 2) being struck by/against an object (15.4%); and 3) motor vehicle accidents (13.7%). For the remaining 23.7%, intentional self-harm, assault/homicide, and other non-specified factors were identified as causative agents (Taylor et al., 2017).

The risk of sustaining a TBI varies with different factors, such as age, sex and occupation. In terms of age, Taylor and colleagues (2017) identified that the most vulnerable populations are children aged 0-4 years (1,591 per 100,000 individuals), older adolescents aged 15-24 years (1,080), and adults older than 75 years (2,232). The increased risk in adolescents could be explained by the hobbies or activities in which they engage (e.g., contact or extreme sports) while in children and the elderly, age-related factors such as skull, brain, and muscle frailty and propensity for falls play an important role (Araki et al., 2017; Gardner et al., 2018; Ilie et al., 2020). Additionally, epidemiological studies have shown that TBI incidence is 1.1-2.2 times higher in males than in females (Faul et al., 2010; Frost et al., 2013; Taylor et al., 2017) due to the likelihood of males engaging in high-risk behaviors more than females (Mollayeva & Colantonio, 2019). With respect to occupation, epidemiological studies have revealed three main vulnerable populations that, due to the physical demands and work responsibilities, are more prone to sustain a TBI: athletes of contact sports (e.g., American football, hockey, boxing) (Theadom et al., 2020); military service
personnel participating in training and deployment (Swanson et al., 2017); and construction workers (Chang et al., 2015).

1.2 Socio-economic impact of TBI

TBI negatively impacts the lives of individuals who have sustained TBI, their families, and society. TBI has the potential to cause permanent cognitive, occupational, and behavioral disability that leads to loss of productivity, unemployment, and diminished quality of life (Boake et al., 2005). Furthermore, the latter three consequences have the potential to destabilize the patient’s family routine and sustainability as they have to adjust to the impairment in day-to-day activities as a consequence of TBI.

The social impact of TBI goes hand in hand with the economic repercussions. Finkelstein and colleagues (2006) estimated that the annual cost of TBI in the USA was $60 billion. In a more comprehensive report, Langlois and colleagues (2011) estimated that the annual cost of TBIs in the US was more than $221 billion including $14.6 billion in medical costs, $69.2 billion due to unemployment, and $137 billion for loss of life quality. These alarming numbers expose the need to reduce such burden.

1.3 Classification of TBI

TBI cases are mainly classified by the physical mechanism of the insult, the severity of the symptoms emerging after TBI, and the pathophysiological changes post-head injury (Saatman et al., 2008).

1.3.1 Classification by physical mechanism of injury

There are three types of TBI under this category (Fig. 1.1): 1) penetrating; 2) closed head; and 3) blast injury.

1) Penetrating traumatic brain injury occurs when an external object pierces the skull and enters the brain causing focal damage (Hawryluk et al., 2022). Penetrating TBI encompasses brain injuries resulting from the transcranial penetration of objects such as bullets and knives (Zyck et al., 2016). Despite its low incidence, it carries a high mortality rate (Aarabi et al., 2015; Laskowitz & Grant, 2016).
2) Closed head injury happens when an external force causes rapid acceleration and deceleration movements of the brain inside an intact skull leading to diffuse damage. Closed head injury is the most common type of TBI (Ginsburg & Huff, 2023; Ng & Lee, 2019).

3) Blast-induced traumatic injury results from exposure to a high-pressure blast wave (Bryden et al., 2019). Blast TBI is commonly reported in members of military services and victims of terrorist attacks (Jorolemon et al., 2023). It has three main mechanisms of brain injury: A) Primary blast injury characterized by the physical brain damage induced by the changes in pressure generated by the shock wave (Guy et al., 1998); B) Secondary blast injury emerges from the impact or penetration of shrapnel or debris to the head resulting in blunt or penetrating brain injury, respectively; C) Tertiary blast injury arising from the displacement of the body by the blast wave causes rapid changes in accelerating forces within the brain resembling a closed head injury (Kluger et al., 2007; Wolf et al., 2009).

**Figure 1.1. Types of TBI according to the physical mechanism of injury.** A) Penetrating traumatic brain injury emerging after an object has penetrated the skull causing focal brain tissue damage. B) Closed-head traumatic brain injury caused by accelerating and decelerating brain movements that elicit diffuse brain damage. C) Blast-induced traumatic brain injury caused by a high-pressure wave that results in diffuse brain damage.
1.3.2 Classification by severity

Based on the neurological signs and symptoms, TBI is classified as mild, moderate or severe. Of all cases reported worldwide, mild TBI accounts for 81%, moderate TBI for 11%, and severe TBI for 8% (Maas et al., 2017; Vadan & Ilut, 2022). Clinicians and researchers assess TBI severity using the widely known Glasgow Coma Scale (GCS) scoring system implemented in 1974. The GCS system evaluates patient consciousness by assessing three main aspects: eye-opening, motor, and verbal responses (Teasdale & Jennett, 1974). Each component of the assessment has individual scores: eye-opening 1-4, motor responses 1-6, and verbal responses 1-5. A total score of 15 indicates “best response”, while a score of 3 suggests total unresponsiveness, see Table 1. For TBI patients, scores of 8 or less suggest that the individual has sustained a “severe” TBI, scores of 9 to 12 are indicative of a “moderate” TBI, while scores of 13 to 15 are characteristic of a “mild” TBI. It is important to mention that, although the GCS scoring system is a helpful prognostic tool (McNett, 2007), it does not necessarily reflect the ultimate level of functioning of the patient. In an attempt to classify TBI in a way that more accurately matches the severity with functional outcome, in 2007, Malec and colleagues (2007) proposed the Mayo TBI classification where, besides the GCS score as part of the criteria, they included other symptomatology such as data on post-traumatic amnesia and loss of consciousness, and neuroimaging analysis (see Table 1.1), the latter being more useful at predicting functional outcomes. More recently, clinicians and researchers have recommended a more objective approach to classify TBI severity. Tenovuo and colleagues (2021) suggest that severity classification criteria, besides neuroimaging analysis, should also incorporate objective, reliable, and measurable variables such as fluid biomarkers (e.g. NFL, S100B, GFAP) that are less likely to be influenced by external factors such as alcohol/drug intake, medication (opioids, sedatives), sleep deprivation and hearing deficits among others that usually would impact the evaluated outcomes on GCS (Tenovuo et al., 2021).
### Table 1.1. Glasgow Coma Scale criteria and Mayo TBI classification system

<table>
<thead>
<tr>
<th>Glasgow Coma Scale</th>
<th>Mayo TBI classification system</th>
</tr>
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<tbody>
<tr>
<td><strong>Eye response</strong></td>
<td><strong>Moderate-Severe/Define TBI</strong></td>
</tr>
<tr>
<td>4=eyes open spontaneously</td>
<td>• Death</td>
</tr>
<tr>
<td>3=eyes opening to verbal command</td>
<td>• Loss of consciousness of 30min or more</td>
</tr>
<tr>
<td>2=eye opening to pain</td>
<td>• Anterograde amnesia of 24h or more</td>
</tr>
<tr>
<td>1=no eye opening</td>
<td>• GCS scores less than 13</td>
</tr>
<tr>
<td><strong>Motor response</strong></td>
<td>• Intracerebral, subdural, or epidural hematoma; cerebral contusion; subarachnoid hemorrhage; penetrating TBI; brain stem injury.</td>
</tr>
<tr>
<td>6=obeys commands</td>
<td><strong>Mild/Probable TBI</strong></td>
</tr>
<tr>
<td>5=localizing pain</td>
<td>• Loss of consciousness less than 30min</td>
</tr>
<tr>
<td>4=withdrawal from pain</td>
<td>• Anterograde amnesia less than 24h</td>
</tr>
<tr>
<td>3=flexion response to pain</td>
<td>• Skull fracture not penetrating.</td>
</tr>
<tr>
<td>2=extension response to pain</td>
<td><strong>Symptomatic/Possible TBI</strong></td>
</tr>
<tr>
<td>1=no motor response</td>
<td>• Blurred vision</td>
</tr>
<tr>
<td><strong>Verbal response</strong></td>
<td>• Confusion</td>
</tr>
<tr>
<td>5=oriented</td>
<td>• Dizziness</td>
</tr>
<tr>
<td>4=confused</td>
<td>• Headache</td>
</tr>
<tr>
<td>3=inappropriate words</td>
<td>• Nausea</td>
</tr>
<tr>
<td>2=incomprehensible sounds</td>
<td>• Focal neurologic symptoms</td>
</tr>
<tr>
<td>1=no verbal response</td>
<td></td>
</tr>
</tbody>
</table>

**Scores:**

- 3-8 = severe TBI
- 9-12 = moderate TBI
- 13-15 = mild TBI

*System utilized to classify TBI cases by severity. Extracted from (Malec et al., 2007; Wijdicks et al., 2005).*
1.3.3 Classification by pathophysiology

According to the physical and physiological changes that arise from a head insult, TBI elicits two types of injury mechanisms. The initial physical changes in response to trauma, also known as primary injury; and the secondary injury comprises all biochemical changes in response to the primary injury that give rise to a myriad of side effects which might contribute to short- and long-term symptoms of TBI. It is important to mention that the pathophysiological features described in the section below vary in severity depending on the location, nature, and severity of the primary injury along with other factors or comorbidities such as age, sex, lifestyle, genetics and multiple trauma (Chan et al., 2017; Xiong et al., 2013).

Primary injury, also referred to as mechanical injury, emerges as an immediate and direct response to trauma, and for that they are regarded as unavoidable injuries. Shearing and tearing forces resulting from brain deformation (acceleration, deceleration or rotation of the brain) during the impact, compromise the integrity of longitudinal structures such as blood vessels and axons giving rise to two of the most common types of primary injury - blood vessel shearing (Chodobski et al., 2011) and diffuse axonal injury (DAI) (MacDonald et al., 2011), respectively. Blood vessel shearing leads to leakage of blood into the brain parenchyma giving rise to brain bruises or hemorrhages (Chodobski et al., 2011; Iwamura et al., 2012). DAI Diffuse axonal injury primarily affects the white matter tracts where bundles of axons run longitudinally (Mesfin et al., 2023). Neuroimaging performed on patients of all TBI severities shows that DAI is a common feature of TBI (Javeed et al., 2021; Messé et al., 2011). DAI results in the impairment of axonal transport, and in some cases axonal degeneration followed by neuronal death leading to neuronal disconnection. Some researchers suggest that the presence of persistent symptoms months and years after the head injury (i.e. Post-Concussive Syndrome) might be caused by diffuse axonal injury due to the loss of connectivity between brain regions resulting in behavioral alterations (Gold & Lipton, 2008; Granacher, 2008).

Secondary injury, also known as biochemical injury, arises from the complication of physical changes of the primary injury phase and occurs in the minutes to years after TBI (Granacher, 2008; Kuo et al., 2022). These injury mechanisms include excitotoxicity, mitochondrial dysfunction, oxidative stress, edema, hypoxia, ischemia, blood-brain barrier (BBB) disturbances,
and neuroinflammation elicited by the dysregulation of the biochemical homeostasis in the brain (Prins et al., 2013). Additionally, because these secondary injury mechanisms evolve after the impact, they are regarded as amenable to therapeutic intervention.

- **Excitotoxicity**: Neuronal glutamate release and dysfunctional astroglial glutamate uptake increases the extracellular synaptic and extra-synaptic concentrations of glutamate that results in the overstimulation of its receptors (NMDA and AMPA) causing persistent depolarization of nearby and distance post-synaptic neurons (Baracaldo-Santamaria et al., 2022). The depolarization triggers the increase in intracellular Ca²⁺ concentration which in turn activates enzymes involved in the degradation of proteins and nucleic acids, leading to neuronal cell death (Baracaldo-Santamaria et al., 2022; Kaur & Sharma, 2018).

- **Mitochondrial Dysfunction and Oxidative Stress**: The substantial increase in Ca²⁺ concentration during excitotoxic neuronal activation causes mitochondria dysfunction represented by the decrease of ATP production, and overproduction of reactive oxygen species, reactive nitrogen species, and free radicals. The former has been reported to compromise cellular function, while the latter overwhelms the antioxidant system; both endanger astrocyte, microglial and neuronal functioning and survival; and exacerbate oxidative damage (Kaur & Sharma, 2018; Nicholls & Ward, 2000; Prins et al., 2013).

- **Edema, hypoxia and ischemia**: Injured blood vessels allow the extravasation of fluid into the interstitial space leading to sequestration of water in the extracellular and intracellular space (Chodobski et al., 2011). The increase in brain water content, edema, causes the brain to swell inside the skull, which increases intracerebral pressure (Kaur & Sharma, 2018). Brain swelling compresses the cerebrovasculature and brain structures impeding normal blood flow and brain physiology. Reduction or lack of perfusion in the brain (i.e., hypoxia or ischemia, respectively) emerging either from blood vessel ruptures or edema leads to impaired neurovascular coupling and thus neuronal cell death (Sendoel & Hengartner, 2014; Wang et al., 2018).

- **Blood-Brain Barrier (BBB) disturbances**: BBB disruptions start with the breakage of cerebral blood vessels at the moment of the impact but persist over time (Rizk et al., 2020) due to either unhealed ruptures, or the presence or absence of pro-inflammatory (e.g.,
TNF, IFNγ) or trophic agents (e.g., TGFβ), respectively that promote the disassembly of basement membranes, and tight, adherens and gap junctions between endothelial cells. As a result of these events, BBB permeability (i.e., passive diffusion and paracellular transport) increases allowing the free passage of not only blood-borne proteins and fluid but also peripheral immune cells.

- **Neuroinflammation**: Neuroinflammation after TBI is primarily characterized by the reactivity of microglia/CNS macrophages and astrocytes in response to inflammatory stimuli such as cytokines, reactive oxygen and nitrogen species, cell death, and BBB leakages (Lozano et al., 2015; Kuo et al., 2022). Astrocyte and microglial reactivity lead to further production of pro- and anti-inflammatory agents including cytokines (van Erp et al., 2023). Beside astroglial and microglial response, neuroinflammation usually encompasses recruitment, infiltration and reactivation of immune peripheral cells; namely monocytes, lymphocytes, and neutrophils. The primary role of astroglial and microglial response and the presence of peripheral immune cells is to remove dangerous stimuli and promote tissue repair (acute neuroinflammation); however, if neuroinflammation is not controlled and persists chronically, it can render the brain environment hostile for all brain resident cells including neurons, endothelial cells, pericytes, smooth muscle cells, oligodendrocytes, astrocytes and microglia (Finnie, 2013). For instance, microglial production of cytokines (e.g., TNF, IL-1β, IFNγ) can induce apoptosis in neurons, promote the disruption of tight junctions further compromising the BBB; and perpetuate ongoing astrocyte and microglial reactivity leading to persistent neuroinflammation (Capaldo & Nusrat, 2009; Harry et al., 2008; Lozano et al., 2015; Wang & Michaelis, 2010).

In addition to the pathophysiological effects of TBI on the brain system, it is important to acknowledge that there is evidence that TBI also has secondary effects on peripheral systems, for instance, the endocrine, immune, and digestive systems (Bollerslev et al., 2013; Hanscom et al., 2021; Hazeldine et al., 2015), which will not be discussed here.
1.4 Mild traumatic brain injury

Mild traumatic brain injury (mTBI), also known as concussion or minor head injury, is the least severe of the three TBI types in terms of acute symptomatology and accounts for 75-90% percent of all TBIs reported every year (Maas et al., 2017; Vadan & Ilut, 2022). Mild TBI is diagnosed when a head injury results in the individual’s loss of consciousness for a period no longer than 30 minutes, impaired memory around the time of injury, confusion, and disorientation accompanied by a GCS score of 13-15. Individuals with mTBI may also experience other disabling physical, cognitive, and behavioral/emotional signs, and symptoms such as seizures, headache, dizziness, poor concentration and memory, depression, and aggression. Although mTBI symptomatology is usually transient and resolves within 10-14 days, it is reported that 10-15% of mTBI cases develop post-concussion syndrome where patients might persistently exhibit one or many symptoms described above even after 1-year post-injury (Bigler, 2008; Katz et al., 2015; Wood, 2004; Permenter et al., 2022). Additionally, a longitudinal study in 2012 reported that disability, cognitive impairment, and psychological issues can persist in mTBI patients up to 14 years after the traumatic event (McMillan et al., 2012). These long-term effects also appear to increase in symptomatology over years to decades. The molecular and cellular pathways that underlie the development of long-term effects in some mTBI cases remain insufficiently characterized (Fehily & Fitzgerald, 2017). However, studies on animal models have suggested that mTBI pathophysiology includes focal ruptures of the BBB, DAI, ionic imbalance, mitochondrial dysfunction, oxidative stress, and transient astrocyte and microglial reactivity.

Furthermore, while epidemiologic studies suggest that moderate or severe TBI increases the risk for neurodegenerative disorders by two to four times (Shively et al., 2012), the risk following a single mTBI encounter is still unclear. However, multiple mild traumatic brain injuries have been associated with neurodegenerative conditions such as Alzheimer’s Disease (AD), Parkinson’s Disease (PD), Amyotrophic Lateral Sclerosis, Frontotemporal Dementia, and Chronic Traumatic Encephalopathy (CTE) (Delic et al., 2020; Franz et al., 2019; Guskiewicz et al., 2005; Jawaid et al., 2009; McKee et al., 2009). This relationship is supported by histopathological evidence of accumulation of potentially pathogenic proteins (e.g., amyloid β, α-synuclein, TDP-43 and
hyperphosphorylated tau) that are closely linked to those diseases. Protein aggregation is not the only feature that repetitive mTBI has in common with other neurodegenerative conditions, repetitive mTBI also shares the presence of pro-inflammatory/anti-inflammatory cytokine imbalance, cerebrovascular impairment and chronic neuroinflammation.

1.5 Repetitive mild traumatic brain injury and its link with neurodegenerative diseases

Due to the relative absence of debilitating acute signs and symptoms in 85-90% of mTBI cases, mTBI patients are at higher risk of sustaining multiple head injuries (Reneker et al., 2019). This might be explained by the possibility that mTBI patients resume their activities without allowing enough time for the brain tissue to properly heal. Several studies on mouse models have demonstrated the cumulative/exacerbated effect of r-mTBI on secondary pathophysiological changes (e.g., BBB disturbance, DAI, ionic imbalance, mitochondrial dysfunction, oxidative stress, astrocyte and microglial reactivity) and cognitive behavior in mice. Other research groups have reported that the cumulative negative effects of r-mTBI are ameliorated when injuries are administered with a longer inter-injury interval (Fehily & Fitzgerald, 2017; Longhi et al., 2005; Prins et al., 2010). Together, these findings were suggestive of a “window of vulnerability” or “recovery phase” where the brain is more susceptible to developing accentuated negative outcomes if a subsequent TBI occurs. It is believed that the recovery phase is where the secondary mechanisms of TBI resolve, thus, if interrupted the failure to heal properly might trigger a greater degree of brain injury (Fig. 1.2) (Blennow et al., 2016; Longhi et al., 2005). Epidemiological studies have identified two populations that are more susceptible to repetitive mTBI (r-mTBI): a) athletes of contact sports (e.g., American football, soccer, hockey, boxing), and b) military personnel (Swanson et al., 2017; Theadom et al., 2020).

The first evidence of the link between repetitive concussions and neurodegenerative conditions dates back nearly a century to analysis of autopsied brains from several boxers which showed general brain atrophy and enlarged ventricles. Those individuals were diagnosed with dementia pugilistica (Martland, 1928). In the modern literature, the term CTE has been favored when
referring to the neurodegenerative condition emerging from repetitive head trauma because it is now understood that repetitive brain injuries, regardless of the source (e.g., contact and non-contact-sports, head-banging behavior, domestic violence, and military-related blasts) might lead to chronic brain tissue degeneration (Hof et al., 1991; McKee et al., 2009; Omalu et al., 2011; Omalu et al., 2005). Clinically, CTE symptomatology is described by memory loss, impaired judgment, impulsive behaviors, aggression, depression, and dementia. CTE neuropathological changes are characterized by a progressive and distinct pattern of hyperphosphorylated tau accumulation in neurons and astrocytes (the latter termed tau astrogliopathy) at the perivascular foci of small blood vessels, and at the depth of the sulci (Bieniek et al., 2021; McKee et al., 2016). It is well-reported in AD (a secondary tauopathy characterized by the accumulation of pathogenic tau in neurons) studies that the accumulation of pathogenic tau in neurons leads to neuronal cell death (Iqbal et al., 2010) which directly contributes to brain atrophy, ventricle enlargement and cognitive decline (Bejanin et al., 2017; Tanner & Rabinovici, 2021), all mentioned features are also observed in CTE cases. However, little is known about the role of accumulated tau within astrocytes in astrocyte physiology and the progression of neurodegenerative disease.
Figure 1.2. Vulnerability window after mild TBI and repetitive mild TBI. After mTBI, the brain undergoes several pathophysiological changes (secondary mechanism of injury) that need some time to resolve. That period is called the “window of vulnerability” or recovery phase. If a second mTBI occurs two outcomes can be triggered depending on the timing of the second mTBI. a) if the second TBI falls outside of the recovery phase and after the normalization of brain homeostasis, the outcomes of the second TBI will be similar to the first encounter. However, b) if a second mTBI occurs during the recovery phase, before the resolution of secondary pathophysiological changes, the outcomes of the second TBI will be exacerbated compared to the effects of a single mTBI. Extracted from (Blennow et al., 2016).
Tau is a microtubule-associated protein encoded by the *Mapt* gene which has 16 exons, and it is located on chromosome 17 (Neve et al., 1986). Through alternative splicing of exons 2, 3, and 10, six main tau isoforms are created. Tau isoforms can contain either 3 or 4 microtubule-binding repeats (3R or 4R tau) in the C-terminal region, and zero, one, or two inserts in the N-terminal tail (0N, 1N, or 2N tau) see Fig. 1.3. Tau is primarily expressed in neurons; however, some studies have shown the presence of tau in astrocytes and oligodendrocytes (Bennett et al., 2016; Clarke et al., 2018). Little is known about the function of tau in astrocytes and oligodendrocytes; however, in neurons, the most widely recognized function of tau is to stabilize and interconnect microtubules in the axons, with recent studies reporting roles of tau in axonal transport, cell signaling and synaptic vesicle release.

![Figure 1.3. Isoforms of microtubule associated protein tau (MAPT).](https://example.com/figure13)

*Figure 1.3. Isoforms of microtubule associated protein tau (MAPT).* Extracted from (Y. Zhang et al., 2022).
Phosphorylation of tau is the best-studied post-translational modification of tau. Increased phosphorylation of tau can reduce tau affinity for microtubules which increases intracellular concentrations of tau that may promote tau oligomerization and aggregation (Alquezar et al., 2021). Aggregation of hyperphosphorylated tau within neurons and astrocytes has been observed in different neurodegenerative diseases known as tauopathies (Reid et al., 2020). Tauopathies are categorized in primary and secondary tauopathies. In primary tauopathies such as Pick’s disease, progressive supranuclear palsy (PSP), aging-related tau astrogliopathy (ARTAG) and CTE, tau accumulation is believed to be the major contributing factor to neurodegeneration (Josephs, 2017). Primary tauopathies are also characterized by the presence of hyperphosphorylated tau within astrocytes in the form of tufted astrocytes, astrocytic plaques, thorn-shaped astrocytes (TSA) or astrocytic tangles, granular fuzzy astrocytes, ramified astrocytes, and globular astrocyte inclusions (Fig. 1.4) (Kovacs, 2020). In secondary tauopathies such as Alzheimer’s disease, tau pathology is thought to be driven or accelerated by other hallmarks of the disease (e.g., amyloid β in AD).

According to McKee and colleagues (2016), there are four stages of CTE (I-IV) based on the spread of phosphorylated tau throughout the brain. Tau astrogliopathy in CTE cases has been reported to progressively increase from Stage II onwards. Tau astrogliopathy in CTE is represented by the presence of TSAs in the perivascular area at the depths of the sulci and in the subpial area (Butler et al., 2022). It is relevant to mention that Butler and colleagues (2022) made certain observations about CTE tau astrogliopathy a) in terms of abundance tau astrogliopathy is inconspicuous compared to neuronal tauopathy; b) tau astrogliopathy, contrary to neuronal tau, increases in relation to age only and not in relation to the years of repeated head injury. Such findings led the authors to suggest that CTE neurological changes may originate as a direct consequence of neuronal tauopathy and not by tau astrogliopathy. Interestingly, TSAs are also present in ARTAG. ARTAG is a term used to describe unclassified tau astrogliopathies characterized by the presence of TSA in different brain regions such as white matter, grey matter, perivascular area, subpial and subependymal areas. ARTAG has been identified in one third of autopsied brains of elderly individuals over 70 years old (Kovacs et al., 2016). The presence of ARTAG is rarely found in isolation from other neurodegenerative diseases such as AD, PD or PSP, which complicates the
association of ARTAG to clinical symptoms (Liu et al., 2016). However, single study on cases with isolated diagnoses of ARTAG showed no cognitive impairment or dementia (Lace et al., 2012). Thus, the overlap of subpial tau astrogliopathy (i.e., subpial TSAs), the positive association of CTE and ARTAG astrocytic p-tau with age, and the lack of evidence regarding the clinical relevance of tau astrogliopathy in ARTAG cases, have generated great debate in the scientific field as to the involvement of tau astrogliopathy in the pathological progression of, for instance, CTE, PSP, CBD and ARTAG.

![Figure 1.4. Tau accumulation within astrocytes in neurodegenerative diseases.](Image)

Neurodegenerative diseases classified as primary tauopathies are characterized by the presence of tau astrogliopathy that has different features depending on the disease: Progressive Supranuclear Palsy (PSP) shows the presence of tufted astrocytes. Corticobasal Degeneration (CBD) exhibits astrocytic plaques. Globular Glial Tauopathies (GGT) are characterized by the presence of globular astroglial inclusions. Pick’s Disease (PiD) shows ramified astrocytes. Chronic Traumatic Encephalopathy (CTE) exhibits thorn-shaped astrocytes or astrocytic tangles. Age-related Tau Astrogliopathy (ARTAG) is characterized by the presence of thorn-shaped astrocytes and granular fuzzy astrocytes. Adapted from (Kovacs et al., 2016).
Moreover, the origin of the aggregated tau inside astrocytes remains to be elucidated. In a detailed review of astroglia and tau, Kovacs (2020) postulates two possible theories that are highly debated in the field: 1) Astrocytes express subtle levels of endogenous tau that are upregulated and hyperphosphorylated possibly by BBB dysfunction, brain trauma, or what the author called an “undefined neurodegeneration-inducing” event. This theory might explain why tau aggregates are found inside ARTAG astrocytes independent of neuronal tau pathology; 2) Tau aggregation in astrocytes might be the consequence of internalization of neuronal-derived tau as a mechanism of neuroprotection that over time leads to astrocytic overload. (see Fig. 1.5).

Although the origin of tau is a controversial topic, the remaining important question is to know how tau accumulation is astrocytes impacts on astroglial physiology and if as a result of that astrocytes are drivers of pathophysiology in tau astrogliopathies such as CTE, PSP, CBD. Additionally, the role of astrocytes in spreading of tauopathy remains to be elucidated.

Figure 1.5. Theories for tau accumulation in astrocytes. A) Astrocytes express small amounts of tau under normal circumstances. However, under the presence of Blood-Brain Barrier (BBB) dysfunction, brain trauma or an “undefined neurodegenerative-inducing” event, endogenous levels of astrocytic tau might increase. Additionally, tau might undergo abnormal post—translational changes that renders it more prone to aggregation. B) Pathogenic tau leave neurons through the synapses. Neighboring astrocytes might internalize tau for clearance purposes. However, when the clearance system is overwhelmed, it might cause tau aggregation in astrocytes. Adapted from (Kovacs, 2020).
1.7 Role of astrocytes in mTBI/r-mTBI

To understand the role of astrocytes in the pathophysiology of TBI, it is important to first determine astroglial physiology under normal circumstances.

1.7.1 Astrocytes in normal homeostatic physiology

Astrocytes are sponge-like cells of neural origin that account for 20-40% of the glial cell population in the brain (Verkhratsky & Nedergaard, 2018). Astrocytes express a wide variety of ion channels, for instance, potassium, sodium, calcium, and chloride channels on their plasma membrane. The astroglial membrane is also composed of water channels known as aquaporins (AQP1, AQP4, and AQP9), connexons, transporters for neurotransmitters (glutamate, gamma-aminobutyric acid (GABA), etc.) and glucose, and a plethora of receptors for opioids, insulin, proteins of the complement system, cytokines and neurotransmitters. The presence of ion channels, receptors, transporters and connexons on the astroglial membrane along with the ability to secrete a wide range of molecules confers an important and diverse physiology for astrocytes. Astrocytes are multi-faceted cells that participate in several homeostatic events ranging from ion/solute balance to neuronal integrity to immune response (Fig. 1.6). I will discuss some of them in the following paragraphs.

- Ion homeostasis

By expressing several ion channels and transporters on their plasma membrane, astrocytes can maintain CNS ionic homeostasis through bidirectional ion flux. These ion channels include passive, voltage-gated, calcium-dependent channels and ATP-dependent transporters for ions such as potassium, sodium, calcium, and chloride. Furthermore, astrocytes express transporters for protons and bicarbonate through which the extracellular pH is regulated. Systemic ionic homeostasis orchestrated by astrocytes is crucial for the physiology of other brain cells (Simard & Nedergaard, 2004).
• **Regulation of synapses: synaptogenesis, synaptic maturation, and synaptic pruning**

Through their **perisynaptic leaflets**, astrocytes are a key element in the formation, maturation, and elimination of synapses (Chung et al., 2015). The **perisynaptic astrocytic leaflets** enclose the synapse creating an insulated space that comprises the presynaptic terminal, the postsynaptic spine, and the astroglial perisynaptic process. This structure is known as the **multipartite synapse** or more recently has been called the synaptic cradle. At the synaptic cradle, astrocytes secrete multiple factors that are crucial for the generation of new synapses such as cholesterol, thrombospondins, and integrins among others (Baldwin & Eroglu, 2017). Additionally, astrocytes release glypicans 4 and 6 that facilitate synapse maturation by acting on the post-synaptic structure. Lastly, astrocytes also contribute to synaptic elimination or pruning by labeling synaptic terminals with complement factor C1q to mediate microglia or astroglial phagocytosis (Luo & Gao, 2021).

• **Neuronal energy support**

Perhaps one of the most important functions of astrocytes is to provide metabolic support to neurons (Beard et al., 2022). It is known that neurons have a high energy demand. However, neurons are not able to sustain their energy demand on their own, and this is where astrocytes come into action. Astrocytes can internalize glucose directly from the bloodstream through the glucose transporter, GLUT1 highly expressed on their vascular end-feet (Müller et al., 2018). Subsequently, glucose is converted into lactate via aerobic glycolysis. Then lactate is shuttled to neurons through lactate transporters (monocarboxylate transporters: MCT1 and MCT4). Inside neurons, lactate is oxidized to pyruvate which in turn is used as a substrate for energy production (Stobart & Anderson, 2013). Besides, astrocytes can polymerize glucose into glycogen which, during periods of high neuronal activity or in hypoglycemic scenarios, is depleted into glucose-lactate to promote energy sustainability in neurons (Bak et al., 2018; Ruchti et al., 2016). **It is important to note that there is evidence that neurons rely more on intracellular pyruvate than imported lactate to generate ATP** (Patel et al., 2014) which has questioned the contribution of astrocytic lactate to neuronal function.
Neurotransmitter homeostasis and modulation of neuronal transmission

By using transporters located in their perisynaptic processes, astrocytes are essential for the internalization and turnover of neurotransmitters glycine, dopamine, serotonin, norepinephrine, glutamate, and GABA. For example, in the case of glutamate and GABA, astrocytes are also responsible for replenishing their precursor pool in neuronal populations through the so-called glutamate/GABA-glutamine cycle (Bak et al., 2006). Glutamate and GABA are the major excitatory and inhibitory neurotransmitter in the CNS, respectively. Upon glutamate and GABA release into the synaptic cleft after neuronal transmission, astrocytes are responsible for removing glutamate primarily through the expression of excitatory amino acid transporters 1 and 2 also known as GLAST and GLT1, respectively (Pajarillo et al., 2019); and GABA through GABA transporter 3 (Liu et al., 2022). The removal of both neurotransmitters from the synaptic cleft is fundamental for effective neuronal transmission because it allows the re-setting of the synaptic environment for the subsequent signal transmission to occur and prevents both neuronal death via glutamate excitotoxicity or persistent GABA mediated neuronal inhibition. Once glutamate or GABA are internalized by astrocytes, they are both converted into glutamine. Consequently, glutamine is shuttled back into neurons where they can be used as a precursor for glutamate or GABA. This process is known as the glutamate/GABA-glutamine cycle or shuttle. Thus, astrocytes with their perisynaptic processes are crucial for isolating synapses to avoid diffusion of neurotransmitters to neighboring synapses, preventing the build-up in the synaptic cleft and thus allowing effective neuronal transmission and the autonomous regulation and replenishment of the glutamine pool in neurons for conversion to glutamate and GABA through the glutamate/GABA-glutamine cycle.

Transporters for other neurotransmitters include: sodium dependant glycine transporters, nucleoside transporters for adenosine, monoamine transporters for dopamine and norepinephrine (Verkhratsky & Nedergaard, 2018).

Bidirectional molecular crosstalk with microglia

Astrocytes engage on a bi-directional interglial crosstalk with microglia through which astrocytes can regulate microglial function in health and disease (Bhusal et al., 2023). Astrocytes are able to
alter microglial release of pro- and anti-inflammatory agents, as well as modulating their proliferation, phagocytosis, and synaptic pruning using wide range of molecules such as growth factors, cytokines, complement proteins, and miRNAs. Conversely microglia-secreted molecules can modulate astrogial proliferation, border/scar formation, and astrogial release of inflammatory agents.

- **Water homeostasis and CNS clearance**

The polarized expression of aquaporin 4 (AQP4) to the vascular end-feet places astrocytes in the center of water homeostasis control and the CNS clearance system called the “glymphatic system”. AQP4 allows the exchange of water from the blood vessels into the parenchyma to regulate extracellular space volume (Kimelberg, 2004). Regarding to the contribution of astrocytes to the glymphatic system, astrocytes help clear waste using the perivascular space created in between the blood vessel and the astrogial end-feet (Jessen et al., 2015). This clearance system has an important role in removing waste solutes, such as amyloid β and tau, that are potentially harmful to brain cells if accumulated (Iliff et al., 2012).

- **Cerebral blood regulation and Blood-Brain Barrier integrity**

Through their vascular processes, astrocytes are a key element of the of the neurovascular unit, a “donut-shaped” structure consisting of neurons, pericytes, smooth muscle cells, endothelial cells, microglia and astrocytes (Kugler et al., 2021; Verkhratsky & Nedergaard, 2018). Additionally, given their strategic position and their ability to respond to neuronal activity, astrocytes are modulators of blood flow. *In vitro* and *in vivo* experiments on cortical tissue indicate that glutamate in the synaptic cleft triggers the release of arachidonic acid metabolites at the astrogial end-feet leading to the dilation or constriction of arterioles mediating the changes in cerebral blood flow in response to neuronal activity in a process known as functional hyperemia (Takano et al., 2006). In addition to providing structural support, astrocytes are involved in the establishment, maintenance, and repair of the BBB by modulating protein expression on endothelial cells, the primary cellular component of the BBB (Alvarez et al., 2013). For example, astrocytes can regulate the abundance of tight junctions between endothelial cells modulating
BBB permeability (Wang et al., 2008; Wosik et al., 2007), and vascular growth and development (Mizee et al., 2013).

- **Reactive Oxygen Species homeostasis**

Astrocytes are a crucial element of the antioxidant system of the CNS. Astrocytes express high levels of glutathione (glutathione production is highly dependent on cystine/glutamate exchangers(Ottestad-Hansen et al., 2018) and ascorbic acid, both radical scavengers that maintain homeostasis after the production of reactive oxygen species as a consequence of normal neuronal activity (Y. Chen et al., 2020).

1.7.2 Astrocytes in TBI Pathophysiology

There has been an increasing number of studies that have interrogated the molecular changes that astrocytes undergo after different types of CNS injury. Yet, it remains poorly understood what the long-term consequences of astrocytes are in response to mTBI or r-mTBI. Below, we provided a collection of studies that have investigated astroglial pathophysiology following CNS injury of a variety of severities. However, it is noteworthy that most of these were limited to acute or subacute time points post-last injury (hours to days) while chronic time points (months) remain largely understudied.

Astrocyte reactivity is the intricate response to changes in the CNS environment triggered by a myriad of stimuli: mechanical force, inflammatory mediators such as cytokines and reactive oxygen species, hypoxia and glucose deprivation, and protein aggregation. Astrocyte reactivity is characterized by a wide spectrum of changes in gene and protein expression, morphology, and function.

For many years scientists have been working on the characterization of reactive astrocytes to better understand astroglial changes in a wide range of CNS injuries including brain trauma. These studies have collectively revealed at least two different subtypes of reactive astrocytes: 1) proliferative, border/scar-forming reactive astrocytes; and 2) non-proliferative reactive
astrocytes (Burda & Sofroniew, 2014; Sofroniew, 2020; Sofroniew & Vinters, 2010). The proliferative subset forms a physical barrier or border/scar that secludes the injured area from the healthy tissue preventing the spread of tissue damage by neuroinflammation and neurodegeneration (Bush et al., 1999; Frik et al., 2018). Non-proliferating reactive astrocytes undergo variable degrees of molecular and morphological changes. In contrast to proliferating astrocytes, these reactive astrocytes remain in their location and are likely to interact with the same neural elements they interact with in a healthy brain such as synapses, neurons, and blood vessels but potentially they may surrender some of their physiological roles. Additionally, astrocyte reactivity can also be categorized as isomorphic when the astroglial domain is preserved or anisomorphic when the astroglial domain is disrupted (Verkhratsky et al., 2014).

In TBI, astrocyte reactivity depends on the severity, injury type (focal or diffuse), brain region (white or grey matter), and proximity to the injury site (Muñoz-Ballester & Robel, 2023) as well as the time after the injury (hours to years - acute, subacute, or chronic time points).

In the context of mild TBI, in contrast to moderate and severe TBI, there is no presence of proliferative astrocyte reactivity or glial border (Shandra et al., 2019) capable of secluding the injury area. Therefore, non-proliferative reactive astrocytes appear to predominate in mild TBI and promote CNS disruptions. Non-proliferative astrocytes observed in mild TBI display three different responses: 1) classical upregulation of intermediate filaments such as glial fibrillary acidic protein (GFAP) and vimentin accompanied with hypertrophy (i.e., enlargement of main processes and soma), 2) atypical loss of homeostatic proteins (GLT1, Kir4.1, GS) with no intermediate filament upregulation, and 3) reactive astrocytes that are inconspicuous to homeostatic astrocytes with no apparent obvious changes. The implications of molecular, cellular, and morphological changes in non-proliferating astrocytes following mild TBI in the pathophysiology warrants further research.

Information gathered from our search on in-vitro or in vivo studies, we could possibly suggest that astrocyte reactivity after TBI may have three different outcomes in astrocyte physiology: 1) loss of supportive astrocytic physiological functions; 2) gain of detrimental traits/ negative function, for instance, exacerbated pro-inflammatory cytokines release, release of neurotoxins
(e.g., saturated lipids), increased production of reactive oxygen and nitrogen species that exacerbates inflammation and tissue damage (Liddelow et al., 2017; Rizor et al., 2019); and 3) engagement in compensatory protective behaviors such as neutrophic factors release (Becerra-Calixto & Cardona-Gómez, 2017); the secretion of thrombospondins involved in the generation of excitatory synapses (Shan et al., 2021); upregulation of antioxidant enzymes such as glutathione-peroxidase and superoxidase dismutases (Pérez-Sala & Pajares, 2023); and degradation of protein aggregations (Giusti et al., 2024).

1.7.2.1 Immediate response of astrocytes to mTBI/r-mTBI

Astrocytes express mechanosensitive ion channels that respond to membrane deformation (Bowman et al., 1992; Islas et al., 1993) known to occur as a result of accelerating-decelerating forces of head impacts. The activation of such receptors causes the influx of extracellular ions like Ca²⁺ and Na⁺ (Floyd et al., 2005), known to depolarize astrocytes which leads to the release of adenosine triphosphate (ATP) via connexins (Neary et al., 2005). Subsequently, ATP acts on neighboring astrocytes (astrocyte networks/syncytia) surrounding the traumatic lesion triggering the increase in intracellular Ca²⁺ concentrations leading to the polarization of astrocyte processes to the injury site. Additionally, Nayak and colleagues (2014) showed that, initially, the astrocyte-derived ATP modulates the recruitment of microglia (via their purinergic receptors) to promote the survival of brain cells in the lesioned area minutes following the trauma (Nayak et al., 2014). Furthermore, the ATP-mediated increase in intracellular astrocyte Ca²⁺ concentration can also contribute to the release of vasoactive molecules such as nitric oxide (Li et al., 2003) and metallopeptidases (MM9) that act on the brain vasculature (Pan et al., 2012). These events set in motion biochemical, molecular, and functional changes that modulate astroglial physiological response as TBI pathology progresses.

1.7.2.2 Delayed astroglial response to mTBI

TBI can directly influence BBB integrity as a result of the initial primary mechanical injury generated at the moment of head impact or as a result of biochemical damage to endothelial cells elicited by the secondary injury. In mTBI, vessel rupture is rare, however, some recent
evidence has shown that the BBB might become compromised leading to the leakage of small blood-borne factors (approx. <10KDa) (Heithoff et al., 2021). The presence of foreign proteins might trigger the release of matrix metalloproteidase 9 which increases BBB permeability (Lin et al., 2013). Furthermore, reactive astrocytes might also increase BBB permeability through the release of vascular endothelial growth factor A that disrupts the expression of tight junction proteins on endothelial cells (Argaw et al., 2009). On the other hand, studies have supported that TBI-mediated astrocyte reactivity might help ameliorate BBB permeability by upregulating the expression of factors such as angiopoietin 1 and Sonic hedgehog (Michinaga et al., 2020, 2021; Rui et al., 2019). The opposing roles on BBB permeability are suggestive of either time-dependent or context-dependent astroglial responses that have not been studied in r-mTBI at acute or chronic time points and will help elucidate the destructive or protective role of astrocytes on BBB integrity. Moreover, after a TBI, the AQP4 channels undergo re-localization away from the end-feet affecting the waste removal at the perivascular area via the glymphatic system (Sullan et al., 2018). The redistribution of AQP4 might explain why tau protein tends to accumulate in the cases of repeated TBI/CTE (Iliff et al., 2014a; Sullan et al., 2018).

For many decades, microglia were considered the prime effector cells responsible for giving rise to neuroinflammation (Streit and Kincaid-Colton, 1995; Alois, 2001). The identification of microglia as immune cells of the CNS, capable of mounting host defenses within the brain, resulted in the perception that microglia were the unique mediators of neuroinflammation. However, a growing body of evidence is uncovering the contribution of other glial cells, specifically astrocytes, in neurodegenerative traits of neuroinflammation (Farina et al., 2007; Soung and Klein, 2019; Philips et al., 2014). The discovery of toll-like receptors and scavenger receptors in astrocytes changed the well-established idea that microglia are the only defense mechanism of the brain (Gurley et al., 2008). The above-mentioned receptors play an essential role in the recognition of endogenous molecules released from damaged or dying cells (also known as damaged-associated molecular patterns) that trigger the release of proinflammatory cytokines such as TNF, IL-1 and IL-6 (Takeuchi & Akira, 2010) these cytokines have been shown to perpetuate astrocyte and microglial reactivity (interglial crosstalk), and modulate cell death and BBB permeability. To what extent astrocytes contribute to the burden of neuroinflammation, in
the context of r-mTBI, compared to microglia, remains to be elucidated; however, there is strong evidence that suggests that astroglia pathobiology is a field with great potential for new therapeutic targets in not only neuroinflammation but also neurodegeneration.

Since under normal circumstances, astrocytes provide trophic and metabolic support to neurons, studying the role of astrocytes in neuronal fate after r-mTBI-mediated astrocyte reactivity is a relevant research focus. Several studies have shown a possible link of astrocytes in excitotoxicity. One study shows that in TBI, there is an increase in glutamate concentration and a drastic reduction of glutamate transporters (GLT1 and GLAST) which might be suggestive of a loss of function of astrocytes to buffer the increased concentration of glutamate (Landeghem et al., 2006). However, given that neuronal death after TBI can be triggered by multiple factors it is difficult to establish a causation between astrocyte dysfunction and neuronal death. Additionally, studies on human induced pluripotent astrocytes suggest that astrocytes are involved in spread of pathological tau to neighboring neurons (Mothes et al., 2023; Reid et al., 2020) which might indirectly link astrocytes to tau-induced neurodegeneration.

Moreover, despite the fact that chronic neurodegeneration after a history of repeated concussion is unquestionable (e.g., CTE), current mouse models of single or r-mTBI fail to replicate overt neurodegeneration (Fehily & Fitzgerald, 2017; Muñoz-Ballester & Robel, 2023) which has probably contributed to the translatability of therapeutic treatments to human. expose the need of developing Thus, it appears that further investigation on the role of astrocytes in TBI-elicited neurodegeneration requires the generation of new mouse models or the utilization of other animal models.

Increasing evidence shows that after TBI, astrocytes might be involved in repairing mechanisms that facilitate synapse remodeling, neuronal maintenance, and outgrowth by secreting perineuronal net proteins such as chondroitin sulfate proteoglycans, hyaluronan, among others (Alhadidi et al., 2023; Burda et al., 2016; D. Wang & Fawcett, 2012). Nonetheless, little is known about the reparative capacities of astrocytes after r-mTBI.
In health, astrocytes contribute to CNS homeostasis by engaging in a wide variety of functions: Astrocytes are involved in maintaining ion homeostasis of the CNS by utilizing ion channels for K⁺, Na⁺, Ca²⁺, and Cl⁻ (1). Astrocytes provide metabolic support to neurons through lactate shuttle using monocarboxylate transporters (MCT1 and MCT4) (2). The presence of glutamate transporters (GLT1 and GLAST) in the astrocytic perineuronal processes is responsible for the uptake of the excess of glutamate released at the synaptic cleft. In this way, astrocytes modulate neuronal transmission and neurotransmitter homeostasis (3). Astrocytes communicate with microglia to ensure that the CNS environment is protected and ready to mount defense if needed (4). Astrocytes are a key element of the neurovascular unit (NVU) where their role is to maintain water homeostasis via aquaporin 4 channels (AQP4); regulate cerebral blood flow; help maintain blood-brain barrier integrity; and help clear harmful proteins from the brain (5). Finally, astrocytes are the CNS...
antioxidant system by providing glutathione (GSH) and ascorbic acid (6). Soon after TBI, astrocytes lose
their ability to maintain ion homeostatic (a), such disbalance set in action a cascade of changes in astrocyte
physiology. As consequence, astrocytes might lose their ability to provide metabolic support to neurons
(b), reduce the expression of glutamate transporters possibly leading to acute excitotoxicity (c),
downregulate the production of GSH and ascorbic acid which may increase oxidative stress (e), and cause
microglial reactivity through the release of inflammatory molecules (d). Astrocytes may also contribute to
TBI pathophysiology by disrupting the integrity of the BBB by the release of pro-inflammatory agents and
failing to clear toxic proteins from the brain (f). Additionally, lately, it has been reported that astrocytes
may engage in neuroprotective responses such as the remodeling of neuronal networks by releasing
perineural proteins that favor the growth and establishment of newborn neurons (g).

1.8 Treatment for TBI

Despite all the efforts, to date, there is no single treatment that modifies the repeated head
trauma-mediated disease or halts the progression of secondary mechanisms of injury into long-
term disability and the risk of developing neurodegenerative diseases. According to Ahmed
(2022), in 2020 and 2021, there were 44 completed clinical trials where 30 studies failed to
provide an improvement on TBI patients, 7 studies showed improvement in treated patients but
lacked statistical data, 4 studies did not show comparisons between treated and control, 2
studies reported worse outcomes in the treated patients relative to controls, and only 1
demonstrated significant improvement in the treated group compared to the control (Ahmed,
2022). The failed clinical trials were expected to improve symptoms, namely, memory and
attention deficit, aggression, mood swings, neuroendocrine and cerebrovascular dysfunction,
headaches, and alcohol use. The only successful clinical trial was a palliative treatment that
reduced salivation in TBI patients with moderate to severe damage. Several factors have been
identified to contribute to the failure of developing effective TBI treatments. The heterogeneity
of TBI secondary mechanism of injury and the interindividual variation of outcomes are suggested
to be a strong contributing factor to the failure of translatable therapeutic approaches (Marklund
& Hillered, 2011). In 2019, Alves and colleagues pinpointed possible reasons for such failure at
three levels of research. For instance, at the fundamental level, ignoring the complex metabolic changes occurring after TBI by focusing on a single factor or pathway of a molecular or cellular system. At the translational level, the utilization of animal models that do not resemble the reality of the human condition (e.g., craniotomy in some mouse models of TBI) as well as inadequate therapeutic windows. Lastly, at the clinical level, the lack of stratification concerning the type of injury, sex, age and co-morbidities of the cohorts (Alves et al., 2019).

Currently, the only available medical interventions are palliative treatments that help relieve or manage acute symptoms based on the severity of the TBI. Treatments range from analgesics to surgical intervention to rehabilitation (Galgano et al., 2017; Jha & Ghewade, 2022). For instance, for mTBI, over-the-counter pain relievers are utilized to treat headaches. For moderate and severe TBI, anti-seizure drugs, as well as surgical approaches to restore oxygen, blood supply, blood pressure, and intracranial pressure, are employed to minimize additional damage to the brain. Additionally, in cases of moderate to severe TBI, physical, emotional, and psychological rehabilitation might also be needed to relearn basic skills and improve behavioral health (Mayo Clinic, 2021).

Through this literature review, my goal has been to expose several areas of the field that are either understudied or under great debate mainly regarding the roles of astrocytes in the pathophysiology of mTBI and, with a further accentuated deficit, r-mTBI. However, to address all of these areas is beyond the scope of a single thesis. The current thesis focuses on interrogating the impact of tau accumulation in astrocytes (tau astrogliopathy) on astroglial responses as well as its implications in TBI pathophysiology. For addressing our first question, we generated a model of tau astrogliopathy (based on previous reported models by Trojanowski (Dabir et al., 2006; Forman et al., 2005)) where mice where genetically engineered to produce human mutant tau under the astrocyte specific GFAP promoter. These mice will be referred as GFAPP301L mice throughout the thesis. For addressing our second question, we exposed GFAPP301L mice to a closed-head model of r-mTBI, consisting of 20 head injuries over the course of a month. It is worth mentioning that we selected the closed-head injury model over others (e.g., Fluid percussion injury model, controlled cortical impact model, weight-drop injury model and blast injury model) because we wanted to replicate the etiology of the hit in repeated mild head injury human cases.
and because it is a model that has been extensively characterized in our lab (Mouzon et al., 2012, 2014, 2018, 2019; Ojo et al., 2013, 2015, 2016).

1.9 Hypothesis

*Repetitive mTBI promotes intracellular tau accumulation in astrocytes leading to cellular impairment which in turn exacerbates the chronic neuroinflammatory response and tau spread after injury.*

1.10 Aims

1. Describing the chronic histopathological and biochemical changes in mouse models with tau-bearing astrocytes (GFAP$^{P301L}$) and tau-bearing neurons (CaMKIIα$^{P301L}$) compared with WT mice under normal aging and following r-mTBI/sham injury.

2. Describing the transcriptomic changes in tau-bearing astrocytes under normal circumstances and after r-mTBI; and assessing their similarity with gene array changes in human CTE astrocytes.

3. Interrogating the effects of tau-bearing astrocytes on inducing neuroinflammation.
CHAPTER 2: CHRONIC EFFECTS OF REPETITIVE MILD TBI ON ASTROGLIAL, MICROGLIAL AND NEURONAL RESPONSE IN MOUSE MODELS OF TAU-BEARING ASTROCYTES vs NEURONS.

2.1 INTRODUCTION

Repetitive mild traumatic brain injury (r-mTBI) has been associated with an increased risk of neurodegenerative diseases such as Chronic Traumatic Encephalopathy (CTE). Although the pathophysiological changes emerging from TBI are well-described, little is understood about the underlying cellular and molecular mechanisms that lead to neurodegenerative outcomes found in r-mTBI/CTE. Pathognomonic lesions in CTE are characterized by the accumulation of phosphorylated tau in neurons and astrocytes (i.e., 4R-tau astrogliopathy) in the perivascular foci in the depths of the sulci (Bieniek et al., 2021; A. McKee et al., 2016). Accumulation of phosphorylated tau in neurons is well accepted in the field as a hallmark pathological criterion for CTE diagnosis, and it is thought to cause dysfunctional axonal transport and synaptic transmission (Rajmohan & Reddy, 2017). However, tau astrogliopathy in CTE remains a controversial pathological feature linked to chronic consequences of exposure to r-mTBI. It is also unknown whether it is a molecular substrate responsible for driving any of the neurological abnormalities following r-mTBI. Very little is understood about the pathophysiological consequences of aggregated tau within astrocytes in the context of TBI and/or other neurodegenerative diseases. Considering the potential of pathogenic tau to impair cellular function, tau astrogliopathy has become an area of great interest; particularly, because astroglial dysfunction can have substantial effects on CNS homeostasis, BBB integrity, and trophic and metabolic support for neurons. From our search of the current literature, there are only a few studies that provide insight into the response of astrocytes to endogenous overexpression of 4R-tau. In-vitro studies have indicated that astrocytes are more vulnerable to oxidative stress and lose their ability to clear synaptic glutamate which increases the susceptibility of neurons to neuronal hyperexcitability and excitotoxic damage (Ezerskiy et al., 2022; Hallmann et al., 2017). Additionally, a group of scientists led by Trojanowski developed two transgenic mouse models of
astroglial tauopathy (Dabir et al., 2006; Forman et al., 2005). In their models, astrocytes were engineered to produce either human WT tau (\(\text{tau}^\text{WT}\)) or human pathogenic tau with the P301L mutation (\(\text{hTau}^{\text{P301L}}\)) under the astrocyte-specific promoter, glial fibrillary acidic protein (GFAP). Both transgenic models (GFAP\(^\text{WT}\) and GFAP\(^\text{P301L}\), respectively) manifested an age-dependent accumulation of tau within astrocytes with mild axonal degeneration by the age of 20 months. Both models also manifest a significant reduction of glutamate transporters, GLAST and GLT1, as early as 5 months, in regions where astrocytic tau accumulation is robust (brainstem and spinal cord). However, there are no studies that look at the effect of r-mTBI in the context of tau astrogliopathy and the impact of such interaction on astroglial homeostasis and pathobiology. Thus, in the first aim of my thesis, we were interested in interrogating the effects of r-mTBI on promoting, accelerating, or exacerbating tau astrogliopathy and whether this had detrimental consequences on astroglial pathobiology and the promotion of TBI-related neurodegeneration. Because GFAP\(^{\text{P301L}}\) mice were no longer available from Dr. Trojanowski, we had to generate our GFAP\(^{\text{P301L}}\) model (Fig. 2.1A-C). We favored the utilization of human P301L mutant tau over human WT tau due to its preferential aggregation of 4R tau (Strang et al., 2019) which is particularly relevant in various primary tauopathies such as CTE, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and age-related tau astrogliopathy (ARTAG) (Reid et al., 2020).

Moreover, we were also interested in comparing r-mTBI-mediated astroglial responses in astrocytes overexpressing htau\(^{\text{P301L}}\) (GFAP\(^{\text{P301L}}\) model) vs astrocytes that were exposed to neuronal overproduction of htau\(^{\text{P301L}}\). For that comparison, we compared our model of tau astrogliopathy and the well-known model of neuronal tauopathy, CaMKII\(\alpha^{\text{P301L}}/\text{rTg4510}\) mice. These mice were genetically engineered to produce hTau\(^{\text{P301L}}\) under the neuron-specific promoter, CaMKII\(\alpha^{\text{P301L}}\).

This thesis project was designed to fill the current gaps in the literature regarding the link between TBI and tau astrogliopathy, and whether accumulation of pathogenic tau drives astroglial pathobiology and neurodegeneration at sub-chronic (3 months) and chronic (6 months) time points after the last injury. In addition, the very limited literature on the effects of tau accumulation in astroglial pathophysiology under normal circumstances prompted us to also assess the histopathological effects of astroglial tauopathy and compare them to the effects of
neuronal tauopathy without the TBI component by using the r-sham groups (i.e., GFAP\textsuperscript{P301L} vs WT and CaMKII\alpha\textsuperscript{P301L} vs WT).

In this chapter, our experiments sought to test the following hypotheses:

a) GFAP\textsuperscript{P301L} sham mice show an age-dependent increase in tau astrogliopathy. Additionally, early exposure to r-mTBI (pre-onset of pathology) will accelerate and exacerbate tau accumulation in astrocytes and brain-wide tauopathy.

b) In sham mice, human pathogenic tau (htau\textsuperscript{P301L}) overexpression in astrocytes (GFAP\textsuperscript{P301L} model) leads to an increased astroglial and microglial response compared to tau\textsuperscript{WT} astrocytes (i.e., WT and CaMKII\alpha\textsuperscript{P301L} models). Astroglial and microglial responses in the GFAP\textsuperscript{P301L} model are increased by the exposure to r-mTBI.

c) In sham mice, htau\textsuperscript{P301L} -bearing astrocytes undergo dysregulation of astroglial homeostatic markers compared to WT and CaMKII\alpha\textsuperscript{P301L} astrocytes. The dysregulation is more pronounced after r-mTBI.

d) In sham mice, overexpression of htau\textsuperscript{P301L} in astrocytes affects synaptic integrity in the GFAP\textsuperscript{P301L} mice compared to their WT and CaMKII\alpha\textsuperscript{P301L} counterparts. Synaptic integrity will be further affected after r-mTBI exposure.
2.2 METHODS AND MATERIALS

2.2.1 Animals

This study investigated three-month-old male and female C57BL/6 mice \( (n=24) \) (JAX ID: 000664), GFAP\(^{P301L} \) \( (n=24) \) (obtained by crossing GFAP-tTA mice and tetO-MAPT*P301L mice, JAX IDs: 005964 and 015815, respectively), and CaMKII\(^{P301L} \) \( (n=24) \) (obtained by crossing Camk2a-tTA mice and tetO-MAPT*P301L mice, JAX IDs: 007004 and 015815, respectively). GFAP\(^{P301L} \) and
CaMKIIα<sup>P301L</sup> (Tg4510) express the P301L MAPT mutation (4R/0N-human) under the GFAP or CaMKIIα promoter leading to tau expression in astrocytes or neurons, respectively. Both transgenic models have FVB-C57BL/6 background. Mice were housed in a 12h light/dark cycle with food and water <i>ad libitum</i>. All experiments were performed in accordance with Office of Laboratory Welfare and National Institutes of Health guidelines with Roskamp Institute Institutional Animal Care and Use Committee approval.

2.2.2 Traumatic Brain Injury Administration

Our well-characterized closed-head mild TBI (Mouzon et al., 2012, 2014, 2018, 2019) was used in a chronic repetitive injury paradigm involving 20 mTBI over the course of four weeks, as described by Pearson et al., (2023). Briefly, mice subjected to r-mTBI were anesthetized with 1.5L/min and 3% isoflurane for 3 minutes, the injury site was shaved. After shaving, mice were positioned in a stereotaxic frame (Stereotaxic Instrument, Stoelting, Wood Dale, Illinois) attached to an impactor (Impact One Stereotaxic Motorized Impactor, Richmond, Illinois). The hit was administrated above the mid-sagittal suture in the central area of the skull on a shaved head. Mice were placed on a heating pad to maintain animal body temperature at 37°C. The head impact was performed using a 5mm blunt metal impactor tip with 1mm strike depth. The impact was delivered at 5m/s with a force of 72N. Injured mice were allowed to recover on a heating pad set at 37°C until they gained consciousness, to prevent hypothermia. Mice were injured every weekday for 5 days a week for four weeks resulting in 20 hits in a month. The sham counterparts were exposed to isoflurane with the same frequency and duration. Mice were sacrificed 3 months or 6 months post-last injury (3mpi and 6mpi, respectively) for subsequent histopathological, biochemical, or transcriptomic analyses.
Figure 2.2. Study timeline of experiments of Chapter 2. Three-month-old wild-type (WT), GFAP\textsuperscript{P301L}, and CaMKII\textalpha\textsuperscript{P301L} were subjected to mild TBI every weekday for 5 days a week for four weeks resulting in 20 hits in a month. Three and six months post-last injury brain mouse tissue was collected for histopathological and biochemical analysis.

2.2.3 Immunohistochemistry

After transcardiac perfusion, mice were decapitated. Half brains of each mouse were assigned for immunohistochemistry and collected in 4% PFA. After 24-48h brains were dehydrated and paraffin-embedded using the Tissue-Tek\textsuperscript{®} VIP and Tissue-Tek\textsuperscript{®} TEC, respectively. Brains were sectioned at 6μm using a Leica RM2235 microtome and mounted on slides. Sections were deparaffinized using HistoClear\textsuperscript{®} and rehydrated in a decreasing gradient of ethanol before the immunohistochemistry procedures.

Immunofluorescence: Following rehydration, slides were submerged in PBS for 5 minutes to wash off the excess ethanol. For better detection of some antigens, antigen retrieval was performed using an acidic buffer (pH 6). Using a pressure cook, antigen retrieval is warmed up, then slides are immersed and heated up for a further 7 minutes in the microwave. Following antigen retrieval, sections were blocked with 5% normal donkey serum dissolved in 0.1% TritonX-100 phosphate-buffered saline (PBST) at room temperature for 1 hour. Next, sections were immunostained using primary antibodies (see Table 2.1) made on 2% normal donkey serum PBST. After overnight incubation at 4°C, sections were rinsed with PBS and transferred to a solution containing the appropriate fluorophore-conjugated secondary antibody (Alexa Fluor antibodies 648nm, 555nm, 488nm 1:500) at room temperature for 1 hour. Then, autofluorescence was quenched from the sections with an autofluorescence removal reagent (Millipore Sigma). Finally,
sections were mounted using anti-fade mounting media with DAPI. Images of sections were collected using a ZEISS LSM 800 confocal microscope.

**Immunohistochemistry:** Following rehydration, slides were submerged in PBS for 5 minutes to wash off the excess ethanol. Then slides were immersed in hydrogen peroxide for 15 minutes to deactivate endogenous peroxidases. Next, antigen retrieval was performed using an acidic buffer (pH 6). Sections were incubated with 2.5% normal goat serum at room temperature for 1 hour and subsequently incubated with primary antibody at 4°C overnight. The next day, slides were rinsed in PBS and incubated with horse radish peroxidase (HRP)-labeled secondary antibody at room temperature for 1 hour. The staining was developed using 3,3’-Diaminobenzidine (DAB). Finally, after slight counterstaining with hematoxylin, tissue was dehydrated in an increasing gradient of ethanol. Next, slides were mounted using toluene. Images of sections were collected using an Olympus DP72 microscope.

2.2.4 Microscopy and Image Analysis

**Confocal microscopy:** Multiple regions of interest in the cortex and corpus callosum (CC) were analyzed in a standardized fashion for each marker. For each mouse (n=6), a minimum of 20 microscopic fields per region from 4 sagittal sections were collected using the 20x objective lens. Images from individual channels (wavelengths 648nm, 555nm, 488nm, and 450nm) were used independently for subsequent percentage area analysis. The immunoreactivity of cell markers was measured by quantitative image analysis performed blindly by the investigator using ImageJ software. Quantification of GFAP+/RZ3+ astrocytes was performed in the entire cortical and hippocampal areas of the 4 sagittal sections.

**Bright-field microscopy:** Multiple regions of interest in the cortex (underneath the impact site) and corpus callosum (CC) were analyzed in a standardized fashion for each marker. For each mouse (n=6), a minimum of 20 microscopic fields per region from 4 sagittal sections were collected using the 20x/40x/60x objective lenses. The immunoreactivity of cell markers was measured by quantitative image analysis performed blindly by the investigator using ImageJ software. Before quantitative analysis, color deconvolution was applied on bright-field images.
to separate the DAB staining from the hematoxylin staining. Then percentage area analysis was performed using the “DAB” set of images.

2.2.5 Western Blotting

Hippocampal and hippocampus-depleted hemisphere samples were sonicated in 250μl of mammalian protein extraction reagent (plus protease and phosphatase inhibitors) to ensure maximal protein extraction. Samples were centrifuged at 14000rpm for 20 minutes at 4°C to pellet out debris. To ensure that the same amount of protein in every sample was being analyzed through immunoblotting, the total amount of protein was determined using the Bicinchoninic acid assay. Ten micrograms of extracted protein were mixed with a denaturing buffer containing β-mercaptoethanol and boiled at 95°C for 10 minutes. Samples were then loaded on 4-15% SDS-PAGE gels for protein separation. Next proteins were transferred onto PVDF membranes overnight. Transferred membranes were blocked with 5% non-fat milk in 0.05% Tween 20 Tris-buffered Saline (TBST) for 1 hour at room temperature. Subsequently, membranes were incubated with the primary antibodies (see Table 1 for antibody list) at 4°C overnight. Membranes were washed in TBST 3 times (5 minutes each) prior to the exposure of HRP-conjugated secondary antibodies (see Table 1) for 1 hour at room temperature. Membranes were washed in TBST and deionized water and developed by using a chemiluminescent system (ECL or FEMTO) (Thermofisher, USA). Imaging of the revealed markers was done using a ChemoDoc MP imager. Subsequently, densitometry analysis of each marker was done employing Image Lab software. Total protein obtained by Ponceau staining was used to normalize the data.
<table>
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<th>Host species</th>
<th>Vendor</th>
<th>Cat. number</th>
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Table 2.1. Antibodies used. Abbreviations: GFAP, Glial Fibrillary Acidic Protein; Iba1, Ionized calcium-binding adaptor molecule; AQP4, aquaporin 4; GLAST, Glutamate transporter; GLT1, Glutamate transporter 1; PSD95, Post-synaptic density 95. IF, Immunofluorescence; IHC, Immunohistochemistry; WB, Western blotting. *Primary antibody conjugated to 488-fluorophore.
2.2.6 Statistical analyses

The statistical analysis of all presented data, except bioinformatic data, was performed using GraphPad Prism Version 9. Data were tested for normality using the Shapiro-Wilk test. Then statistical analysis was obtained using Two-way ANOVA accompanied by the Benjamini, Krieger, and Yekuteli test to correct multiple comparisons. In the cases where data were not normally distributed, we performed log transformation data. If after transformation, data remained with a non-normal distribution, the Kruskal-Wallis non-parametric test was performed. Data are presented as mean ±SEM. P values <0.05 were considered statistically significant. Graphs in the Results Section show asterisks representing different p-value ranges: < 0.03=*; <0.02=** and <0.01=***.

2.3 RESULTS

Three-month-old WT, GFAP$^{P301L}$, and CaMKIIα$^{P301L}$ mice were subjected to our 20-hit model of r-mTBI (or r-sham), and three and six months after the last injury (at the age of 7 and 10 months, respectively), we performed histopathological and biochemical analyses on brain tissue to investigate the effects that r-mTBI has on tau astrogliopathy and how this interaction affects tauopathy, neuroinflammation (astrocyte and microglial reactivity), astrocytic homeostasis and synaptic integrity. However, before assessing the TBI effect, we found it relevant to first evaluate the histopathological and biochemical effects of tau astrogliopathy alone under normal circumstances. To accomplish this, we utilized the data from the sham cohorts of each genotype at 3 and 6 months post-last isoflurane exposure (7 and 10 months of age, respectively).

2.3.1 Astroglial vs neuronal tau-mediated changes in tauopathy within astrocytes and in the cortex, the hippocampus, and hippocampus-depleted hemisphere at 7 and 10 months of age.

To analyze tau pathology in the regions of interest we have selected several markers of phosphorylated species that had been reported to favor tau hyperphosphorylation and aggregation such as tau phosphorylated at Threonine 231 (RZ3), Serine 202 (CP13) and Serine 396/Serine 404 (PHF1) (Alonso et al., 2004; Gong & Iqbal, 2008). Thus, to examine the effects of astroglial vs neuronal mutant human tau (htau$^{P301L}$) on promoting tau pathogenesis we analyzed the abundance of tau phosphorylation within astrocytes and in the cortex and hippocampus by
immunohistochemistry. First, we counted the amount of double positive GFAP\textsuperscript{+ve}/RZ3\textsuperscript{+ve} astrocytes in 4 sagittal sections of the cortex and hippocampus of our three mouse models. As expected, we did not find GFAP\textsuperscript{+ve}/RZ3\textsuperscript{+ve} astrocytes in WT and CaMKII\textalpha\textsubscript{P301L} mice at any age. In the GFAP\textsuperscript{P301L} mice, we counted 6 ±1.4 and 5 ±1.8 GFAP\textsuperscript{+ve}/RZ3\textsuperscript{+ve} astrocytes in the cortex and hippocampus, respectively, at 7 months of age (Fig. 2.3A). At 10 months of age, we counted 39 ±7 and 8 ±2.5 GFAP\textsuperscript{+ve}/RZ3\textsuperscript{+ve} astrocytes in the cortex and hippocampus, respectively (Fig. 2.3B). The two-way ANOVA revealed a significant age effect in the cortex [F(1,16)=7.696, p=0.014] and hippocampus [F(1,16)=4.862, p=0.042].

Next, we assessed RZ3 immunoreactivity in the cortex of our mouse models. At 7 months of age, tau phosphorylation was not affected in the brains harboring tau-bearing astrocytes vs WT, however, there was a significant ~12-fold increase in CaMKII\textalpha\textsubscript{P301L} mice compared to both WT and GFAP\textsuperscript{P301L} (Fig. 2.3C). On the contrary, at 10 months of age, there was a significant ~3-fold and ~6-fold increase in tau phosphorylation in the GFAP\textsuperscript{P301L} and CaMKII\textalpha\textsubscript{P301L} compared to the WT, respectively (Fig. 2.3D) because there was a reduction in RZ3 immunoreactive tau phosphorylation in the CaMKII\textalpha\textsubscript{P301L} mice at 10 months of age compared to their younger counterparts (CaMKII\textalpha\textsubscript{P301L} 7 months of age).

Lastly, we evaluated using immunoblotting the effects of astroglial vs neuronal tau on brain-wide tauopathy by assessing tau phosphorylation (RZ3, CP13 and PHF1) and total tau (DA9), separately analyzing the hippocampus (HIPPO) and the hippocampus-depleted hemisphere for each of our three mouse models. Because tau abundance in the CaMKII\textalpha\textsubscript{P301L} model was disproportionately increased compared to the other models, we performed a separate Two-Way analysis using WT and GFAP\textsuperscript{P301L} data only, for all markers of interest.
Figure 2.3: RZ3 immunoreactivity in the cortex and hippocampus of WT, GFAP<sup>P301L</sup> and CaMKII<sub>α</sub><sup>P301L</sup> mice at 3- and 6-months post-last injury. RZ3/GFAP+ cells in the cortex, CTX (A) and Hippocampus, HIPP (B) from 4 serial sagittal sections at 3-months and 6-months post-last injury. RZ3 immunoreactive percent area in the cortex at 3-months (C) and 6-months (D) post-last injury. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses. Representative images of the immunofluorescent staining are shown in Fig. 2.3E.
Figure 2.3 (continuation): RZ3 immunoreactivity in the cortex and hippocampus of WT, GFAP<sup>P301L</sup> and CaMKII<sub>a</sub><sup>P301L</sup> mice at 3- and 6-months post-last injury. (E) Image at the top indicates the region of interest (yellow box) where the images were taken (red dot indicates impact site). Qualitative images of RZ3 (red) immunoreactivity in the cortex of WT, GFAP<sup>P301L</sup> and CaMKII<sub>a</sub><sup>P301L</sup> mice 3-months after r-mTBI/sham injury. Top-two panels depict RZ3 in WT, GFAP<sup>P301L</sup> and CaMKII<sub>a</sub><sup>P301L</sup> 3mpi (sham and TBI). Bottom-two panels depict RZ3 in WT, GFAP<sup>P301L</sup> and CaMKII<sub>a</sub><sup>P301L</sup> 6mpi (sham and TBI). Images were captured at x20 magnification. Scale bar 50μm.
At 7 months of age, in the hemisphere (without HIPPO), there was a very significant 82-fold increase in phosphorylated tau assessed by RZ3 levels in CaMKIIα<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(2,22)=14.14, p=<0.001]) (Fig. 2.4A). In contrast, there was no difference in RZ3 levels in the hemisphere (no HIPPO) of GFAP<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(1,17)=0.03, p=0.861]) (Fig. 2.4B). CP13 levels showed a significant 48-fold and 10-fold increase in CaMKIIα<sup>P301L</sup> mice (two-way ANOVA: genotype effect [F(2,24)=10.64, p=<0.001]) and GFAP<sup>P301L</sup> mice (two-way ANOVA: genotype effect [F(1,17)=7.88, p=0.12]), compared to WT, respectively (Fig. 2.4C-D). PHF1 levels showed a significant 50-fold increase in CaMKIIα<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(2,25)=9.18, p=0.001]) (Fig. 2.4E) while there was a significant 3-fold genotype effect towards an increase in GFAP<sup>P301L</sup> compared to WT (two-way ANOVA: genotype effect [F(1,18)=6.018, p=0.025]) (Fig. 2.4F). In the case of total tau abundance, there was a significant ~16-fold and ~2.5-fold increase in DA9 levels in CaMKIIα<sup>P301L</sup> (two-way ANOVA: genotype effect [F(2,22)=71.66, p=<0.001]) and GFAP<sup>P301L</sup> (two-way ANOVA: genotype effect [F(1,17)=21.02, p=<0.001]) compared to WT, respectively (Fig. 2.4G-H).

In the hippocampus, there was a significant 40-fold increase in RZ3 in the CaMKIIα<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(2,26)=26.91, p=<0.001]) (Fig. 2.5A) while there was a significant 2-fold genotype effect towards an increase in GFAP<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(1,19)=5.72, p=0.027]) (Fig. 2.5B). CP13 levels showed a significant ~2.5-fold increase in CaMKIIα<sup>P301L</sup> compared to WT (two-way ANOVA: genotype effect [F(2,26)=55.44, p=<0.001]) while there was no difference GFAP<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(1,18)=2.44, p=0.135]) (Fig. 2.5C-D). PHF1 levels showed a significant 270-fold increase in CaMKIIα<sup>P301L</sup> compared to WT (two-way ANOVA: genotype effect [F(2,26)=110.5, p=<0.001] (Fig. 2.5E) and a significant 17-fold increase in GFAP<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(1,18)=7.96, p=0.011]) (Fig.2.5F). In the case of total tau abundance, there was a significant 68-fold increase in DA9 levels in CaMKIIα<sup>P301L</sup> compared to WT (two-way ANOVA: genotype effect [F(2,26)=101.2, p=<0.001]) (Fig. 2.5G) and a significant 18-fold increase in GFAP<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(1,19)=28.19, p=<0.001]) (Fig. 2.5H).
Figure 2.4: Changes in Tau species (phosphorylated and total tau) in the half hemispheres of WT, GFAP<sup>P301L</sup> and CaMKI<sub>a</sub><sup>P301L</sup> mice at 3 months post-last injury. Levels of RZ3 (A, B), CP13 (C, D), PHF1 (E, F) and DA9 (G, H) in the hemi-brain (hemisphere minus the hippocampus) at 3 months post-last injury. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses. Note: Graphs from B, D, F and H are from WT and GFAP<sup>P301L</sup> cohorts alone. Representative immunoblots from the hippocampus-depleted hemisphere are depicted on the left of the graphs.
Figure 2.5: Changes in Tau species (phosphorylated and total tau) in the hippocampus of WT, GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice at 3 months post-last injury. Levels of RZ3 (A, B), CP13 (C, D), PHF1 (E, F) and DA9 (G, H) in the hippocampus (HIPPO) at 3 months post-last injury. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses. Note: Graphs from B, D, F and H are from WT and GFAP<sup>P301L</sup> cohorts alone. Representative immunoblots from the hippocampus are depicted on the left of the graphs.
At 10 months of age, in the hemisphere (without HIPPO), there was a significant 10-fold increase in RZ3 levels in CaMKIIα<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(2,25)=55.64, p=<0.001]) (Fig. 2.6A) and a significant 2-fold increase in GFAP<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(1,18)=11.41, p=0.003]) (Fig. 2.6B). CP13 levels showed a significant 100-fold increase in CaMKIIα<sup>P301L</sup> mice (two-way ANOVA: genotype effect [F(2,24)=31.54, p=<0.001]) and no changes in GFAP<sup>P301L</sup> mice (two-way ANOVA: genotype effect [F(1,16)=0.46, p=0.505]) compared to WT, respectively (Fig. 2.6C-D). PHF1 levels showed a significant 8-fold increase in CaMKIIα<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect [F(2,24)=70.96, p=<0.001]) (Fig. 2.6E) while there was no difference in GFAP<sup>P301L</sup> compared to WT (two-way ANOVA: genotype effect [F(1,16)=4.32, p=0.054]) (Fig. 2.6F). In the case of total tau abundance, there was a significant 7-fold and 3-fold increase in DA9 levels in CaMKIIα<sup>P301L</sup> (two-way ANOVA: genotype effect [F(2,26)=74.24, p=<0.001]) and GFAP<sup>P301L</sup> (two-way ANOVA: genotype effect [F(1,18)=13.74, p=0.002]) compared to WT, respectively (Fig. 2.6G-H).
Figure 2.6: Changes in Tau species (phosphorylated and total tau) in the half hemispheres of WT, GFAP\textsuperscript{P301L} and CaMKII\textsuperscript{αP301L} mice at 6 months post-last injury. Levels of RZ3 (A, B), CP13 (C, D), PHF1 (E, F) and DA9 (G, H) in the hemi-brain (hemisphere minus the hippocampus) at 6 months post-last injury. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-Way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses.

Note: Graphs from B, D, F and H are from WT and GFAP\textsuperscript{P301L} cohorts alone. Representative immunoblots from the hippocampus are depicted on the left of the graphs.
In the hippocampus, there was a significant 26-fold increase in RZ3 in the CaMKIIα<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect \([F(2,26)=70.07, p=<0.001]\)) (Fig. 2.7A) while no changes in GFAP<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect \([F(1,18)=0.378, p=0.546]\)) (Fig. 2.7B). CP13 levels showed a significant 161-fold increase in CaMKIIα<sup>P301L</sup> compared to WT (two-way ANOVA: genotype effect \([F(2,24)=33.78, p=<0.001]\)) while no difference between GFAP<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect \([F(1,16)=1.06, p=0.318]\)) (Fig. 2.7C-D). PHF1 levels showed a significant 17-fold increase in CaMKIIα<sup>P301L</sup> compared to WT (two-way ANOVA: genotype effect \([F(2,25)=156.5, p=<0.001]\) (Fig. 2.7E) but no difference in GFAP<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect \([F(1,17)=0.106, p=0.748]\)) (Fig. 2.7F). In the case of total tau abundance, there was a significant 27-fold increase in DA9 levels in CaMKIIα<sup>P301L</sup> compared to WT (two-way ANOVA: genotype effect \([F(2,26)=184.5, p=<0.001]\)) (Fig. 2.7G) and a significant 3-fold in GFAP<sup>P301L</sup> mice compared to WT (two-way ANOVA: genotype effect \([F(1,18)=20.05, p=<0.001]\)) (Fig. 2.7H).
Figure 2.7: Changes in Tau species (phosphorylated and total tau) in the hippocampus of WT, GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice at 6 months post-last injury. Levels of RZ3 (A, B), CP13 (C,D), PHF1 (E, F) and DA9 (G, H) in the hippocampus (HIPPO) at 6 months post-last injury. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses. Note: Graphs from B, D, F and H are from WT and GFAP<sup>P301L</sup> cohorts alone. Representative immunoblots from the hippocampus are depicted on the left of the graphs.
2.3.2 Astroglial vs neuronal tau-mediated changes on astrocyte and microglial reactivity in the cortex and corpus callosum at 7 and 10 months of age.

To investigate the effect of astrocyte-derived and neuronal-derived tau on astrocyte and microglial morphological phenotype at baseline, we quantified the percentage area of GFAP/S100B and IBA1 immunoreactivity in the cortex and the corpus callosum (CC) to evaluate astrocyte and microglial reactivity, respectively (Fig. 2.8.1-2.8.2). At 7 mo. of age, the presence of tau within astrocytes in the GFAP<sup>P301L</sup> model did not influence S100B, GFAP, and IBA1 immunoreactivity levels in the cortex or GFAP and IBA1 immunoreactivity in the CC (Fig. 2.9) compared to WT. However, the presence of neuronal mutant tau in the CaMKII<sub>α</sub><sup>P301L</sup> mice led to a significant 3-fold increase in s100b immunoreactivity in the cortex (two-way ANOVA: genotype effect [F(2,25)=11.78, p=<0.001]), (Fig. 2.9A); a significant 10-fold and a 3-fold increase in GFAP immunoreactivity within the cortex and CC, respectively (two-way ANOVA: genotype effect [F(2,23)=40.54, p=<0.001]; and genotype effect [F(2,25)=8.63, p=0.001], respectively), compared to both WT and GFAP<sup>P301L</sup> models (Fig. 2.9C&G). Additionally, IBA1 immunoreactivity in the cortex and CC of the CaMKII<sub>α</sub><sup>P301L</sup> was significantly greater compared to the other two genotypes. Those changes were represented by a 2-fold and 4-fold increase in the cortex and CC, respectively (two-way ANOVA: genotype effect [F(2,23)=28.55, p=<0.001]) (Fig. 2.9E&H).
Figure 2.8.1: Qualitative images of astrocyte and microglial activation markers in the cortex of WT, GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice 3-months after r-mTBI/sham injury. Top right image indicates the region of interest (yellow box) where the images were taken (red dot indicates impact site). Top-two (WT mice), Middle-two (GFAP<sup>P301L</sup>) and Bottom-two (CaMKIIα<sup>P301L</sup>) panels depicts S100β, GFAP and IBA1 immunostaining in the cortex (CTX). S100 and GFAP images were captured at x20 magnification; IBA1 images were captured at x40 magnification. Scale bars 50μm.
Figure 2.8.2: Qualitative images of astrocyte and microglial activation markers in the corpus callosum of WT, GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice 3-months after r-mTBI/sham injury. Top right image indicates the region of interest (yellow box) where the images were taken (red dot indicates impact site). Top-two (WT mice), Middle-two (GFAP<sup>P301L</sup>) and Bottom-two (CaMKIIα<sup>P301L</sup>) panels depicts S100β, GFAP and IBA1 immunostaining in the corpus callosum (CC). GFAP images were captured at x20 magnification; IBA1 images were captured at x60 magnification. Scale bars 50μm and 20μm, respectively.
Figure 2.9: Quantification of astrocyte reactivity (GFAP, S100B) and microglial reactivity (IBA1) in the cortex (CTX) and corpus callosum (CC) of WT, GFAP<sup>P301L</sup> and CaMKII<sup>α</sup>P301L mice 3-months after r-mTBI/sham injury. Percentage area of S100B (A,B), GFAP (C,D) and IBA1 (E,F) in the cortex tissue. Percentage area of GFAP (G) and IBA1 (H) in the corpus callosum tissue. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses. Note: Graphs from B, D and F are from WT and GFAP<sup>P301L</sup> cohorts alone.
At 10 months of age (Fig. 2.10.1 & 2.10.2), there were no changes in cortical S100B immunoreactivity across genotypes (two-way ANOVA: genotype effect [F(2,22)=4.37, p=0.025]), (Fig. 2.11A). Additionally, there were no changes in GFAP and IBA1 immunoreactivity between WT and GFAP\(^{P301L}\) mice (two-way ANOVA: genotype effect [F(1,12)=1.21, p=0.293], respectively), while both markers were significantly increased in the CaMKII\(\alpha\)^{P301L} cohort in the cortex and CC. In the cortex of CaMKII\(\alpha\)^{P301L} mice, we observed a significant 8-fold increase in GFAP immunoreactivity and a significant 4.5-fold increase in IBA1 immunoreactivity compared to WT and GFAP\(^{P301L}\) (two-way ANOVA: genotype effect [F(2,21)=, p=<0.001]; genotype effect [F(2,21)=50.45, p=<0.001], respectively) (Fig. 2.11C&G). In the CC of CaMKII\(\alpha\)^{P301L} mice, there was a significant 3-fold increase and a significant 4-fold increase in GFAP and IBA1 immunoreactivity compared to WT and GFAP\(^{P301L}\), respectively (two-way ANOVA: genotype effect [F(2,22)=10.17, p=<0.001]; genotype effect [F(2,24)=27.17, p=<0.001], respectively) (Fig. 2.11E&I).
Figure 2.10.1: Qualitative images of astrocyte and microglial activation markers in the cortex of WT, GFAP\textsuperscript{P301L} and CaMKII\textsubscript{αP301L} mice 6-months after r-mTBI/sham injury. Top right image indicates the region of interest (yellow box) where the images were taken (red dot indicates impact site). Top-two (WT mice), Middle-two (GFAP\textsuperscript{P301L}) and Bottom-two (CaMKII\textsubscript{αP301L}) panels depicts S100\textbeta, GFAP and IBA1 immunostaining in the cortex (CTX). Images were captured at x20 magnification. Scale bars 50μm.
Figure 2.10.2: Qualitative images of astrocyte and microglial activation markers in the corpus callosum of WT, GFAP\textsuperscript{P301L} and CaMKII\textsuperscript{αP301L} mice 6-months after r-mTBI/sham injury. Top right image indicates the region of interest (yellow box) where the images were taken (red dot indicates impact site). Top-two (WT mice), Middle-two (GFAP\textsuperscript{P301L}) and Bottom-two (CaMKII\textsuperscript{αP301L}) panels depicts GFAP at x20 magnification and IBA1 immunostaining at x10 magnification in the corpus callosum (CC). Scale bars 50μm.
Figure 2.11: Quantification of astrocyte (GFAP, S100B) and microglial (IBA1) reactivity in the cortex (CTX) and corpus callosum (CC) of WT, GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice 6-months after r-mTBI/sham injury. Percentage area of S100B (A, B), GFAP (C,D) and IBA1 (G, H) in the cortex tissue. Percentage area of GFAP (E, F) and IBA1 (I,J) in the corpus callosum tissue. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses. Note: Graphs from B, D, F, H and J are from WT and GFAP<sup>P301L</sup> cohorts alone.
2.3.3 Astroglial vs neuronal tau-mediated changes on astrocyte homeostatic markers in the hippocampus and hippocampus-depleted hemisphere at 7 and 10 months of age.

To investigate the influence of astroglial and neuronal tau on markers of astrocyte homeostasis, we assessed the protein levels of three common homeostatic markers by immunoblotting – the water channel aquaporin4 (AQP4) and the glutamate transporters (GLT1 and GLAST) – in the cortex and hippocampus of our three mouse models. At 7 months of age, no changes in AQP4, GLT1, and GLAST levels were observed in the hemisphere (without HIPPO) across the mouse...
models (Fig. 2.1A-D) (two-way ANOVA: genotype effect \(F(2,22)=2.83, p=0.08\); genotype effect \(F(2,26)=4.1, p=0.028\); genotype effect \(F(2,24)=2.50, p=0.103\), respectively). However, a significant reduction in AQP4 and GLT1 protein levels was observed in the hippocampus of both tau models (GFAP\(^{P301L}\) and CaMKII\(^{αP301L}\)) compared to the WT (Fig. 2.1E-G) (two-way ANOVA: genotype effect \(F(2,24)=24.43, p=<0.001\); genotype effect \(F(2,25)=49.14, p=<0.001\), respectively). GLAST levels in the hippocampus remained unchanged across genotypes (Fig. 2.1H) (two-way ANOVA: genotype effect \(F(2,27)=2.25, p=0.125\)). Since, hippocampal levels of AQP4 and GLT1 showed a dramatic decrease in our humanized tau models at 7 months of age, we performed a Pearson's correlation analysis to examine the relationship between hippocampal AQP4/ GLT1 levels and hippocampal tau protein levels (RZ3, PHF1, and DA9) (Fig. 2.13). There was a weak, negative correlation between AQP4 and both phosphorylated tau species (RZ3 and PHF1) (Fig. 2.13A-B); however, the relationship was not significant. In contrast, there was a weak, negative correlation between AQP4 and total tau \((r=-0.39)\); the relationship was significant \((p=0.035)\) (Fig. 2.13C). There was a weak negative correlation between GLT1 and RZ3 that was not significant (Fig. 2.13D). However, a weak negative correlation between GLT1 and PHF1 was observed \((r=-0.39)\); this relationship was significant \((p=0.03)\) (Fig. 2.13E). Besides, there was a moderate, negative correlation between GLT1 levels and DA9 levels \((r=-0.44)\); the relationship was significant \((p=0.016)\) (Fig. 2.13F).
Figure 2.12: Changes in astrocyte homeostatic protein markers in WT, GFAP<sup>P301L</sup> and CaMK<sub>I</sub><sup>δ</sup>P301L mice at 3 months post-last injury. Qualitative (A) and quantitative immunoblotting levels of aquaporin 4 (AQ4) (B) and glutamate transporters GLT1 and GLAST (C, D) in the hemi-brain (without the hippocampus). Qualitative (E) and quantitative immunoblotting levels of AQ4 (F) and glutamate transporters GLT1 and GLAST (G, H) in the hippocampus. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses.
Figure 2.13: Correlation between AQP4 or GLT1 and tau species (RZ3, PHF1 and DA9) in WT, GFAP\textsuperscript{P301L} and CaMKII\textsuperscript{P301L} mice at 3 months post-last injury. Correlation between AQP4 vs RZ3(pTau-Thr231) [A], PHF1(pTau-Ser396/404) [B], DA9-total tau [C] in the hippocampus. Correlation between GLT1 vs RZ3(pTau-Thr231) [D], PHF1(pTau-Ser396/404) [E], DA9-total tau [F] in the hippocampus. Data analyzed by Pearson’s correlation analysis, and simple linear correlation was used to determine the slope.
At 10 months of age, interestingly, there was a significant 4-fold increase in AQP4 levels in the hemisphere (without HIPPO) in GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice compared to WT (Fig. 2.1A-B) (two-way ANOVA: genotype effect [F(2,27)=9.48, p=<0.001]). Furthermore, there were no changes in GLT1 and GLAST levels across the mouse models (Fig. 2.1C-D) (two-way ANOVA: genotype effect [F(2,24)=0.97, p=0.393]; genotype effect [F(2,27)=4.10, p=0.028], respectively). In contrast to the 7-month-old mice, at 10 months of age, there were no changes in AQP4, GLT1, and GLAST protein levels in the hippocampus across genotypes (Fig. 2.1E-H) (two-way ANOVA: genotype effect [F(2,25)=2.12, p=0.141]; genotype effect [F(2,26)=1.21, p=0.312]; genotype effect [F(2,26)=0.004, p=0.996], respectively).
Figure 2.14: Changes in astrocyte homeostatic protein markers in WT, GFAP<sup>P301L</sup> and CaMKII<sup>α</sup><sub>P301L</sub> mice at 6 months post-last injury. Qualitative (A) and quantitative immunoblotting levels of aquaporin 4 (AQ4) (B) and glutamate transporters GLT1 and GLAST (C, D) in the hemi-brain (without the hippocampus). Qualitative (E) and quantitative immunoblotting levels of AQ4 (F) and glutamate transporters GLT1 and GLAST (G, H) in the hippocampus. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekutieli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses.
2.3.4 Astroglial vs neuronal tau-mediated changes in synaptic integrity changes in the hippocampus and hippocampus-depleted hemisphere at 7 and 10 months of age.

To investigate whether the cell-specific expression of pathogenic tau impairs synaptic integrity, we analyzed the protein levels of pre-synaptic proteins (synapsin 1 and synaptophysin) and a post-synaptic protein (PSD95) in cortical and hippocampal lysates of our mouse models. At 7 months of age, the levels of the pre-synaptic marker, synapsin 1, in the hemisphere (without HIPPO) of GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice revealed a significant ~2-fold and a 4.5-fold decrease, respectively, compared to WT (Fig. 2.15A-B) (two-way ANOVA: genotype effect [F(2,21)=27.91, p=<0.001]). There were no changes in the levels of synaptophysin and PSD95 across genotypes (Fig. 2.15C-D) (two-way ANOVA: genotype effect [F(2,24)=3.33, p=0.53]; genotype effect [F(2,24)=1.86, p=0.177], respectively). In the hippocampus, we observed a significant ~2-fold and a 3-fold decrease in synapsin of GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice compared to WT (Fig. 2.15E-F) (two-way ANOVA: genotype effect [F(2,26)=9.37, p=<0.001]). There were no differences in synaptophysin and PSD95 levels across the genotypes (Fig. 2.15G-H) (two-way ANOVA: genotype effect [F(2,27)=5.80, p=0.008]; genotype effect [F(2,26)=4.31, p=0.024], respectively). Since synapsin 1 protein levels showed a significant decrease in the brain of humanized tau models, we assessed Pearson’s correlation between synapsin 1 and tau protein levels (Fig. 2.16). In the hemisphere (without HIPPO), synapsin 1 and phosphorylated tau (RZ3 and PHF1) showed weak, negative correlation; they were not significant (Fig. 2.16A-B). In the case of the relationship between synapsin 1 and total tau, we observed a significant moderate, negative correlation (r=-0.52, p=0.004) (Fig. 2.16C). In the hippocampus, there was no correlation between synapsin 1 and tau species (Fig. 2.16D-F).
Figure 2.15: Changes in synaptic integrity protein markers in WT, GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice at 3 months post-last injury. Qualitative (A) and quantitative immunoblotting levels of synapsin (B), synaptophysin (C) and PSD95 (D) in the hemi-brain (without the hippocampus). Qualitative (E) and quantitative immunoblotting levels of synapsin (F), synaptophysin (G) and PSD95 (H) in the hippocampus. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses.
Figure 2.16: Correlation between synapsin and tau species (RZ3, PHF1 and DA9) in the hemi-brain or hippocampus of WT, GFAP\textsuperscript{P301L} and CaMKII\textsuperscript{αP301L} mice at 3 months post-last injury. Correlation between synapsin vs RZ3(pTau-Thr231) [A], PHF1(pTau-Ser396/404) [B], DA9-total tau [C] in the hemi-brain (without the hippocampus). Correlation between synapsin vs RZ3(pTau-Thr231) [D], PHF1(pTau-Ser396/404) [E], DA9-total tau [F] in the hippocampus. Data analyzed by Pearson’s correlation analysis, and simple linear correlation was used to determine the slope.
At 10 months of age, synapsin 1 levels in the hemisphere (without HIPPO) in the GFAP\textsuperscript{P301L} cohort showed a significant 3-fold increase compared to WT (Fig. 2.17A-B) (two-way ANOVA: genotype effect [F(2,27)=5.58, p=0.009). While there was no change between CaMKII\textsubscript{α}\textsuperscript{P301L} and WT mice. Additionally, synaptophysin and PSD95 remained unchanged (Fig. 2.17C-D) (two-way ANOVA: genotype effect [F(2,25)=1.92, p=0.167]; genotype effect [F(2,27)=0.08, p=0.919], respectively). In the hippocampus, there were no differences in synapsin 1, synaptophysin, and PSD95 protein levels in the hippocampus across the genotypes (Fig. 2.17E-H) (two-way ANOVA: genotype effect [F(2,26)=0.73, p=0.491]; genotype effect [F(2,27)=0.944, p=0.402]; genotype effect [F(2,27)=0.55, p=0.581], respectively).
Figure 2.17: Changes in synaptic integrity protein markers in WT, GFAP\textsuperscript{P301L} and CaMKII\alpha\textsuperscript{P301L} mice at 6 months post-last injury. Qualitative (A) and quantitative immunoblotting levels of synapsin (B), synaptophysin (C) and PSD95 (D) in the hemi-brain (without the hippocampus, HIPPO). Qualitative (E) and quantitative immunoblotting levels of synapsin (F), synaptophysin (G) and PSD95 (H) in the hippocampus. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses.
2.3.5 Tau astrogliopathy and tau phosphorylation in the cortex, hippocampus and hippocampus-depleted hemisphere three and six months post-last mTBI exposure.

Next, we sought to investigate the effect of r-mTBI on brain-wide tauopathy. First, we evaluated the abundance of GFAP^{+ve}/RZ3^{+ve} astrocytes in the cortex and hippocampus. Our analysis revealed that there was no TBI-mediated effect on the number of astrocytes positive for phosphorylated tau at 3mpi and 6mpi compared to their shams (2.3A-B) (two-way ANOVA: injury effect [F(1,16)=1.68, p=0.213], no injury*age interaction). Then, we analyzed RZ3 immunoreactivity in the cortical area underneath the impact site in our three mouse models. At 3mpi, r-mTBI did not cause an increase in phosphorylated tau (p-tau) in the WT cohort; however, the presence of astroglial pathogenic tau (GFAP^{P301L} mice) and neuronal pathogenic tau (CaMKII^{αP301L}) resulted in a significant increase in p-tau immunoreactivity in the cortex compared to their respective sham counterparts (Fig. 2.3C) (two-way ANOVA: injury effect [F(1,22)=4.87, p=0.038], no injury*age interaction). However, by 6mpi, no TBI effect was observed across the three different mouse models (Fig. 2.3D) (two-way ANOVA: injury effect [F(1,12)=0.07, p=0.788], no injury*age interaction).

We then assessed the TBI-mediated changes in tau phosphorylation (RZ3, CP13 and PHF1) as well as the total abundance of tau (DA9) in the hippocampus-depleted hemisphere. At 3mpi, r-mTBI did not elicit alterations in the levels of any marker of tau phosphorylation or the total amount of tau compared to sham in any of the three mouse models (Fig. 2.4A-H) (two-way ANOVA: RZ3 injury effect [F(1,22)=0.72, p=0.791]; CP13 [F(1,24)=0.86, p=0.362]; PHF1 [F(1,25)=0.024, p=0.877]; DA9 [F(1,22)=0.0001, p=990], no injury*age interaction). In contrast, in the hippocampus, we observed that exposure to r-mTBI in the CaMKII^{αP301L} cohort increased tau phosphorylation levels, but not total tau abundance compared to their sham controls (Fig. 2.5A-H) (two-way ANOVA: RZ3 injury effect [F(1,26)=5.76, p=0.024]; genotype*injury [F(2,26)=5.36, p=0.011]; CP13 [F(1,26)=11.65, p=0.002]; genotype*injury [F(2,26)=19.74, p=0<0.001]; PHF1 [F(1,26)=10.44 p=0.003]; genotype*injury [F(2,26)=8.97, p=<0.001]; DA9 [F(1,26)=0.24, p=0.623]). At 6mpi, in the HIPPO-depleted hemisphere, r-mTBI did not cause changes in tau phosphorylation or total tau abundance in the WT and GFAP^{P301L} mice compared to their sham counterparts. The only cohort to show a TBI-mediated effect on tau phosphorylation (RZ3 and
PHF1 but not CP13) and total tau abundance compared to their sham control was the CaMKIIαP301L cohort (2.6A-H) (two-way ANOVA: RZ3 injury effect [F(1,25)=2.95, p=0.09]; CP13 [F(1,24)=0.7, p=0.4]; PHF1 [F(1,24)=1.71, p=0.203]; DA9 [F(1,26)=0.212, p=0.157]). In the hippocampus, we did not observe TBI-dependent changes in tau phosphorylation in the WT and GFAPP301L-cohorts (Fig. 2.6A-H). The CaMKIIαP301L cohort manifested a TBI-mediated decrease in PHF1-linked tau phosphorylation (but no RZ3 or CP13) that compromised a significant ~1.5-fold decrease of PHF1 levels in the TBI group compared to the sham group (Fig. 2.6E) (two-way ANOVA: RZ3 injury effect [F(1,26)=0.027, p=0.869]; CP13 [F(1,24)=0.013, p=0.908]; PHF1 [F(1,25)=2.14, p=0.155]; DA9 [F(1,26)=0.74, p=0.397], no injury*age interaction).

The lack of a TBI-mediated effect on tau phosphorylation in the HIPPO and HIPPO-depleted hemisphere (compared to the GFAPP301L mice at 3mpi might be the result of including distal brain regions to the impact site.

2.3.6 Persistent astrocyte and microglial reactivity in the cortex and corpus callosum three and six months post-last mTBI exposure.

To investigate whether astroglial tau exacerbated chronic neuroinflammatory outcomes three and six months after r-mTBI, we analyzed astrocyte reactivity (S100B and GFAP immunoreactivity) and microglial reactivity (IBA1 immunoreactivity) in the cortex and CC of our three mouse models at 3mpi and 6mpi (Fig 2.9 & 2.11).

Three months post-last injury, there was no TBI-dependent difference in S100B immunoreactivity in the cortex of WT and GFAPP301L cohorts (Fig 2.9A-B). However, there was a significant ~1.5-fold increase in S100B in the CaMKIIαP301L r-mTBI group compared to sham controls (two-way ANOVA: injury effect [F(1,25)=10.17, p=0.004]; genotype*injury [F(2,25)=0.87, p=0.430]). Additionally, there was a significant 1.5-fold increase in GFAP immunoreactivity in the cortex of the CaMKIIαP301L r-mTBI group compared to their sham controls (two-way ANOVA: injury effect [F(1,23)=3.88, p=0.061]; genotype*injury [F(2,23)=0.86, p=0.435]) (Fig. 2.9C). Because astrocyte reactivity in the CaMKIIαP301L model (both r-sham and r-mTBI mice cohorts) was disproportionately exacerbated compared to the other models, we performed a separate Two-Way analysis using only the r-mTBI/sham WT and GFAPP301L data, as shown in Fig. 2.9D. Our analysis revealed that r-mTBI induced a significant 2-fold increase in GFAP immunoreactivity in
both WT and GFAP\textsuperscript{P301L} cohorts when compared to their sham counterparts (two-way ANOVA: injury effect \([F(1,15)=15.62, p=0.001]\); genotype*injury \([F(1,15)=0.63, p=0.437]\)). Moreover, unlike exposure to exogenous tau (i.e., CaMKII\textalpha\textsuperscript{P301L} model), the presence of tau inside astrocytes (i.e., the GFAP\textsuperscript{P301L} model) was not sufficient to evoke a worsened TBI response compared to WT counterparts. In the corpus callosum (CC), GFAP immunoreactivity in WT and GFAP\textsuperscript{P301L} cohorts was increased 3 mpi in the r-mTBI group compared to shams (two-way ANOVA: injury effect \([F(1,25)=12.13, p=0.002]\); genotype*injury \([F(2,25)=1.64, p=0.213]\]). In the case of the CaMKII\textalpha\textsuperscript{P301L} cohort, there was no TBI effect on astrocyte reactivity in the CC (Fig. 2.9G). Furthermore, no augmented TBI effect was noted in the GFAP\textsuperscript{P301L} cohort compared to WT counterparts.

IBA1 immunoreactivity in the cortices of our mouse models demonstrated no changes in microglial reactivity in the CaMKII\textalpha\textsuperscript{P301L} cohort in the TBI group compared to the sham (two-way ANOVA: injury effect \([F(1,23)=5.14, p=0.033]\); genotype*injury \([F(2,23)=0.47, p=6.27]\)) (Fig. 2.9E). As mentioned above, due to the disproportionate levels of IBA1 immunoreactivity between the CaMKII\textalpha\textsuperscript{P301L} cohort and other genotypes we again excluded the CaMKII\textalpha\textsuperscript{P301L} cohort data and ran a Two-Way statistical analysis on only the GFAP\textsuperscript{P301L} and WT groups. This analysis revealed that microglial reactivity was significantly increased in the TBI groups of both models compared to their sham counterparts (two-way ANOVA: injury effect \([F(1,15)=11.98, p=0.003]\); genotype*injury \([F(2,15)=0.432, p=0.521]\)) (Fig. 2.9F). Additionally, the effect of r-mTBI exposure was not worsened in mice harboring tau-bearing astrocytes (GFAP\textsuperscript{P301L} cohort) compared to WT. In the CC of all cohorts, microglial reactivity was significantly increased in the TBI groups compared to the shams (Fig. 2.9H) but with no difference in the TBI response in the GFAP\textsuperscript{P301L} cohort compared to the WT (two-way ANOVA: injury effect \([F(1,23)=18.88, p=<0.001]\); genotype*injury \([F(2,23)=0.245, p=0.784]\)).

Six months post-last injury, no TBI effect on GFAP and S100b immunoreactivity was observed across genotypes (Fig. 2.11A-C) when all three genotypes were considered; when CaMKII\textalpha\textsuperscript{P301L} data were excluded a significant 1.5-fold increase was observed in GFAP immunoreactivity in the r-mTBI group of both WT and GFAP\textsuperscript{P301L} compared to their respective sham counterparts (Fig. 2.11D). GFAP immunoreactivity in the CC unexpectedly showed a significant decrease in the
CaMKIIαP301L TBI group compared to its sham counterparts (Fig. 2.11E). After the CaMKIIαP301L data set was removed, we observed a significant 2-fold increase in GFAP immunoreactivity in the WT TBI group compared to sham controls (two-way ANOVA: injury effect [F(1,12)=14.25, p=0.003]; genotype*injury [F(2,12)=1.59, p=0.231]). No TBI-related difference was observed in the GFAPP301L cohort (Fig. 2.11F). Furthermore, there were no TBI-mediated changes in microglial reactivity in the cortex across the cohorts (two-way ANOVA: injury effect [F(1,21)=0.379, p=0.544]; genotype*injury [F(2,21)=0.577, p=0.570]), (Fig. 2.11G-H). In the CC, the TBI effect was evident in the WT cohort represented by a significant 5-fold increase in the r-mTBI group compared to the r-sham (two-way ANOVA: injury effect [F(1,24)=8.55, p=0.007]; genotype*injury [F(2,24)=4.56, p=0.021]), while in the GFAPP301L and CaMKIIαP301L cohorts, there was no TBI effect on microglial reactivity (Fig. 2.11I-J). Moreover, there was no synergistic effect of TBI and tau astrogliopathy on astrocyte and microglial reactivity in the cortex or CC compared to the WT cohort.

Altogether these data suggested that the presence of pathological tau within astrocytes does not exacerbate the effect of r-mTBI on chronic astrocyte and microglial reactivity at 3mpi and 6mpi. However, r-mTBI elicited an augmented astroglial and microglial response in CaMKIIαP301L mice (with neuronal tau pathology) at 3mpi, only. The failure to trigger increased astrocyte and microglial reactivity in TBI mice compared to sham at the later timepoint (6mpi) might be explained by a possible plateau of astroglial and microglial response to increased levels of pathology by the age of 10 months.

2.3.7 Changes in astrocyte homeostatic markers in the hippocampus and hippocampus-depleted hemisphere three and six months post-last mTBI exposure.

To investigate the influence of tau astrogliopathy on astrocyte homeostasis after r-mTBI, we assessed the levels of proteins AQP4, GLT1, and GLAST in the cortex and hippocampus of our mouse models. These analyses revealed an absence of TBI-dependent changes in the levels of AQP4, GLT1, and GLAST in both regions of interest across the three genotypes at 3mpi and 6mpi (Fig. 2.12 and 2.14, respectively) (HEMISPHERE 3mpi two-way ANOVA: injury effect [F(1,22)=0.934, p=0.344]; injury effect [F(1,26)=0.004, p=0.950]; injury effect [F(1,24)=1.80, p=0.192], respectively, HIPPO 3mpi injury effect [F(1,24)=0.668, p=0.422]; injury effect
[F(1,25)=0.10, p=0.752]; injury effect [F(1,27)=6.42, p=0.017], respectively, none of them had genotype injury interaction) (HEMISPHERE 6mpi two-way ANOVA: injury effect [F(1,27)=1.14, p=0.295]; injury effect [F(1,24)=3.85, p=0.061]; injury effect [F(1,27)=1.92, p=0.177], respectively, HIPPO 6mpi injury effect [F(1,25)=3.19, p=0.086]; injury effect [F(1,26)=0.33, p=0.569]; injury effect [F(1,26)=0.013, p=0.908], respectively, none of them had genotype injury interaction).

2.3.8 Changes in synaptic integrity in the hippocampus and hippocampus-depleted hemisphere three and six months post-last mTBI exposure.

To examine whether r-mTBI differentially impacts synaptic integrity in our htauP301L-bearing astrocyte vs neuronal models, we analyzed the protein levels of synapsin 1, synaptophysin and PSD95 in the hemisphere (without HIPPO) and hippocampal lysates of these mouse models. Three months post-last injury, no TBI-dependent changes in synapsin-1, synaptophysin or PSD95 were observed for any of the three genotypes in the HIPPO-depleted hemisphere or hippocampus (Fig. 2.15) (HEMISPHERE 3mpi two-way ANOVA: injury effect [F(1,21)=0.75, p=0.394]; injury effect [F(1,24)=0.34, p=0.564]; injury effect [F(1,24)=0.0001, p=0.971], respectively, HIPPO 3mpi injury effect [F(1,26)=0.73, p=0.398]; injury effect [F(1,27)=0.47, p=0.498]; injury effect [F(1,26)=2.87, p=0.102], respectively, none of them had genotype injury interaction). Similarly, at 6mpi, there was no TBI effect on the immunoreactivity levels of synaptic markers in the hemisphere across all three genotypes (Fig. 2.17) (HEMISPHERE 6mpi two-way ANOVA: injury effect [F(1,27)=0.93, p=0.343]; injury effect [F(1,25)=0.63, p=0.434]; injury effect [F(1,27)=0.005, p=0.940], respectively, HIPPO 6mpi injury effect [F(1,26)=0.24, p=0.625]; injury effect [F(1,27)=0.005, p=0.940]; injury effect [F(1,26)=0.013, p=0.908], respectively, none of them had genotype injury interaction).

2.4 DISCUSSION

Tau astrogliopathy is a pathological hallmark of primary tauopathies such as CTE. However, how tau astrogliopathy contributes to the pathophysiological changes in the disease warrants investigation. Using our GFAP\textsuperscript{P301L} mouse model, harboring tau-bearing astrocytes, we attempted to decipher the effects of endogenous htau\textsuperscript{P301L} accumulation on astroglial pathobiology as well as the effect of r-mTBI in tau accumulation and how the interaction might affect astroglial pathological response by assessing histopathological and biochemical changes. We hypothesized
that understanding the tau-dependent effects on astroglial pathobiology would provide new cell-specific research avenues that could potentially reveal new disease-modifying therapeutics not only for CTE but possibly for other 4R-tau astrogliopathies like ARTAG, PSP, and CBD. In the current study, we have collected evidence revealing that astroglial overexpression of htau$^{301L}$ does not promote changes in classic astrocyte reactivity markers but elicits a significant transient reduction in homeostatic astrocyte-specific markers (AQP4 and GLT1) involved in regulating water movement across cell membranes and glutamate homeostasis. Additionally, r-mTBI does not augment or accelerate the effect on tau astrogliopathy.

As described in Chapter 1, astrocytes carry important functions in the CNS that include neurotransmitter homeostasis, CNS clearance, BBB support, and metabolic and trophic support to neurons. Investigation of the effect of tau accumulation on astroglial physiology is scarce possibly because in primary tauopathies such as PSP, CBD, and CTE the presence of neuronal tau accumulation is dominant compared to tau astrogliopathy. From studies on neurons, we know that pathogenic tau promotes cell dysfunction by compromising axonal transport and synaptic transmission (Rajmohan & Reddy, 2017). Thus, if pathogenic tau causes dysfunction within neurons, it has the potential also to cause functional impairment in astrocytes. An ex-vivo experiment with primary rat astrocytes suggested that overexpression of tau$^{WT}$ led to unfavorable changes in the astroglial intermediate filament network which disrupted organelle transport and provoked cell death (Yoshiyama et al., 2003). Knowing that a single human astrocyte can extend its processes up to 2 million synapses and with at least one process (perivascular end-feet) get in contact with a blood vessel (Hösli et al., 2022; Oberheim et al., 2009), it is conceivable that astroglial dysfunction or death can have a great impact on CNS homeostasis. To understand the substantiative role of tau astrogliopathy in clinical conditions, researchers should utilize animal models that recapitulate either or both neuronal and astroglial tauopathy. Currently, there is no single model with analogous levels of neuronal and astroglial tau pathology, thus, to decipher the contribution of each tau pathology to the pathophysiology of astrocytes following r-mTBI/CTE we investigated them in isolation. To accomplish that we used a model that overexpresses tau within astrocytes (GFAP$^{301L}$) and a model that overexpresses tau within neurons (CaMKIIα$^{301L}$/Tg4510).
Pathological analyses of human r-mTBI cases show a significant increase in astrocyte and microglial reactivity mainly in the white matter and grey-white matter interface (Babcock et al., 2022). From our findings, we know that as opposed to astrocytic tau$^{P301L}$ overexpression, neuronal tau$^{P301L}$ elicits significantly more astrocyte and microglial reactivity in the white matter (i.e., corpus callosum, CC) and grey matter (i.e. cortex) compared to WT mice at 7 and 10 months of age. At the age of 7 months, CaMKII$^{\alpha P301L}$ mice already exhibit substantial tau pathology (H. Wang et al., 2018) while GFAP$^{P301L}$ mice do not manifest robust increases in tau pathology until the age of 24 months (Forman et al., 2005). Additionally, Foreman and colleagues (2005), reported that in areas where tau astrogliopathy was robust, astrocyte reactivity was exacerbated. This suggests that tau astrogliopathy at 7 and 10 months of age, as in our study, is not robust enough to cause significant astrocyte and microglial reactivity, as opposed to neuronal tauopathy. From our preliminary data, and data retrieved from other models of tauopathy (Dabir et al., 2006; Forman et al., 2005), we know that by the age of 3 months, GFAP$^{P301L}$ mice do not exhibit obvious astroglial tauopathy; thus, we opted for inducing r-mTBI (our 20-hit model), in parallel, to assess whether r-mTBI accelerates the onset and exacerbate tau astrogliopathy and its potential pathological interactions. Repetitive mTBI evoked a transient region-specific increase in cortical levels of tau phosphorylation at 3mpi but it did not increase the number of astrocytes bearing phosphorylated tau (tau astrogliopathy) at 3mpi and 6mpi. Three months post-last injury (at 7 months of age), we detected increased astrocyte and microglial reactivity in the cortex and CC of GFAP$^{P301L}$ mice (TBI vs sham), however, the TBI-mediated effect on astrocyte and microglial reactivity was not exacerbated compared to WT counterparts. At 6mpi, WT and GFAP$^{P301L}$ mice presented sustained cortical astrocyte reactivity compared to shams while CaMKII$^{\alpha P301L}$ mice no longer showed a TBI effect. At the 6mpi time point, all models failed to show sustained TBI-induced microglial reactivity in the cortex, and only the WT mice showed TBI-induced microglial reactivity in the CC. We hypothesize that the absence of a TBI effect on astrocyte and microglial reactivity, and tauopathy in CaMKII$^{\alpha P301L}$ mice at 6mpi is because, by the age of 10 months, these mice already show robust tauopathy that might trigger high levels of astroglial and microglial response (Wang et al., 2018) in sham mice, thus masking any exacerbation by r-mTBI possible. However, the same explanation cannot be used for why the
GFAP$^{P301L}$ mice failed to show a TBI microglial reactivity in the later time point; the reason for that lack of response is unclear at this time.

Altogether, these results suggest that at 7 and 10 months of age, overexpression of tau$^{P301L}$ in astrocytes is not enough to cause significant astrocyte and microglial reactivity compared to WT, as opposed to neuronal tauopathy. In addition, r-mTBI does not increase tau astrogliopathy but triggers a transient increase of tau phosphorylation in the cortical area underneath the impact. Such an increase in tau phosphorylation is not robust enough to trigger exacerbated TBI-mediated astrocyte and microglial reactivity compared to the WT cohort. In the case of the CaMKIIα$^{P301L}$ mice, a substantial presence of phosphorylated tau might be the reason why astrocyte and microglial responses are greater than in WT mice. By our findings, we can suggest that TBI-dependent astrocyte and microglial reactivity in human cases might be orchestrated by the overt increase in neuronal tauopathy rather than tau astrogliopathy. Further investigation on aged GFAP$^{P301L}$ mice, could potentially help us understand the contribution of robust tau astrogliopathy in astrocyte and microglial responses.

Astrocyte-specific molecular and cellular changes occurring in r-mTBI/CTE brains have been explored to a limited degree. Our biochemical analysis of homeostatic astrocyte markers in our mouse models of tau astrogliopathy and neuronal tauopathy revealed a region-specific reduction of AQP4 and GLT1 in the hippocampus but not in the cortex of shams when compared to WT shams. AQP4 is a water channel particularly expressed on the astrocytic perivascular end feet. AQP4 is the essential channel of the brain water flux and waste clearance system also known as the glymphatic system (Nagelhus & Ottersen, 2013). AQP4 is also present in perineuronal astrocytic processes where it is involved in maintaining water volume and ion/neurotransmitter buffering in response to synaptic transmission (MacAulay & Zeuthen, 2010). Behavioral analyses were beyond the scope of our current studies; therefore we do not have behavioral data in our two transgenic models. However, Skucas and colleagues (2011) reported that AQP4/-/- mice have impaired memory consolidation linked to impairments in long-term potentiation and long-term depression (Skucas et al., 2011). Thus, based on the dramatic reduction of hippocampal AQP4 in our tauopathy models, we could expect that our GFAP$^{P301L}$ mice might also have impaired memory capabilities. In the case of the CaMKIIα$^{P301L}$ mice, it is well-known that they exhibit
behavioral changes at the age of 5 months (Maurin et al., 2014), however, those changes are more likely to be linked to the substantial accumulation of pathogenic tau. Moreover, our analyses revealed that r-mTBI did not elicit changes in AQP4 levels or cortical perivascular AQP4 polarization in the brain of any of our mouse models. This coincides with other studies that also report no differences in AQP4 abundance after TBI (Iliff et al., 2014; Zhao et al., 2017). We also reported that in the cortex of 10 months old GFAP\textsuperscript{P301L} and CaMKII\textalpha P301L mice, there is a significant increase in cortical AQP4 levels compared to WT. Our findings are in line with a mouse study in that reports that cortical levels of AQP4 are increased in 8.5 months old CaMKII\textalpha P301L mice (Harrison et al., 2020); and a human study that reports that in Globular Glial Tauopathy (with neuronal and astroglial tau pathology), there is an increase in AQP4 abundance in the frontal cortex (Ferrer et al., 2020). However, there is a lack of understanding of why and how this occurs as well as why at 10 months of age and not at 7 months of age. The other astrocyte-specific marker that our data showed to be affected by the presence of tau overexpression in astrocytes or neurons is the glutamate transporter 1 (GLT1) in hippocampal astrocytes only. GLT1 is responsible for preventing excitotoxicity by recycling 85% of glutamate excess at the synaptic cleft after synaptic transmission (Petr et al., 2015). Thus, a significant reduction of GLT1 expression would lead to a significant increase in extracellular glutamate concentration which ultimately can cause neuronal loss (Ezerskiy et al., 2022). Dabir and colleagues (2006) reported that GFAP\textsuperscript{P301L} mice had an age-dependent decrease in GLT1 levels in the spinal cord and brainstem but not in the cortex (Dabir et al., 2006). This might explain why we see effects in the hippocampal GLT1 levels but not in the cortical. As mentioned before, reduction in glutamate transport has been linked to neuronal death, studies on CaMKII\textalpha P301L mice show ~55% loss of hippocampal neurons by the age of 6 months, which might suggest a relation between the drastic reduction of hippocampal GLT1 and neuronal loss potentially through excitotoxicity. Further experiments assessing glutamate concentration and neuronal electrophysiology might help establish that relationship. Moreover, r-mTBI did not cause changes in GLT1 or GLAST at 3 or 6 months compared to controls. In the case of GLT1, a previous study showed significant downregulation of GLT1 in the cortical astrocytes after TBI (Shandra et al., 2019). The authors also noted that the GLT1 downregulation was observed in astrocytes directly below the impact
site. The region-dependent changes of proteins might also explain why we do not see TBI-mediated changes in GLT1 in our analysis of the hemisphere lysate. Our results suggest that a decrease in hippocampal AQP4 and GLT1 levels weakly correlated with the increase in hippocampal mutant tau abundance and that r-mTBI does not alter this correlation. Additional experiments are essential to assess the mechanism and the functional effects of alteration in glutamate transporter and AQP4 abundance. Through our search in the literature, we did not find rmTBI/CTE studies that focus on hippocampal levels of homeostatic markers, however, given that in those conditions astroglial and neuronal tauopathies co-exist, it is highly suggestive that at a given time in the course of the human condition, AQP4 and GLT1 could potentially be dysregulated, however, histopathological analyses are warranted in order to make a conclusion.

In our models of tauopathy, compared to WT mice, we also observed significantly diminished cortical and hippocampal synapsin 1 with no changes in synaptophysin or PSD95 at 7 months of age. Helboe and colleagues (2017) also reported the same findings in the CaMKIIαP301L model, those changes coincided with the presence of hippocampal neurodegeneration (Helboe et al., 2017). An in vivo study suggested the correlation of pathogenic tau with the reduction of synapsin 1 when intracerebral injections of tau led to synapsin 1 reduction in hippocampal neurons (Piacentini et al., 2017). The decrease in cortical synapsin 1 level is weakly correlated with increased levels of tau abundance. The reduction in synapsin 1 has been shown to negatively impact inhibitory synaptic transmission (Gitler et al., 2004) which may be considered as an alternative mechanism of neuronal loss. Additionally, it is well known that CaMKIIαP301L mice develop neurodegeneration as early as 6 months of age; further characterization, for instance, NeuN stereological and electrophysiological analyses will need to be done in our GFAPP301L model to conclude that the dramatic reduction of synapsin1 is related to neurodegeneration at 7 or 10 months of age. Lastly, the fact that there is a region-specific response shows the differential astroglial vulnerability to pathological tau. Differences in neuronal vulnerability/susceptibility to pathogenic proteins such as amyloid-β, α-synuclein, and tau have been widely reported to occur in neurodegenerative conditions. For instance, in AD, basal forebrain cholinergic neurons have increased vulnerability to amyloid-β, while in PD, dopaminergic neurons in the substantia nigra are more vulnerable to α-synuclein (Chen et al., 2022; Sulzer & Surmeier, 2013). We also observed
that r-mTBI does not cause changes in synaptic markers. This might be explained by the remodeling capacity that synaptic proteins have after TBI as shown in a study in mice where 48h after r-mTBI (daily hits for 5 days on the closed skull), pre-frontal cortical levels of synaptophysin were downregulated and PSD95 were upregulated, but those changes resolved by 5mpi (Xu et al., 2021). We cannot discard that after 20 hits we would also observe dysregulation in synaptic markers at more acute time points.

Several findings published by a group of scientists led by Dr. McKee have suggested that tau astrogliopathy in CTE as well as in ARTAG is developed as a byproduct of age. However, the fact that ARTAG is only found in a third of the population beyond 75 years old, led researchers to think that those individuals might have encountered a “event” that triggered astrocytic tau accumulation such as a cerebrovascular event, genetic pre-disposition, or head trauma (G. G. Kovacs, 2020). Opposed to McKee’s theory, Dr. Stewart and colleagues suggest that tau astrogliopathy is not a mere byproduct of aging but that it has emerged in response to repeated head trauma, and it is involved in the pathophysiological changes of the disease. Dr. Stewart has based his theory on evidence of tau astrogliopathy in a young cohort of CTE patients. Our studies support the idea that age on its own might not be responsible for the accumulation of pathogenic tau within astrocytes, instead, a triggering event (in our GFAP<sup>P301L</sup> model is the genetic manipulation) might have to be present to elicit tau astrogliopathy in an age-dependent manner. Our GFAP<sup>P301L</sup> model shows the increased presence of tau astrogliopathy by the age of 10 months, with no obvious TBI effect on tau astrogliopathy. The possible reason why we do not see TBI effects on tau astrogliopathy at our later timepoint (6mpi) might be explained by the predominance of the genetically driven model over head trauma itself. However, this does not exclude the possibility that in the human condition, repeated head trauma can be a “triggering event” for tau astrogliopathy. Despite the discrepancies and limitations of our study, this important topic still warrants more investigation. The major existential question that remains unanswered is to determine the origin of tau astrogliopathy which we could not address in this study. Moreover, we could not oversee the negative impacts that tau astrogliopathy has on astroglial physiology, with our studies we were able to reveal that despite that tau astrogliopathy is not as dominant as neuronal tauopathy, it was able to cause profound transient region-
dependent changes in astrocyte-specific markers known to participate in the homeostasis of glutamate-mediated synaptic transmission and water transport and clearance system. The dramatic reduction of AQP4 and GLT1 in the hippocampus but not in the cortex of our tau mouse models could be possibly explained by the heterogeneous nature of astrocytes depending on the area of the brain they reside. Astrocyte-specific single-cell RNA sequencing, in vitro and in vivo studies, has shown how hippocampal and cortical astrocytes differ in their transcriptome composition and their response to the same stimulus (Batiuk et al., 2020; Oberheim et al., 2012). Furthermore, because the changes in AQP4 and GLT1 were transient we can speculate that there are intrinsic reparative mechanisms that allow the restoration of essential homeostatic astroglial proteins, however, further analysis is required to assess the functionality of the restored proteins. Besides, it remains to be elucidated why human pathological tau negatively affected GLT1 but not GLAST levels, as well as synapsin 1 but no other studied synaptic markers. Besides, is it possible that other astroglial glutamate transporters might be engaging in compensatory mechanisms to uptake glutamate from the synaptic cleft preventing massive excitotoxicity?

2.5 LIMITATIONS OF THE STUDY

Data presented in this study provides useful insights into the effects of overexpression of pathogenic tau inside astrocytes at baseline and after r-mTBI, however, this study also had several limitations that should be noted. Firstly, the expression of pathogenic tau in astrocytes in our GFAP\textsuperscript{P301L} model occurs from embryonic day 14 (Brenner & Messing, 2021) and persists throughout their lives which leads to an age-dependent increase in tau astrogliopathy. In humans, according to Kovacs (2020), the accumulation of tau within astrocytes is more likely to appear with age only after a triggering event has been encountered (e.g., cerebrovascular accidents, gene predisposition, head trauma) that either increases the expression and accumulation of astrocytic endogenous tau, or the internalization and aggregation of secreted neuronal tau Fig. 1.4 (Kovacs, 2020). However, in mice, genetic manipulation is the only way we can obtain models of tau astrogliopathy that would help us understand more the contribution of this disease hallmark to the pathophysiology of diseases such as CTE. Additionally, we also acknowledge that the origin of tau could also elicit different astroglial responses.
Secondly, tau expression in our model is controlled by the GFAP promoter whose expression in the brain is region-dependent and known to increase in response to TBI (Jurga et al., 2021). The region-dependent nature of GFAP expression leads to differential expression/accumulation of tau within astrocytes throughout the brain which might affect regional astroglial response in sham and r-mTBI condition. Whereas the increase of GFAP expression in response to TBI is likely to confound the effects of TBI itself on tau pathology. Furthermore, GFAP is also expressed in non-CNS cells such as enteric glia, liver cells, and fibroblasts (Middeldorp & Hol, 2011), however, the contribution to our findings of tau overexpression in the gut (i.e., the “gut-brain axis”) or liver to our findings remains to be elucidated. However, the GFAP^{P301L} model was the only tool available to us at the time of starting this study. In the future, we will consider the utilization of an astrocyte-specific promoter that is known to not change in response to TBI such as aldehyde dehydrogenase 1, ALDH1.

Thirdly, although we use comparisons among the sham mice to refer to baseline effects of tau astrogliopathy, we are aware that repeated exposures to anesthetics over 1 month may have elicited changes in brain functioning including astroglial biology that are not seen in naïve mice (Stollings et al., 2016; Szrama et al., 2022). Lastly, for our genotype comparisons, we are utilizing mouse models with slightly different background strains: tau models are on an FVB-C57 background (50/50) while our wildtype controls are on the C57BL/6J background. Several studies have shown slight differences in cytokine composition between FVB and C57 (Szade et al., 2015), different susceptibility to anesthetics except isoflurane (Sato et al., 2006), and diabetes (Haluzik et al., 2004). However, there are no comparative analyses between C57 and FVB-C57 mice. Because our tau models are on a 50/50 background, we considered that there might be even subtler changes in the cellular, molecular, and chemical composition of the brain which might not overtly affect our outcomes.

2.6 SUMMARY

In summary, the results provided in this section of the thesis show that our hypotheses are partially upheld. Our GFAP^{P301L} mice, similar to other models of tau astrogliopathy, show an age-dependent increase in tau astrogliopathy and brain-wide tauopathy, however, r-mTBI failed to accelerate or exacerbate tau astrogliopathy but show a transient increase in tau phosphorylation.
in the cortical area underneath the impact; thus, our first hypothesis is partially supported. Next, overexpression of htau\textsuperscript{P301L} within astrocytes does not elicit increased astrocyte and microglial reactivity compared to WT astrocytes at 7 or 10 months of age, besides, r-mTBI does not exacerbate astrocyte and microglial reactivity compared to WT cohorts at 3 and 6mpi; thus, our second hypothesis is rejected. Then, our biochemical analysis revealed that 4R-tau-bearing astrocytes undergo region-specific changes that alter levels of homeostatic markers important for optimal neuronal transmission and water transport, however, there was no TBI effect. These findings partially support our third hypothesis. Finally, partially supporting our fourth hypothesis, GFAP\textsuperscript{P301L} mice exhibit temporal dysregulation of synaptic integrity that is not exacerbated by r-mTBI exposure.
CHAPTER 3: EFFECTS OF ASTROCYTIC VS NEURONAL TAU IN ASTROCYTE-SPECIFIC TRANSCRIPTIONAL CHANGES FOLLOWING REPETITIVE MILD TBI.

3.1 INTRODUCTION

In the previous chapter, we assessed histopathological and biochemical changes of astrocytes in response to overexpression of endogenous tau versus exposure to neuronal tau under normal circumstances (after sham exposures) and after r-mTBI. However, astroglial morphological changes in astrocytes do not comprehensively capture the heterogeneity of astroglial response to any stimulus including TBI (Muñoz-Ballester & Robel, 2023). Evaluation of transcriptomic changes can give insights into ongoing cellular responses that can impact cellular dysfunction and injury recovery. Astrocyte-specific transcriptional analysis has advanced the understanding of possible underlying cellular mechanisms in neurodegenerative diseases including Alzheimer’s Disease, Parkinson’s disease, and Amyotrophic Lateral Sclerosis (Galea et al., 2022; Miller et al., 2017; Qian et al., 2023) opening new routes of investigation that have great therapeutic potential. Taking advantage of transcriptomic analysis, various groups have investigated astroglial changes in response to single TBI (different models; FPI and blast injury) at acute time points post-injury (24h up to 7 days) while the evaluation of chronic astroglial transcriptomic changes after a closed single mTBI and r-mTBI is scarce. Studies of acute astroglial response show the time-dependent nature of astroglial response. At 24h post-FPI, mouse TBI astrocytes show signs of mitochondrial metabolic depression and dysregulation of calcium signaling compared to their sham counterparts (Arneson et al., 2018). Those changes appear to diminish at 48h post-blast injury, where mouse TBI astrocytes exhibit a neuroprotective state that favors neurogenesis as well as astroglial development (Zhang et al., 2023). Seven days post-FPI, mouse TBI astrocytes show the upregulation of the immune response (Todd et al., 2021). Nonetheless, since the pathology of repeated mild head trauma manifests over many years and decades, there is a need to incorporate the longitudinal assessment of astrocyte-specific transcriptomic changes at acute and chronic time points following r-mTBI. A study performed with astrocytes harvested from the
white matter of CTE cases (Stage II-III) indicates that there is a downregulation of normal functioning, and an upregulation of dysfunctional mitochondrial metabolism accompanied by increased neuroinflammation with a “neurotoxic profile” (Chancellor et al., 2021). Additionally, there is limited information about the transcriptional changes to astrocytes exposed to endogenous/exogenous tau overexpression at baseline and in the context of r-mTBI. Thus, we assessed transcriptional changes of isolated primary astrocytes harvested from our three experimental models (WT, GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup>) at 3 and 6 months after r-mTBI/sham (7 and 10 months of age, respectively). In the present chapter, we seek to understand the pathophysiology of tau astrogliopathy under normal circumstances and after r-mTBI through transcriptome analysis.

Experiments in this chapter sought to test the following hypotheses:

a) Tau<sup>P301L</sup>-bearing astrocytes show greater transcriptional dysregulation compared to astrocytes from WT and CaMKIIα<sup>P301L</sup>; this dysregulation becomes more pronounced over time.

b) Repetitive mTBI causes increased transcriptional changes in astrocytes harboring human mutant tau (GFAP<sup>P301L</sup>) compared to astrocytes that do not express human tau (WT and CaMKIIα<sup>P301L</sup> mouse models).

c) Transcriptomic changes in astrocytes harvested from CTE cases will demonstrate stage-dependent detrimental changes compared to controls.

d) Tau<sup>P301L</sup>-bearing astrocytes manifest changes in astroglial biology that resemble pathological changes of astrocytes extracted from CTE cases.

### 3.2 METHODS AND MATERIALS

#### 3.2.1 Human brain tissue

Hippocampi of 34 male athletes who participated in American football, hockey, and mixed martial arts with a history of repetitive mild TBI were obtained through Boston University School of Medicine for the present study. The cohort was composed of n=12 CTE stage II (CTE-II), n=11 CTE-III, and n=11 CTE-IV. Additionally, we had n=9 control brains. It is important to mention that at the moment of analysis, we only had access to a limited number of control cases, and as such
our control group has two main discrepancies from CTE cases (see Table 3.1): 1) there is age discrepancy between the control cases and CTE cases at stage II and III where CTE cases are significantly younger compared to control cases; and 2) there is a significant difference in the male:female ratio in the control cases compared to CTE cases. However, with a qualitative analysis of the transcriptome profile of each case (Fig. S3.1), we were able to reveal that there are no prominent differences between female and male control samples which allowed us to move forward with the CTE vs control transcriptome comparisons. It is also worth mentioning that despite the age discrepancy between the controls and CTE-II/CTE-III cases we still performed transcriptome comparisons to have an insight of astrocyte-specific changes as the disease progresses. Additionally, given that our control cohort was age-matched to CTE-IV cases we utilized this data (i.e., CTE-IV vs control) to assess murine-human similarities.

<table>
<thead>
<tr>
<th></th>
<th>NDC</th>
<th>CTE II</th>
<th>CTE III</th>
<th>CTE IV</th>
</tr>
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<tr>
<td>N</td>
<td>9</td>
<td>12</td>
<td>11</td>
<td>11</td>
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<tr>
<td>Sex (M/F)</td>
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<td>12/0</td>
<td>11/0</td>
<td>11/0</td>
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<tr>
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<td>12/0</td>
<td>7/4</td>
<td>9/2</td>
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<tr>
<td>Sport Played (Soc/Rug/Hoc/Foot)</td>
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<td>0/0/1/10</td>
<td>0/0/0/11</td>
</tr>
<tr>
<td>Sport Level (am/co/semipro/pro)</td>
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<td>2/2/0/8</td>
<td>0/2/0/9</td>
<td>0/3/0/8</td>
</tr>
<tr>
<td>Military Service (Y/N)</td>
<td>N/A</td>
<td>1/11</td>
<td>0/11</td>
<td>2/9</td>
</tr>
<tr>
<td>Age begun</td>
<td>N/A</td>
<td>12.0(±3.35)</td>
<td>12.7(±1.95)</td>
<td>11.3(±2.24)</td>
</tr>
<tr>
<td>Years of play</td>
<td>N/A</td>
<td>14.0(±5.08)</td>
<td>12.7(±1.95)</td>
<td>11.3(±2.24)</td>
</tr>
<tr>
<td>Age of symptom onset</td>
<td>N/A</td>
<td>43.1(±21.29)</td>
<td>44.7(±18.6)</td>
<td>59.9(±14.58)</td>
</tr>
<tr>
<td>Years retirement to symptom onset</td>
<td>N/A</td>
<td>16.6(±18.77)</td>
<td>15.7(±19.11)</td>
<td>31.9(±16.28)</td>
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<tr>
<td>Years symptom onset to death</td>
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<td>10.7(±9.95)</td>
<td>20.6(±12.33)</td>
<td>17.4(±10.96)</td>
</tr>
<tr>
<td>Age at death</td>
<td>84.3(±12.25)</td>
<td>54.9(±22.03)*</td>
<td>65.3(±11.08)*</td>
<td>77.3(±8.26)</td>
</tr>
</tbody>
</table>

**Table 3.1. Demographics for human CTE cases** used for microdissection and gene array analysis of hippocampal astrocytes. NDC, non-demented controls; ND, not determined.

3.2.2 CTE pathological staging

CTE classification was based on the criteria proposed by McKee and Daneshvar in 2015 (McKee & Daneshvar, 2015). Briefly, in Stage I, brains exhibit isolated perivascular foci of p-tau NFTs, neuropil threads, and astrocytic tangles mostly in the depths of cerebral sulci of the superior, dorsolateral, lateral, and inferior frontal cortices as well as p-tau NFT degeneration in the locus coeruleus,
amygdala, entorhinal cortex, hippocampus, medulla, and cingulate gyrus. In Stage II, there is the presence of multiple foci of tau pathology at the depths of the sulci mostly in the superior, dorsolateral, lateral, and inferior frontal, anterior inferior and lateral temporal, inferior and superior parietal, insular and septal cortices, and substantia innominata. Stage III exhibits NFTs diffusely in the frontal, temporal and parietal cortices, mainly around small vessels and within the depths of sulci. Additionally, the hippocampus, entorhinal cortex, amygdala, nbM, and locus coeruleus show extensive neurofibrillary pathology as well as frequent NFTs in the hypothalamus, substantia nigra, and dorsal and median raphe nuclei. Stage IV shows a significant loss in brain weight and pronounced frontal and temporal lobe atrophy. At the light microscopic level, there is severe spongiosis of cortical layer II and widespread neuronal loss, including the substantia nigra, patchy widespread myelin loss, extensive astroglial tangles, and small NFTs in a patchy irregular distribution throughout frontal, temporal, and parietal cortices as well as extensive NFT degeneration in the insula, septa, temporal cortex, amygdala, hippocampus, entorhinal cortex, substantia nigra, and locus coeruleus. In Stage IV, p-tau pathology also involves the cerebellum, medial lemniscus, and inferior olives of the medulla. CTE stage correlates with the progression of clinical symptoms (McKee & Daneshvar, 2015).

3.2.3 Animals

The animals used for this chapter were the same cohorts used in Chapter 2. Briefly, we used three-month-old male and female C57BL/6 mice (n=24), GFAP^{P301L} (n=24) and CaMKIIα^{P301L} (n=24). Mice were housed in a 12h light/dark cycle with food and water ad libitum. All experiments were performed in accordance with Office of Laboratory Welfare and National Institutes of Health guidelines with Roskamp Institute Institutional Animal Care and Use Committee approval.

3.2.4 Injury protocol

Repetitive mild injury (r-mTBI) was administered using the same 20hit paradigm as previously described in Chapter 2. Briefly, mice were administered a closed head injury daily from Monday through Friday for four weeks. Mice were sacrificed 3 months or 6 months post-last injury (3mpi and 6mpi, respectively) for subsequent transcriptomic analyses.
3.2.5 Astroglial Isolation

Following transcardial perfusion with chilled (4°C) PBS during euthanasia, mice were decapitated, the brain was removed, and placed into a petri dish on ice. Enzymatic tissue digestion was performed using the Adult Brain Dissociation Kit (Miltenyi Biotec). The brain was cut into small pieces using a sterile scalpel blade, and samples were transferred to a 15ml falcon tube where 1950 μL of enzyme mix 1 (enzyme P and buffer Z), and then 30 μL of enzyme mix 2 (enzyme A and buffer Y) were added, brains were incubated in the enzyme mix for 30 minutes. Brains were then further dissociated using repeated pipetting. Samples were briefly centrifuged and filtered through a 70μm cell strainer to achieve a single cell suspension. Single cells were resuspended in 900μL of Debris removal solution and mixed with 3.1mL of PBS containing 0.5% fetal bovine serum (PBS buffer), samples were then transferred to a fresh 15mL falcon tube and 4ml of PBS buffer was carefully overlayed. Samples were then centrifuged at 3000XG for 10 minutes. Cells were rinsed with PBS buffer to remove any remaining debris removal solution and centrifuged for a further 10 minutes. The supernatant was aspirated, and cells were labelled with FcR blocking Reagent (Miltenyi Biotec) for 10 min at 4°C to avoid the unspecific binding of the astrocyte-specific magnetic bead-conjugated ASCA2 antibody (Miltenyi Biotec). Samples were subsequently incubated with ASCA2 antibody for 15 minutes at 4°C. Then, the samples were loaded onto a pre-conditioned LS separation column and rinsed three times to remove unlabelled cells. To elute ACSA2⁺ve cells, the LS column was removed from the magnet and PBS buffer was used to elute the sample. Enriched ACSA2⁺ve cells were briefly centrifuged and store at -80°C in Trizol until RNA extraction.

It is worth mentioning that for performing astrocyte isolation, we utilized the complementary hemispheres from mice in Chapter 2. Two left hemispheres (depleted of the hippocampus) from two different mice that belonged to the same group were combined to collect sufficient material for one RNA sample. In total we had an n=3 RNA samples per group per genotype.
3.2.6 RNA Isolation and Sequencing

**RNA isolation:** RNA was extracted from astrocytes using a Trizol-based protocol (Chomczynski and Mackey, 1995). Briefly, 500µl of Trizol was added to cell isolates obtained from the astrocyte isolation procedure. Samples were sonicated and allowed to dissociate at room temperature for 5 min. Samples were centrifuged at 14000rpm for 15 minutes. Post-centrifugation, three layers were formed: the top-most layer was the aqueous phase and the one containing RNA. The aqueous layer was transferred to a centrifuge tube for further processing. Additionally, 1µl of glycogen, per sample, was added to enhance RNA extraction. Serial centrifugation steps accompanied by ethanol washes yielded the RNA pellet that was used for subsequent RNA sequencing.

**RNA sequencing:** RNA samples were quantified and sent to GENEWIZ LLC (south Plainfield, NJ, USA) for integrity evaluation (average RIN values =5), library preparation and sequencing using the Illumina Nextseq 500 sequencer. RNA sequencing libraries were prepared from 100ng of RNA using the NEB Next Ultra RNA Library Prep Kit for Illumina. Briefly, mRNA was first enriched with Oligod(T) beads and fragmented, with the 1st and 2nd strand cDNA subsequently synthesized. cDNA fragments were end-repaired and adenylated, and universal adaptor ligated to fragments, followed by index addition and library enrichment with limited cycle PCR. The sequencing library
was validated on the Agilent TapeStation and quantified using the Qubit 20 Fluometer as well as qPCR. The sequencing libraries were clustered on one lane of a flowcell. After clustering, the flowcell was loaded on the Illumina HiSeq4000 according to the manufacturer’s instructions. The samples were sequenced using a 2×150 Paired End configuration. Raw sequence data from Illumina HiSeq (.bcl files) were converted into Fastq files and de-multiplexed using the Illumina's bcl2fastq 2.17 software. One mismatch was allowed for index sequence identification.

3.2.7 Bioinformatic analyses on RNA Sequencing of mouse astrocytes

Bioinformatic analyses were obtained using the public platform usegalaxy.org. RNA sequences received from GENEWIZ are uploaded to the platform where raw data undergoes a quality check (FastQC 0.73+galaxy 0 followed by MultiQC 1.11+galaxy 1), and then the sequence reads were trimmed to remove adapter sequences, poly-N-containing reads and low quality reads (Phred score <25) (Trimmomatic 0.38.1 followed by MultiQC 1.11+galaxy 1), and mapped to the Mus musculus GRCm38 reference genome (HISAT2 2.2.1+galaxy 1). Output files were subjected to labeling of duplicated molecules (MarkDuplicates 2.18.2.4) for their subsequent removal when measuring the gene expression in FPKM (fragments per kilobase million) (featureCounts 2.0.3+galaxy 1). Differential gene expression analysis was conducted to determine relationships between gene expression levels in the injury group versus their sham counterparts (DESeq2 2.11.40.7+ galaxy 2). The IDs of the genes were obtained using annotateMyIDs. The covariates were included in all models to adjust for any potential confounding influence on gene expression between main group effects. This was conducted using the Wald test (in DESeq2Genes with FDR adj p<0.05 classified as differentially expressed genes (DEG). Values with an adjusted -P value of <0.05 were processed for pathway enrichment analysis using Ingenuity Pathway Analysis (IPA, QIAGEN). In exceptional cases where DEGs exceeded 5000 in number, we added another cut-off parameter for the expression log2 fold change ±1.2.

3.2.8 Single-cell gene expression analysis on human hippocampal CTE astrocytes

This analysis was performed by one of our collaborators, Professor Elliott Mufson at Barrow Neurological Institute, Arizona.
Single GFAP-immunolabeled hippocampal astrocytes (a total of 50-100 astrocytes per CTE case pooled/per assay) from CTE Stages II, III, and IV cases (n = 12, 11, 11, respectively) were microaspirated by laser capture microdissection (PALM MicroBeam C IP, Carl Zeiss MicroImaging Inc., Thornwood, NY). Microdissected astrocytes were homogenized in Trizol solution and RNAs were reverse transcribed in the presence of the poly d (T) primer (100 ng/mL) and TC primer (100 ng/mL) in 1 × first strand buffer, 500 μM deoxyribonucleotide triphosphate (dNTP)s, 5 mM dithiothreitol (DTT), 20 U of SuperRNase Inhibitor, and 200 U of reverse transcriptase. Single-stranded complementary DNAs (cDNAs) were digested with RNase H and re-annealed with the primers in a thermal cycler: RNase H digestion step at 37°C, 30 min; denaturation step at 95°C, 3 min; and primer reannealing step at 60°C, 5 min. This step generated cDNAs with double-stranded regions at the primer interface. Samples were then purified by column filtration (Montage PCR filters; Millipore, Billerica, MA). RNAs hybridization probes were synthesized by in vitro transcription with the use of P incorporation in 40 mmol/L Tris (pH 7.5); 6 mmol/L MgCl2; 10 mmol/L NaCl; 2 mmol/L spermidine; 2.5 mmol/L DTT; 125 μmol/L adenosine triphosphate (ATP), guanosine triphosphate (GTP), and cytidine triphosphate (CTP); 2.5 μmol/L cold uridine triphosphate (UTP); 20 U of RNase inhibitor; 2 kU of T7 RNA polymerase (Epicentre, Madison, WI); and 60 μCi of 33P-UTP (PerkinElmer, Waltham, MA). The labeling reaction was performed at 37°C for 4 h. Radiolabeled terminal continuation RNA probes were hybridized to custom-designed microarrays without further purification. Single-cell gene array expression was run in triplicate to increase the sequence depth per sample.

Custom-designed microarray platforms and data analysis: Platforms consist of 1 μg of linearized cDNA purified from plasmid preparations adhered to high-density nitrocellulose (Hybond XL, GE Healthcare, Piscataway, NJ). cDNAs were verified by sequence analysis and restriction digestion. Approximately 864 cDNAs were used on our custom array platform. Arrays were hybridized for 24 h in a solution consisting of 6 × saline-sodium phosphate-ethylenediaminetetraacetic acid, 5 × Denhardt's solution, 50% formamide, 0.1% sodium dodecyl sulfate (SDS), and denatured salmon sperm DNA (200 μg/mL) at 42°C in a rotisserie oven. Following the hybridization protocol, arrays were washed sequentially in 2 × saline sodium citrate/0.1% SDS, 1 × SSC/0.1% SDS, and
0.5 × SSC/0.1% SDS for 15 min each at 37°C. Arrays were placed in a phosphor screen for 24 h and developed on a phosphor imager (Storm 840, GE Healthcare, Piscataway, NJ).

Hybridization signal intensity was determined utilizing ImageQuant software (GE Healthcare). Briefly, each array was compared with negative control arrays utilizing the respective protocols without any starting RNA. Expression of terminal continuation amplified RNA bound to each target minus background was expressed as a ratio of the total hybridization signal intensity of the array (a global normalization approach). Global normalization effectively minimizes variation because of differences in the specific activity of the synthesized probe and the absolute quantity of the probe. Data analyzed in this manner does not allow the absolute quantification of mRNA levels generated. However, an expression profile of relative changes in mRNA levels was generated. Relative changes in total hybridization signal intensity and individual mRNAs were analyzed by One-way ANOVA with post hoc Neumann-Keuls test analysis. The level of significance was set at p<0.01 for individual comparisons.

3.3 RESULTS

Three-month-old WT, GFAP^{P301L}, and CaMKIIα^{P301L} mice were subjected to our 20-hit model of r-mTBI/sham, and three and six months after the last injury, we performed transcriptomic analyses on isolated ex-vivo primary astrocytes to investigate the contribution of tau astrogliopathy to changes in pathobiology under sham conditions (comparison among sham groups of each genotype) and whether r-mTBI exposure exacerbates the effects that astrocyte-specific tau overexpression has on cellular pathological response.

3.3.1 Astrocyte-specific transcriptional changes in response to astroglial vs neuronal tau at 7 and 10 months of age.

To determine the changes in astrocytic gene expression across our mouse models in response to astroglial or neuronal tau, we extracted the RNA from astrocytes isolated at 7 and 10 months of age.
age (Fig. 3.2A). To demonstrate astrocyte enrichment in our samples we investigated protein expression of cell-specific markers as well as the transcriptional expression of selected several cell-specific markers published in several studies (J. Pan et al., 2020). We observed a clear enrichment of astrocytic markers, at protein and transcriptomic levels, compared to microglia, endothelial cells, oligodendrocytes, and neurons (Fig. 3.2B-C). Having validated the enrichment for astrocyte RNA, we determined the number of genes that are differentially expressed in astrocytes in the three mouse models (WT, GFAP$^{P301L}$ and CaMKII$\alpha^{P301L}$) at both time points. Considering only significantly altered genes (adjusted p-value<0.05), we identified that at 7 months of age, the presence of tau within astrocytes of our GFAP$^{P301L}$ model induced the dysregulation of 11401 astroglial genes (5799 upregulated and 5602 downregulated) compared to WT. In comparison, the presence of neuronal tau in the brain caused the dysregulation of 7900 genes (4298 upregulated and 3602 downregulated) in CaMKII$\alpha^{P301}$ astrocytes compared to WT astrocytes (Fig. 3.3A).
Figure 3.2: Schematic representation of astrocyte isolation technique for RNA sequencing and astrocyte purity post-isolation. (A) Represents the steps for isolation of RNA from magnetically assisted cell sorting of astrocytes harvested from mouse brain tissue by ACSA2+ beads. (B) Qualitative immunoblots of cell specific markers in the brain following MACS sorting of ACSA2+ astrocytes from WT mouse brain tissue - OLIG2 (oligodendrocyte marker), MAP2/synapsin-1 (neuronal markers), IBA1 (microglial marker) and GFAP (astrocyte marker). (C) Heat map depicts RNA counts for cell specific markers following RNA sequencing of ACSA2+ astrocytes obtained from WT, GFAPP301L and CaMKIIαP301L mice at 3- and 6-months following r-mTBI/sham injuries.
Figure 3.3: Astrocyte specific pathways that are dysregulated in GFAP\textsuperscript{P301L} and CaMKII\textalpha\textsuperscript{P301L} mice compared to Wild-type mice at 7-month-old. Venn diagram of differentially expressed genes (DEGs) are shown in A (i.e. entire DEGs, overlapping DEGs and unique DEGs). Histogram in B and C depicts results of IPA pathway analyses after analyzing the entire DEG list between GFAP\textsuperscript{P301L} vs WT and CaMKII\textalpha\textsuperscript{P301L} vs WT, respectively. Histogram in D depicts results of IPA pathway analysis after analyzing the overlapping DEG list between GFAP\textsuperscript{P301L} vs WT. Histogram in E and F depicts results of IPA pathway analyses after analyzing the unique DEG list between GFAP\textsuperscript{P301L} vs WT and CaMKII\textalpha\textsuperscript{P301L} vs WT, respectively. Upregulated and downregulated pathways in B-F are depicted in red and blue, respectively. Heat-bar in B-F represents -log 10 of the P value (yellow – Topmost significant; purple – least significant). Threshold for obtaining the DEGs: adj. p-value $\geq$0.05 with its respective $-$log value $\geq$1.3. N=3 technical replicates/group.
Figure 3.4: Astrocyte specific pathways that are dysregulated in GFAP<sup>P<sub>301L</sub></sup> and CaMKIIα<sup>P301L</sup> mice compared to Wild-type mice at 10-month-old. Venn diagram of differentially expressed genes (DEGs) are shown in A (i.e. entire DEGs, overlapping DEGs and unique DEGs). Histogram in B and C depicts results of IPA pathway analyses after analyzing the entire DEG list between GFAP<sup>P<sub>301L</sub></sup> vs WT and CaMKIIα<sup>P301L</sup> vs WT, respectively. Histogram in D depicts results of IPA pathway analysis after analyzing the overlapping DEG list between GFAP<sup>P<sub>301L</sub></sup> vs WT. Histogram in E and F depicts results of IPA pathway analyses after analyzing the unique DEG list between GFAP<sup>P<sub>301L</sub></sup> vs WT and CaMKIIα<sup>P301L</sup> vs WT, respectively. Upregulated and downregulated pathways in B-F are depicted in red and blue, respectively. Heat-bar in B-F represents -log 10 of the P value (yellow – Topmost significant; purple – least significant). Threshold for obtaining the DEGs: adj. p-value ≥0.05 with its respective –log value ≥1.3. N=3 technical replicates/group.
Next, we performed pathway enrichment analysis on the significantly dysregulated genes (adjusted p-value<0.05 and IPA fold change cut-off=±1.2). At 7 months of age, interestingly, two opposing profiles were observed. Tau-bearing astrocytes show an immunosuppressed phenotype, characterized by the downregulation of *interleukin signaling (IL-8, IL-15, IL-1)*, *NFKB and STAT3 signaling*, as well as *production of nitric oxide (NO), and reactive oxygen species (ROS)* (compared to WT astrocytes (Fig. 3.3B). On the contrary, CaMKIIαP301L astrocytes showed the upregulation of the above-mentioned pathways when compared to WT astrocytes (Fig. 3.3C). Other important pathways identified in GFAPP301L astrocytes compared to WT include the upregulation of *calcium signaling* and *antioxidant action of Vitamin C (ascorbic acid)*. CaMKIIαP301L astrocytes also exhibit downregulation of *neurovascular coupling and antioxidant action*. Overall, changes seen in our mouse models suggest that GFAPP301L astrocytes have an immunosuppressed phenotype with enhanced antioxidant role and dysregulated calcium signaling, while CaMKIIαP301L astrocytes have a proinflammatory phenotype and impaired antioxidant defense.

At 10 months of age, we identified that GFAPP301L astrocytes had 11198 DEGs (5759 upregulated and 5439 downregulated) compared to WT, while CaMKIIαP301L astrocytes had 10086 DEGs (5178 upregulated and 4908 downregulated) compared to WT (Fig. 3.4A). Pathway enrichment analysis of significant genes (adjusted p. value<0.05, FC cutoff ±1.2) revealed that gene alteration in GFAPP301L astrocytes compared to WT indicate upregulation of *calcium signaling, glutamate receptor signaling and mitochondrial dysfunction* as well as a sustained but less dominant immunosuppressed phenotype (Fig. 3.4B) compared to the data 7 months of age. In the case of the CaMKIIαP301L astrocytes compared to WT, we identified upregulation of *mitochondrial dysfunction and neuroinflammation* and downregulation of *synaptogenesis, neurovascular coupling, and oxidative phosphorylation* (Fig. 3.4C).

To examine if there were genes common to astrocytes expressing human pathogenic tau (GFAPP301L) and those exposed to neuronal-derived htauP301L (CaMKIIαP301L), we searched for commonalities in differentially expressed genes (DEGs) obtained from the GFAPP301L vs WT and the CaMKIIαP301L vs WT comparison. At 7 months of age, we identified 2138 common/overlapping genes (862 upregulated and 1276 downregulated) with this comparison (Fig. 3.3A). Enriched
pathway analysis identified that those overlapping genes were associated with the upregulation of chemokine signaling, synaptogenesis, neuroinflammation, and mitochondrial dysfunction; and the downregulation of neurovascular coupling and detoxification function (glutathione-mediated) (Fig. 3.3D) [It is important to mention that because our purpose was to show pathways GFAPP301L and CaMKIIαP301L models have in common we only presented the 3.3D corresponding to the dysregulation in the GFAPP301L vs WT cohort only. The magnitude of the dysregulation varies slightly in the CaMKIIαP301L vs WT]. In addition, we performed enrichment pathway analysis on DEGs that were unique to each sham comparison (referred as unique DEGs): this revealed 9263 and 5762 DEGs in GFAPP301L vs WT and CaMKIIαP301L vs WT comparisons, respectively (Fig. 3.3A). We observed that some pathways no longer appear as dysregulated in the “unique DEGs” analysis compared to the “entire set of DEGs” analysis (unique and overlapping DEGs) in the GFAPP301L vs WT comparison such as the NFκB signaling, chemokine signaling, and inhibition of angiogenesis (Fig. 3.3E compared to Fig. 3.3B). In the CaMKIIαP301L vs WT comparison, we observed that chemokine signaling and inhibition of angiogenesis are lost in the “unique DEGs” analysis but found in the “overlapping DEGs” analysis while the dysregulation in IL-1 signaling is no longer significantly dysregulated in the “unique DEGs” analysis. Additionally, neurovascular coupling dysregulation changed directionality (Fig. 3.3C & 3.3F). The absence of NFκB signaling and IL-1 signaling in their respective analyses might arise from the fact that we are removing overlapping DEGs that, in conjunction with unique DEGs have a synergistic effect on signaling pathways. Regarding chemokine signaling, and inhibition of angiogenesis no longer identified in the unique DEGs analysis of both transgenic vs WT “unique DEGs” analysis but appear in overlapping DEGs analysis, we could suggest that those pathways are uniquely dysregulated in astrocytes only in response to mutant tau irrespective of its origin. Lastly, the change in the directionally of neurovascular coupling from downregulation in the “entire set of DEGs” analysis to upregulation in the “unique DEGs” analysis might show the dominant inhibitory effect of tau on neurovascular function.

At 10 months of age, we identified twice as many overlapping genes (4541 DEGs: 1998 upregulated and 2543 downregulated) in the GFAPP301L vs WT and CaMKIIαP301L vs WT comparison compared to the 7 months analysis (Fig. 3.4A). Enriched pathway analysis identified
that those overlapping genes were associated with the upregulation of chemokine signaling, synaptogenesis, neuroinflammation, and mitochondrial dysfunction; and the downregulation of cholesterol biosynthesis and detoxification function (glutathione-mediated and superoxide degradation) (Fig. 3.4D) [it is important to mention that because our purpose was to show pathways GFAPP301L and CaMKIIαP301L models have in common we only presented the 3.4D corresponding to the dysregulation in the GFAPP301L vs WT cohort only. The magnitude of the dysregulation varies slightly in the CaMKIIαP301L vs WT]. In addition, we performed enrichment pathway analysis on DEGs that were unique to each sham comparison: 6657 and 5545 DEGs in GFAPP301L vs WT and CaMKIIαP301L vs WT comparisons, respectively (Fig. 3.4A). We observed that some pathways no longer appear as dysregulated in the “unique DEGs” analysis compared to the “entire set of DEGs” analysis in the GFAPP301L vs WT comparison such as the mitochondrial dysfunction, chemokine signaling, VEGF signaling, detoxification function (glutathione-mediated), and endothelin-1 signaling (Fig. 3.4E compared to Fig. 3.4B). Those pathways, now appear in the “overlapping DEGs” analysis only. Additionally, in the “unique DEGs” analysis we observed the upregulation of potassium channels not seen in the other comparisons. In the CaMKIIαP301L vs WT comparison, we observed that chemokine signaling, mitochondrial dysfunction, endothelin-1 signaling, VEGF signaling, opioid signaling, calcium signaling and cholesterol biosynthesis were not present in the “unique DEGs” analysis but found in the “overlapping DEGs” (Fig. 3.4C & 3.4F). As mentioned in the paragraph above, pathways that are no longer significant in the “unique DEGs” analysis but are identified as significant dysregulated pathways in the “overlapping DEGs” analysis might indicate that those pathways are uniquely dysregulated in response to mutant tau irrespective of its origin.

Lastly, because we identified opposing states of immune response in GFAPP301L and CaMKIIαP301L astrocytes compared to WT, we were prompted to assess the differences of identified neuroinflammation-related pathways across time points for both GFAPP301L and CaMKIIαP301L astrocytes compared to WT (Fig. 3.5). Our analysis revealed that at both timepoints (7 and 10 months of age) GFAPP301L astrocytes tend to have an immunosuppressed phenotype compared to WT astrocytes. This phenotype was more prominent at 7 months of age with 10 out of 28 inflammatory pathways downregulated including IL-1, IL-8 and NFκB signaling. At 10 months of
age, GFAP$^{\text{P301L}}$ astrocytes showed a less dominant immunosuppressive phenotype with the downregulation of 3/28 pathways including the signaling of pro-inflammatory IL-12 and production of pro-inflammatory IL-15. Additionally, GFAP$^{\text{P301L}}$ astrocytes at 10 months of age also manifested the upregulation of 4/28 inflammatory pathways compared to WT. In contrast, CaMKII$^{\text{P301L}}$ astrocytes showed a progressive increased inflammatory state compared to WT astrocytes with 21/28 and 27/28 pathways upregulated at 7 and 10 months of age, respectively.
Figure 3.5: Inflammatory specific pathways that are altered in astrocytes of GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice compared to Wild-type mice at 7- and 10-month-old. Heat-map depicts results of IPA pathway analyses (stratified for inflammatory pathways alone) after analyzing DEGs between GFAP<sup>P301L</sup> vs WT and CaMKIIα<sup>P301L</sup> vs WT. Upregulated and downregulated pathways are depicted in red and blue, respectively. Heat-bar represents activation Z score following Fischer’s test of identified DEGs present in the IPA knowledge base. The 7- and 10-month-old mice above correspond with 3- and 6-month post-last injury sham mice from our original study. The four values on the heatmap indicate the small Z-score of those wells. The Z-score on all the other white wells is 0.
3.3.2 Astroglial transcriptome changes in response to r-mTBI three and six months post-last mTBI exposure.

Next, the changes in astrocytic gene expression across our mouse models in response to r-mTBI were evaluated by comparing the transcriptomic data for injured vs sham mice for each model. Three months post-last injury (3mpi), r-mTBI caused the dysregulated expression of 57 genes (51 downregulated and 6 upregulated) in WT, 175 genes (63 downregulated and 112 upregulated) in CaMKIIαP301L, and 441 genes (157 downregulated and 284 upregulated) in the GFAPP301L mice (Fig. 3.6A-D). This demonstrated that the presence of endogenous htauP301L in astrocytes causes greater transcriptomics gene dysregulation in response to r-mTBI compared to the exposure of astrocytes to exogenous neuronal tau in the CaMKIIαP301L or the absence of pathogenic human tau as in the WT mice. Additionally, we did not find a single gene that is dysregulated across the cohorts in response to TBI (Fig. 3.6A), which exposed the context-dependent nature of the astroglial response.

Next, we performed pathway enrichment analysis on the significantly dysregulated genes. In the WT cohort, the 57 significantly regulated genes were analyzed by IPA to predict the functional consequences of their dysregulation, and 5 significantly modulated pathways were identified. These were phagosome formation, insulin secretion, c-AMP signaling, synaptogenesis and dendritic cell maturation (Fig. 3.6E), all of them downregulated. Regarding the GFAPP301L cohort, 441 DEG’s mapped to 52 significant pathways including the downregulation of oxidative phosphorylation, synaptic long-term potentiation, and opioid signaling; and the upregulation of neuroinflammation, complement system signaling, and ERK5-mediated cell activation (Fig. 3.6F). In the CaMKIIαP301L cohort, 175 differentially expressed genes (DEG’s) were mapped to 17 significant pathways. Those included the downregulation of phagosome formation and neuroprotective erythropoietin signaling, the upregulation of nitric oxide and reactive oxygen species production, and senescence (Fig. 3.6G). These datasets revealed that in GFAPP301L astrocytes, r-mTBI elicits more pronounced changes that compromise mitochondrial functioning and neuronal supportive mechanisms; and favors a pro-inflammatory state compared to that observed in WT and CaMKIIαP301L.
Figure 3.6: Astrocyte specific pathways that are dysregulated in WT, GFAP\(^{P301L}\) and CaMKII\(\alpha^{P301L}\) mice at 3-month post-last injury. Venn diagram of injury dependent differentially expressed genes (DEGs) across all 3 genotypes are shown in A (i.e. entire DEGs, overlapping DEGs and unique DEGs). Volcano plot of injury dependent DEGs are shown in B (WT), C (GFAP\(^{P301L}\)) and D (CaMKII\(\alpha^{P301L}\)). Top 10 DEGs are highlighted on the volcano plots. Upregulated DEGs are in red, Downregulated DEGs are in blue. Histogram in E (WT), F (GFAP\(^{P301L}\)) and G (CaMKII\(\alpha^{P301L}\)) depicts results of IPA pathway analyses after analyzing the entire DEG list between r-mTBI vs sham groups for each of the 3 different genotypes. Upregulated and downregulated pathways in E-G are depicted in red and blue, respectively. Heat-bar in E-G represents -log 10 of the P value (yellow – Topmost significant; purple – least significant). Threshold for obtaining the DEGs: adj. p-value\(\geq0.05\) with its respective – log value \(\geq1.3\). \(N=3\) technical replicates/group.
Six months post-last injury (6mpi), r-mTBI caused the dysregulated expression of 5 genes (3 downregulated and 2 upregulated) in the GFAP\textsuperscript{P301L} astrocytes, and 555 genes (298 downregulated and 257 upregulated) in CaMKI\textalpha\textsuperscript{P301L} astrocytes (Fig. 3.7A-C). It is important to note that data from the WT cohort has been omitted because our internal validation revealed contamination of neuronal markers in the r-mTBI samples. Similar to 3mpi data, no common TBI-gene alterations were identified across the two genotypes analyzed (Fig. 3.7A). Pathway enrichment analysis on the significantly TBI-dysregulated genes from GFAP\textsuperscript{P301L} astrocytes was not possible to perform due to the low number of DEGs. In the CaMKI\textalpha\textsuperscript{P301L} cohort, IPA analysis on the 579 DEGs revealed 191 significantly modulated pathways that include the upregulation of\textit{phagosome formation, neuroinflammation, NO and ROS production, and leukocyte extravasation}; and the downregulation of\textit{antioxidant action, oxidative phosphorylation, and neurovascular coupling} (Fig. 3.7D). Collectively, these enriched pathways suggested that at 6mpi CaMKI\textalpha\textsuperscript{P301L}-derived astrocytes exhibit a progressive and persistent injury-associated phenotype with a diminished bioenergetic activity and pro-inflammatory state compared to GFAP\textsuperscript{P301L} astrocytes.
3.3.3 Transcriptome changes in human astroglia from CTE individuals

We also assessed gene array changes in hippocampal astrocytes harvested from CTE brains at stages II, III, and IV compared to non-demented controls (NDC). Our analysis shows that compared to controls, GFAP$^{+}$ astrocytes microaspirated from CTE brains stage II (CTE-II) had 74 downregulated DEGs, CTE-III derived astrocytes had 55 DEGs (54 downregulated and 1 upregulated), and CTE-IV derived astrocytes had 156 DEGs (153 downregulated and 3 upregulated) (Fig. 3.8D). We looked for similarities in astrocyte-specific genes across CTE stages.

Figure 3.7: Astrocyte specific pathways that are dysregulated in GFAP$^{P301L}$ and CaMKII$^{P301L}$ mice at 6-month post-last injury. Venn diagram of injury dependent differentially expressed genes (DEGs) across the two Tau mouse genotypes are shown in A (i.e. entire DEGs, overlapping DEGs and unique DEGs). Volcano plot of injury dependent DEGs are shown in B (GFAP$^{P301L}$) and C (CaMKII$^{P301L}$). Top 10 DEGs are highlighted on the volcano plots. Upregulated DEGs are in red, Downregulated DEGs are in blue. Histogram in D depicts results of IPA pathway analyses after analyzing the entire DEG list between CaMKII$^{P301L}$ r-mTBI vs sham groups. Upregulated and downregulated pathways in D are depicted in red, respectively. Heat-bar in D represents -log 10 of the P value (yellow – Topmost significant; purple – least significant). Threshold for obtaining the DEGs: adj. p-value≥0.05 with its respective -log value ≥1.3. N=3 technical replicates/group. Note: IPA analyses was not performed for GFAP$^{P301L}$ r-mTBI vs sham group due to the low number of DEGs.
vs control comparisons, and we found that CTE-II through IV share the downregulation of 21 genes such as Kcnj6, Atg3/aAg4a, and Slcga12 involved in potassium ion homeostasis, autophagy, and GABA uptake, respectively. Pathway enrichment analysis on DEGs revealed that CTE-II astrocytes (vs NDC astrocytes) sustained downregulation of 22 pathways including calcium signaling, neurovascular coupling, synaptogenesis, and autophagy (Fig. 3.8A). In the case of CTE-III (vs NDC), astrocytes underwent downregulation of 2 pathways only: sirtuin signaling and estrogen receptor signaling (Fig. 3.8B). In CTE-IV astrocytes (vs NDC astrocytes), the pathway analysis identified 100 dysregulated pathways including the upregulation of thrombin signaling, and sphingosine signaling, and the downregulation of neuroinflammation, interleukin signaling (IL-6, 8, 17), endothelin 1 signaling, calcium signaling, synaptogenesis, and insulin-like growth factor signaling (Fig. 3.8C). Thus, these data revealed that CTE astrocytes are compromised and exhibit loss of neurorestorative function(s) and in the most severe CTE stage, CTE-IV, such changes are prominently accompanied by an immunosuppressed state compared to controls.
Figure 3.8: Astrocyte specific pathways that are dysregulated in human CTE cases vs non-demented controls after laser microdissection of GFAP+ astrocytes and gene array analyses. Venn diagram of entire, overlapping, and unique differentially expressed genes (DEGs) between staged CTE cases (II, III and IV) vs non-demented controls (NDC) are shown in D. Histogram in A, B and C depicts results of IPA pathway analyses after analyzing the entire DEG list between CTE stage II vs NDC, CTE stage III vs NDC and CTE stage IV vs NDC, respectively. Uregulated and downregulated pathways in A-C are depicted in red and blue, respectively. Heat-bar in A-C represents -log 10 of the P value (yellow – Topmost significant; dark blue – least significant). Threshold for obtaining the DEGs: adj. p-value≥0.05 with its respective –log value ≥1.3. Approximately 50-100 astrocytes were micro-dissected and subjected to customized gene array analyses to interrogate >850 genes with >20 gene ontology groups.
Additionally, we looked for similarities in astrocyte-specific signaling pathway changes in response to repeated head injury between humans and mice. Because of the comparable ages between CTE-IV cases and non-demented controls (NDC), we selected CTE-IV vs NDC transcriptional data to assess similarities between humans and mice. Firstly, CTE-IV astrocytes (vs NDC) showed slightly more similarities with sham GFAP$^{P301L}$ astrocytes (vs WT) than with sham CaMKII$\alpha^{P301L}$ astrocytes (vs WT) at 7 months of age with 21 and 12 identified pathways in common, respectively (Table 3.2). Similarities with sham GFAP$^{P301L}$ astrocytes included the significant immunosuppressed response characterized by the downregulation of IL-8 signaling and NO and ROS production. Similarities with sham CaMKII$\alpha^{P301L}$ astrocytes included decreased neurovascular coupling signaling. At the age of 10 months, CTE-IV astrocytes (vs NDC) had as many common pathways with sham GFAP$^{P301L}$ astrocytes (vs WT) as with sham CaMKII$\alpha^{P301L}$ astrocytes (vs WT) (Table 3.3). Similarities with sham GFAP$^{P301L}$ astrocytes included significant immune response suppression. Similarities with sham CaMKII$\alpha^{P301L}$ astrocytes included decreased neurovascular coupling signaling, synaptogenesis, and calcium signaling. On the other hand, CTE-IV astrocytes (vs NDC) showed 4 common pathways with WT r-mTBI astrocytes (vs sham) 3mpi including the downregulation of phagosome formation, insulin secretion, cAMP-mediated signaling and Synaptogenesis (Table 3.3). Additionally, CTE-IV astrocytes (vs NDC) revealed three times more pathways in common with GFAP$^{P301L}$ r-mTBI astrocytes (vs sham) than CaMKII$\alpha^{P301L}$ r-mTBI astrocytes (vs sham) at 3mpi (Table 3.4). CTE-IV-GFAP$^{P301L}$ TBI similarities included the downregulation in synaptogenesis and calcium signaling. CTE-IV-CaMKII$\alpha^{P301L}$ TBI similarities included downregulation of erythropoietin signaling. Six months post-last injury, CTE-IV astrocytes (vs NDC) had 9 pathways in common with CaMKII$\alpha^{P301L}$ r-mTBI astrocytes (vs sham) including the downregulation of neurovascular coupling and synaptic long-term depression. Since IPA analysis on GFAP$^{P301L}$ r-mTBI astrocytes (vs sham) was not possible due to the low number of affect DEGs (5DEGs), we could not perform the comparison with human astrocytes.
### Table 3.2: Overlap between genotype dependent pathways in mouse (at 7 months-old) and CTE dependent pathways in human cases.

**Top panel** shows list of IPA generated pathways that *overlap* between GFAP\textsuperscript{P301L} vs WT mice at 7-months of age and CTE stage IV cases vs NDC.

**Bottom panel** shows list of IPA generated pathways that *overlap* between CaMKII\alpha\textsuperscript{P301L} vs WT mice at 7-months of age and CTE stage IV cases vs NDC.

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<td>Semaphorin Neuronal Repulsive Signaling Pathway</td>
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<th>CaMKII\alpha\textsuperscript{P301L} vs WT 3M</th>
<th>CTE4 VS CTRL</th>
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<td>Protein Kinase A Signaling</td>
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Table 3.3: Overlap between genotype dependent pathways in mouse (at 10-months old) and CTE dependent pathways in human cases.

**Top panel** shows list of IPA generated pathways that overlap between GFAP<sup>P301L</sup> vs WT mice at 10-months of age and CTE stage IV cases vs NDC.

**Bottom panel** shows list of IPA generated pathways that overlap between CaMKIIα<sup>P301L</sup> vs WT mice at 10-months of age and CTE stage IV cases vs NDC.
Table 3.4: Overlap between injury dependent pathways across all models and CTE dependent pathways in human cases. **Top panel** shows list of IPA generated pathways that **overlap** between WT r-mTBI vs sham mice at 3-months post-last injury and CTE stage IV cases vs NDC. **Second panel** shows list of IPA generated pathways that **overlap** between GFAP<sup>P301L</sup> r-mTBI vs sham mice at 3-months post-last injury and CTE stage IV cases vs NDC. **Third panel** shows list of IPA generated pathways that **overlap** between CaMKII<sup>α</sup>P301L r-mTBI vs sham mice at 3-months post-last injury and CTE stage IV cases vs NDC. **Bottom panel** shows list of IPA generated pathways that **overlap** between CaMKII<sup>α</sup>P301L r-mTBI vs sham mice at 6-months post-last injury and CTE stage IV cases vs NDC.
3.4 DISCUSSION

Through pathway analysis of differentially expressed genes (DEGs), we were able to shed some light on potential dysfunctional changes in astrocytes in response to endogenous vs exogenous (neuronal) tau overexpression under sham and injury conditions. Under sham conditions, astrocytic tau overexpression drove greater transcriptional changes in astrocytes compared to neuronal tau overexpression at 7 and 10 months of age. Tau-bearing astrocytes (GFAP\textsuperscript{P301L} astrocytes) showed a clear immunosuppressed phenotype at 7 months which was accompanied by mitochondrial dysfunction at the age of 10 months. On the contrary, CaMKII\textalpha\textsuperscript{P301L} astrocytes exposed to neuronal tau and robust microglial reactivity showed a progressive pro-inflammatory phenotype and lost their neurovascular coupling ability as well as their antioxidant function.

Under injury conditions, GFAP\textsuperscript{P301L} astrocytes showed greater transcriptional dysregulation compared to WT and CaMKII\textalpha\textsuperscript{P301L} astrocytes 3mpi. However, at 6mpi, the TBI effect on GFAP astrocytes is substantially decreased compared to 3mpi. The pronounced decrease of astrocytic DEGs in GFAP\textsuperscript{P301L} TBI vs sham analysis from 441 DEGs at 3mpi to 5 DEGs at 6mpi led us to hypothesize that at 6mpi (or 10 months of age) sham astrocytes have increased the accumulation of tau which masks the TBI or that astrocytes underwent recovery/resolution of reactive states.

To shed some light on those possibilities, we plan to investigate how different sham GFAP\textsuperscript{P301L} astrocytes are at 7 and 10 months of age as well as how different TBI GFAP\textsuperscript{P301L} astrocytes are at both ages. Moreover, through our collaborator - Dr. Elliott Mufson - at Barrow Neurological Institute (Arizona) we were able to evaluate gene array profiles in astrocytes harvested from CTE at stages II-IV and non-demented brains. Our comparisons between mouse and human astrocyte-specific transcriptomic changes showed that human CTE-IV derived astrocytes (vs NDC) manifest similarities with mouse sham tau-bearing astrocytes (vs WT) such as an immunosuppressive phenotype and also with mouse astrocytes exposed to neuronal tau pathology (plus substantial microglial reactivity) such as downregulation of neurovascular coupling and synaptogenesis, which may help understand the contribution of astrocytic and neuronal tauopathy in tauopathies.

Overexpression of tau\textsuperscript{P301L} caused an immunosuppressed state in sham astrocytes at 7 months of age which became less apparent at the age of 10mo. This might indicate that with age and
phenotype development (more tau astrogliopathy, Fig. 2.2), GFAP\textsuperscript{P301L} sham astrocytes start to manifest an immune response toward the progressive build-up of endogenous tau which might reduce the negative magnitude of neuroinflammatory suppression. In future studies, we plan to incorporate an aged GFAP\textsuperscript{P301L} cohort to delve into the effects of robust astroglial tau aggregation. As to our finding on astroglial transcriptional changes in response to neuronal tau and extensive microglial reactivity, CaMKII\textalpha\textsuperscript{P301L} sham astrocytes showed robust and progressive cell reactivity compared to WT at 7 and 10 months of age. Our findings are in line with an \textit{in-vitro} study on astrocyte-neuron co-culture studies where neurons manifest tauopathy and astroglial responses include increased and progressive cell reactivity, astroglial development, and stress response pathways suggestive of astrocyte reactivity (Rickner et al., 2022).

Furthermore, through our transcriptional analysis of GFAP\textsuperscript{P301L} and CaMKII\textalpha\textsuperscript{P301L} vs WT astrocytes, we observe a progressive mitochondrial dysfunction in response to overexpression of htau\textsuperscript{P301L} independently of its origin, astroglial or neuronal. Multiple studies in neurodegenerative diseases such as AD and PD have shown that mitochondrial impairment might cause astrocytes to engage in a neurotoxic behavior which renders neurons vulnerable to cell death (Bantle et al., 2021; Chen et al., 2022; Clasin, 2022; Fiebig et al., 2019; McConnell et al., 2019; Mulica et al., 2021).

With respect to human CTE astrocytes, our transcriptomic analysis of hippocampal CTE-IV astrocytes (vs NDC) showed significant immune response suppression and decreased neurovascular coupling signaling but, unlike murine astrocytes (GFAP\textsuperscript{P301L} and CaMKII\textalpha\textsuperscript{P301L} vs WT sham astrocytes), human astrocytes did not show changes in oxidative phosphorylation or dysregulated antioxidant function. The downregulation of neuroinflammatory pathways such as IL-12 and IL-15 in GFAP\textsuperscript{P301L} sham astrocytes at both time points (7 and 10mo) might cause the suppression of astrocyte-microglia bidirectional crosstalk because microglia respond to both interleukins to mount immune responses (Gaviglio et al., 2022; Gomez-Nicola et al., 2010). These findings might, to some extent, explain why in Chapter 2 (Fig. 2.7&2.9) we failed to observe a genotype effect in astrocyte and microglial reactivity between GFAP\textsuperscript{P301L} and WT sham groups. CTE-IV astrocytes (vs NDC) also manifest an immunosuppressed phenotype characterized by the downregulation of IL-8 signaling which was also found in GFAP\textsuperscript{P301L} sham astrocytes (vs WT) at 7
months of age. IL-8 signaling is also involved in microglial reactivity (McLarnon, 2016), thus, its downregulation alters the astrocyte-microglia communication to mount immune responses. The downregulation of neurovascular coupling signaling of CTE-IV (vs NDC) and CaMKIIαP301L astrocytes (vs WT) at both ages might cause failure in the autoregulation of the cerebral blood flow as well as BBB dysfunction (Clasin, 2022; McConnell et al., 2019; Toth et al., 2016). In contrast to our findings, Chancellor et al., 2021, showed that CTE white matter astrocytes exhibit a neuroinflammation state and the downregulation of mitochondrial functioning. The observed discrepancies in transcriptional changes might emerge from spatial-dependent astroglial responses to repeated head trauma (hippocampus vs white matter). In 2018, Hsu and colleagues found that in the white matter of CTE cases, there was prominent astroglial degeneration which might explain why astrocytes of the white matter exhibit those metabolic changes. Thus, in future studies, it would be interesting to consider the differences between grey matter (protoplasmic) and white matter (fibrous) astrocytes in response to tau astrogliopathy at baseline and following r-mTBI.

Additionally, transcriptional analysis reveals that CTE-IV astrocytes (vs NDC) share similarities with GFAP\textsuperscript{P301L} and CaMKIIαP301L astrocytes (vs respective sham counterparts) after r-mTBI including the downregulation of synaptogenesis (3mpi) and upregulation of thrombin signaling (6mpi), respectively. Our findings align with other studies on hippocampal changes post-TBI, which have shown a reduction in synaptogenesis (Jamjoom et al., 2021; Wen et al., 2017). In addition, in brain injury, thrombin signaling has been involved in BBB disruption, brain edema, neuroinflammation, and neuronal death. Thus, at chronic time points after r-mTBI, astrocytes appear to facilitate the generation of an unfavorable environment for neurons.

Altogether, these findings suggest that tau astrogliopathy in CTE might result in a “loss of function” phenotype where hippocampal astrocytes fail to mount an immune response and communicate to microglia, lose their ability to support synaptogenesis and maintain neurovascular coupling. In the future, we plan to validate the dysregulation of transcripts to corroborate the changes displayed by the transcriptome analysis. Additionally, the prominent pathways identified in the present study would warrant further investigation to understand and determine if the molecular changes dictating their dysregulation might represent therapeutic
targets. Given that tau-bearing astrocytes from our sham GFAP mice (vs WT) also showed a similar immunosuppressed state we could suggest that our GFAP$^{P301L}$ model is a potential mouse model candidate for studying tau astrogliopathy which might help increase our understanding of the effects of astrocytic tau accumulation on astroglial pathobiology in tauopathies such as CTE, PSP, CBD, and GGT. Moreover, in the present study, we showed temporal astroglial changes in response to r-mTBI. Thus, the transient astroglial changes in the course of the disease should be taken into consideration when developing therapeutic approaches for r-mTBI/CTE.

3.5 LIMITATIONS OF THE STUDY

Our mouse transcriptomic analysis of astrocytes was performed using a heterogeneous mixture of astroglial populations harvested from the hippocampi-depleted brain to ensure optimal quantities of RNA material. As such, we should consider the possibility that region-specific TBI-dependent changes and potentially important signals from the most vulnerable astroglial population might have been masked. Additionally, emerging evidence is showing how different astroglial populations exhibit unique changes in response to any stimuli including TBI (Shandra et al., 2019; Sofroniew, 2020). Thus, in future studies, we will consider single-cell RNA analysis to better understand how different populations of astrocytes respond to r-mTBI. Additionally, we could complement transcriptomic analysis with in-situ hybridization to reveal the spatial location of affected astroglial populations.

Moreover, it is important to acknowledge that the homogenization and digestion of brain tissue required to harvest astrocytes might initiate cellular inflammatory responses that could potentially contribute to the changes we report in this section. According to Marsh and colleagues (2022), the utilization of transcriptional and translational inhibitors may help preserve the in-vivo phenotype of isolated cells. Additionally, we recognize that our samples from the GFAP$^{P301L}$ and CaMKIIα$^{P301L}$ cohorts show some contamination of neuronal and microglial markers, respectively, which might mask the actual contribution of astrocytes to the changes reported in the genotype vs WT, and TBI vs Sham comparisons of GFAP$^{P301L}$ and CaMKIIα$^{P301L}$ mouse models. Since all samples were isolated using the same technique and parameters, we hypothesize that the contamination of neuronal and microglial markers might depend on the phenotype of the transgenic models. For instance, the increase of neuronal marker in the
GFAP$^{P301L}$ astroglial pellet might be a consequence of tau insertion and expression in the early days of development which might alter the lineage of astrocyte progenitors leading to an incomplete differentiation of neural progenitor cells into astrocytes/neurons (this is mere speculation due to the lack of literature on animal models of tau astrogliopathy). In regards to the presence of microglial markers on the astroglial pellet harvested from CaMKII$^{\alpha}$P301L mice, it could be explained by the increase of FcR receptors in microglial in response to inflammation (microglial activity) which is abundant in the CaMKII$^{\alpha}$P301L model as shown in Chapter 2. Besides, despite that we showed astrocyte-enriched samples after magnetic isolation Fig.3.2B, we did not perform validation for every single cohort or sample to ensure optimal material. Thus, we cannot discard the possibility that cell contamination in our samples originates from a technical issue (e.g, low volume of FcR blocking reagent for CaMKII$^{\alpha}$P301L astrocytes).

As mentioned in Chapter 2, in the current studies we were utilizing mouse models with slightly different strain backgrounds, WT that were on the C57 background while both humanized tau mouse models were on the FVB-C57 background (50/50 background). Several studies have shown slight differences in cytokine composition between FVB and C57. However, there is not a comparative analysis between C57 and FVB-C57 mice. Because our tau models are on a 50/50 background, we considered that there might be even subtler changes in the cellular, molecular, and chemical composition of the brain which should not overtly affect our outcomes.

With respect to our transcriptomic analysis on CTE vs NDC astrocytes, it is important to mention that our control samples are not sex-matched as they compromised 2 males and 7 females while the CTE samples were all male. We performed a qualitative analysis that consisted of generating a heatmap with the abundance of transcript reads for each gene in each sample which showed that female and male astrocyte-specific transcriptomic profiles (microarray genes) do not differ substantially (Fig. S3.1A). Instead, we observed that the APOE allele might have a predominant effect on the astroglial transcriptome given that only the APOE 2/4 case showed evident overall transcriptional downregulation compared to APOE 3/3 and APOE 3/4 cases. This finding on its own is worthy of further research. Since there were no apparent differences between male and female samples, CTE (II-IV) vs CTRL transcriptomic comparisons were feasible. Additionally, the mean of age is significantly different between some CTE groups and the non-demented control.
group (CTE-II vs CTRL: \(p<0.01\); CTE-III vs CTRL: \(p=0.009\)). However, those control cases were the ones available to us at the time of performing the experiments. For future analyses, we hope to correct the sex and age discrepancies which will increase the reliability of our analysis.

Additionally, there are a few aspects to take into consideration while interpreting the astroglial functional changes in the human-mouse comparison. Human and mouse transcriptomic data were obtained using different RNA sequencing tools. Human RNA data was obtained by a customized array that evaluated changes in 864 genes in hippocampal astrocytes, while our mouse data was obtained by unbiased bulk RNA sequencing on astrocytes harvested from the entire brain excluding the hippocampi. Previous reports from our labs have shown differential spatial responses of astrocytes to TBI where the cortical area directly below the impact and CC exhibit greater astrocyte reactivity compared to other brain areas (Mouzon et al., 2012, 2019; Ojo et al., 2015). Besides, other groups have shown that astrocytes exhibit region-dependent transcriptional changes (Batiuk et al., 2020; Clarke et al., 2021). Thus, we should use caution to not generalize the current findings to all astrocyte populations of every brain region.

Finally, despite our attempts to understand the contribution of astrocytic tau vs neuronal tau in astroglial pathobiology by individually comparing astrocyte-specific murine (GFAP\(^{P301L}\) and CaMKII\(^{αP301L}\)) to human transcriptomic changes (CTE-IV) either with relation to genotype (transgenic mice vs WT) or injury (TBI vs Sham), we acknowledge that astrocytic and neuronal tau aggregation are present in the same environment and, as such, it might have a synergistic effect on astroglial pathobiology. In addition, we should keep in mind that the utilization of mouse models allows us to have control over the area, strength, and inter-injury interval of the impact as well as the time points post-last injury that leads to the collection of less variable and more reliable results. However, with human cases, the researcher does not have control over the above-mentioned variables that have been shown to have an impact on the astroglial response. Despite all the caveats presented above, we were still able to obtain valuable information regarding the behavior of tau-bearing astrocytes under normal circumstances and following TBI. We were also able to find similarities between mouse and human tau-bearing astrocytes which shows the clinical potential that our finding might have upon further investigation.
3.6 SUMMARY

In summary, the results provided in this section of the thesis show that our hypotheses are majorly upheld. First, when compared to WT, tau-bearing astrocytes have ~1.5 and ~1.1 more significantly DEGs compared to astrocytes exposed to robust neuronal tau pathology and microglial reactivity, respectively, at 7 and 10 months of age. Tau-bearing murine astrocytes exhibit a compromised bioenergetic system which might interrupt their physiological roles in neuronal trophic and metabolic support, and ion homeostasis. We also observed that in both humanized tau mouse models the dysregulated pathways get more pronounced with age. These findings upheld our first hypothesis. Second, 3mpi, tau-bearing astrocytes show ~8 and ~2.5 times more transcriptional dysregulation compared to WT and CaMKIIαP301L astrocytes, respectively. However, such changes are diminished by the 6mpi thus our second hypothesis is partially upheld. Third, analysis of human astroglial transcriptomic changes shows that astrocytes harvested from CTE stage IV manifest in ~2 and ~3 times more transcriptional dysregulation than stages II and III, respectively, when compared to controls. However, IPA analysis does not show a progressive impairment in CTE astrocytes. Thus, our third hypothesis is partially upheld. Finally, at the latest stage of CTE (CTE-IV) astrocytes appear to develop an immunosuppressed phenotype comparable to the effects of tau overexpression in murine astrocytes from our GFAPP301L mouse model at 7 and 10 months of age. Therefore, our fourth hypothesis is upheld.

Lastly, the immunosuppressed state of tau-bearing astrocytes and its commonality with CTE-IV-derived astrocytes at both ages prompted us to further investigate the effects of the immunosuppressed phenotype. Because astrocytes and microglia are the two main cell populations in charge of mounting immune response in the CNS, our goal with the experiments described in Chapter 4, was to try to understand the astrocyte contribution in the bidirectional molecular communication with microglia in isolation. Additionally, because CTE-IV-derived astrocytes share more similarities with astrocytes harvested from our GFAPP301L model at 7 months of age either under normal circumstances or after r-mTBI we chose to analyze the astroglial-microglial interaction at 7 months of age (3mpi).
Supplementary Figures.

Supplementary Figure 3.1: Heat map revealing expression levels of all genes in the microarray of all non-demented (NDC) control cases and brief clinical demographics of CTE and NDC cohorts. (A) Heat map depicts relative intensity score (i.e., expression levels) of all genes in the microarray from all non-demented control (NDC) cases. Upregulated and downregulated genes are depicted in red and blue, respectively.
CHAPTER 4: INFLUENCE OF ENDOGENOUS VS EXOGENOUS TAU ON ASTROCYTE REACTIVITY AND THEIR INTERACTION WITH MICROGLIA AFTER R-MTBI.

4.1 INTRODUCTION
As mentioned in Chapter 3, the resemblance of GFAP<sup>P301L</sup> sham astrocytes at the age of 7 months and hippocampal CTE-IV astrocytes regarding the immunosuppressed state and the greater response of tau-bearing astrocytes 3mpi prompted us to further study the possible roles of an immunosuppressed astroglial state of sham GFAP<sup>P301L</sup> astrocytes and the reactive state of tau-bearing astrocytes after r-mTBI in astrocyte reactivity and the homeostatic bidirectional communication with microglia. Given that CaMKII<sub>α</sub> astrocytes exhibited an opposing immune state compared to GFAP<sup>P301L</sup> astrocytes, we were also interested in understanding how astrocytes exposed to exacerbated tau pathology and microglial reactivity impacts on astroglial functional state and the astrocyte-microglia cross-talk. In the CNS, astrocytes and microglia are in constant communication to maintain neuronal health and the integrity of neuronal circuits. Crosstalk between astrocytes and microglia is possible via secreted messengers such as cytokines, chemokines, growth factors, and metabolic mediators (Matejuk & Ransohoff, 2020). Under homeostatic conditions, for instance, astrocyte-derived cytokines (e.g. IL-33, a member of the IL-1 family) trigger microglial reactivity that results in synaptic refinement (Vainchtein et al., 2018). Under neuroinflammatory conditions, microglial-derived cytokines such as IL-1β, TNF, and IL-6 are thought to regulate astrocytic responses that include tissue remodeling and repair (Shinozaki et al., 2017) or trigger a neurotoxic astroglial profile via microglial release of IL-1α, TNF, and C1q (Liddelow et al., 2017). Following brain trauma, the expression of inflammatory genes is regulated by transcription factors such as NFκB and STAT3 (Nonaka et al., 1999; Oliva et al., 2012; Pennypacker et al., 2000). Chronic NFκB activation in reactive astrocytes and microglia has been associated with favoring a pro-inflammatory environment in the brain and provoking neuronal cell death (Dresselhaus & Meffert, 2019). STAT3 activation in astrocytes, in the context of CNS injury, has been reported to be a critical regulator of astrocyte reactivity including GFAP
expression, secretion of immunomodulator agents such as chemokines and cytokines, and proliferation typical of scar-forming astrocytes (Ceyzériat et al., 2016; Herrmann et al., 2008).

In the present chapter, first, we wanted to investigate the reactive state of primary astrocytes harvested from our three mouse models (WT, GFAP\textsuperscript{P301L} and CaMKII\textalpha\textsuperscript{P301L} mice; TBI and Sham) at baseline, then look at how cellular priming with LPS changed the reactive state of primary astrocytes. Then, we sought to evaluate the \textit{in-vitro} microglial response to astrocyte-derived secretome or astrocyte-conditioned media (ACM) across our three mouse models and under r-sham and r-mTBI conditions. Furthermore, we were also interested in assessing the \textit{in vivo} reaction of microglia and astrocytes to intracerebral injection of ACM derived from our mouse models.

Experiments in this chapter sought to test the following hypotheses:

a) \textit{Tau}\textsuperscript{P301L}-bearing astrocytes from GFAP\textsuperscript{P301L} sham mice manifest reduced levels of cell activation markers compared to WT and CaMKII\textalpha\textsuperscript{P301L} sham astrocytes. However, cell activation markers in tau-bearing astrocytes will be increased after r-mTBI compared to the astrocytes harvested from the other models. Additionally, when exposed to a secondary inflammatory stimulus (\textit{post-last} injury), tau overexpression in GFAP\textsuperscript{P301L} TBI astrocytes will lead to an increased reactive state compared to WT and CaMKII\textalpha\textsuperscript{P301L} TBI astrocytes.

b) In a secretome-to-transcriptome analysis, \textit{tau}\textsuperscript{P301L}-bearing TBI astrocytes harvested three months following r-mTBI (plus live ex \textit{vivo}) manifest a more reactive secretome profile compared to WT/CaMKII\textalpha\textsuperscript{P301L} TBI astrocytes.

c) \textit{In-vitro} exposure of microglia to astroglial secretome from sham astrocytes harboring human mutant tau (GFAP\textsuperscript{P301L} astrocytes) will not cause changes in microglial reactivity at baseline conditions, however, astrocytic secretome collected from GFAP\textsuperscript{P301L} TBI astrocytes will elicit an increased microglial response that leads to a greater secretion of pro-inflammatory cytokines compared to WT and CaMKII\textalpha\textsuperscript{P301L} TBI ACM.
d) Intracerebral injection of secretome collected from tau^{P301L}-bearing TBI astrocytes causes increased astrocyte and microglial reactivity in the cortex of naïve mice compared to their sham counterparts and WT TBI astrocytes.

4.2 METHODS AND MATERIALS

4.2.1 Animals
The animals used for this chapter were the same cohorts used in Chapter 2. Briefly, we used three-month-old male and female C57BL/6 mice (n=24), GFAP^{P301L} (n=24) and CaMKII<α^{P301L} (n=24) Mice were housed in a 12h light/dark cycle with food and water *ad libitum*. All experiments were performed in accordance with Office of Laboratory Welfare and National Institutes of Health guidelines with Roskamp Institute Institutional Animal Care and Use Committee approval.

4.2.2 Injury protocol
Repetitive mild injury (r-mTBI) was administered using the same 20hit paradigm as previously described in Chapter 2. Briefly, mice were administered a closed head injury daily from Monday through Friday for four weeks. Mice were sacrificed 3 months or 6 months post-last injury (3mpi and 6mpi, respectively) for subsequent transcriptomic analyses.

4.2.3 Primary astrocyte culture
Astrocyte cultures were prepared from perfused brains of 3-month-old GFAP^{P301L}, CaMKII<α^{P301L} and non-carrier littermates (n=6 per genotype, n=3 Sham and n=3 TBI). Entire brains were collected using stringent aseptic conditions, minced, dissociated using an enzyme-based brain disassociation kit (MACS, Miltenyi Biotec), and passed through sterile 40µm nylon sieves. Cell pellets enriched in astrocytes and microglia were seeded in T75 flasks containing Dulbecco modified Eagle medium-F12 (DMEM-F12) complemented with anti-bacterial and antimycotic reagent, and 10% fetal bovine serum. Cells were allowed to grow in a humidified chamber provided with 5% CO₂ and 95% air at 37°C. When cells reached 80% confluency at day 24 *in-vitro*, microglial cells and oligodendrocyte precursor cells were removed through shaking as detailed in
Pure astrocyte cell pellets were re-seeded at $1 \times 10^4$ in 24-well plates for further experiments (see Fig. 4.1)

**Figure 4.1. Study timeline of experiments of Chapter 4.** Three-month-old wild-type (WT), GFAP$^{P301L}$, and CaMKII$^{\alpha}$P301L were subjected to mild TBI every weekday for 5 days a week for four weeks resulting in 20 hits in a month. Three months post-last injury (3mpi), astrocytes were harvested from and cultured for 25 days (25 DIV) until a confluency of 80% was reached. Primary astrocytes and their media (i.e., astrocyte-conditioned media (ACM)) were utilized for subsequent experiments as described in the figure. Details of the experiments can be found in their respective section.

### 4.2.4 LPS exposure to primary astrocytes

Primary astrocytes harvested from WT, GFAP$^{P301L}$ and CaMKII$^{\alpha}$P301L mouse brain (n=3 per group, Sham and TBI) and were re-seeded into 24-well plates and cultured until 80% confluency was
reached 5 days later. On DIV30, astrocytes were exposed to 50ng/ml LPS made up in DMEM-F12. Cells were exposed to the stimulus for 8 hours. After the 8h LPS exposure, media in the wells was vacuum-absorbed and cells were exposed to mammalian protein extraction reagent containing protease and phosphatase inhibitors and stored at -80°C until required for western blotting analysis. The LPS exposure time and the concentration were selected based on preliminary analyses performed on TauKI sham primary astrocytes where we exposed TauKI primary astrocytes to LPS for 15min, 30min, 1h, 4h, and 8h. As opposed to cell line astrocytes, our primary astrocytes had a delayed response to LPS, 8h being the only timepoint at which we observed differential cellular reactivity between TauKI Sham and TBI primary astrocytes.

4.2.5 Secretome-to-transcriptome analysis

To perform the secretome-to-transcriptome analysis we looked for commonalities between significantly dysregulated genes (adj. p value=0.05) of our astrocyte-specific bulk RNA analysis (TBI vs Sham) from our three mouse models and the secretome profile on an online database of astroglial secretome www.gliome.org. Because following TBI, astrocytes are exposed to an increased presence of cytokines including IL-1β, IFNg, and TNF (Longhi et al., 2013; Malik et al., 2023; Roselli et al., 2018; Vincent et al., 2023), we opted for using the secretome profile of C8D1A stimulated with IL-1β (0.2 ng/ml) + IFNg (1 ng/ml) + TNF (10 ng/ml). This profile has 1691 identified secreted molecules.

4.2.6 Astrocyte-conditioned media (ACM) media collection and exposure

One day after astrocytes were re-seeded in the 24-well plates, fresh media was added. After 5 days, ACM from wells was collected and stored at -80°C until needed for subsequent experiments. Immortalized microglia (IMG cell line; EF4001 Kerafast Biotech Company) were seeded into 24-well plates at 5x10⁵ cells per well with DMEM-F12 media (supplemented with 10% Fetal Bovine Serum and 1%antibiotic/anti-mycotic) and placed in a humidified chamber provided with 5% CO₂ and 95% air at 37°C. Once cells reached 100% confluence, they were exposed to 30ul of ACM for 30min ( time was selected based on primary studies where microglia seemed to respond better at 30min compared to 1h or 24h of exposure). After ACM exposure,
media was collected and stored at -80°C until needed for cytokine analysis. Cells on the wells were exposed to mammalian protein extraction reagent containing protease and phosphatase inhibitors and stored at -80°C until required for western blotting analysis.

4.2.7 Western Blotting

Cell lysates were obtained by adding 100μl of mammalian protein extraction reagent (plus protease and phosphatase inhibitors). Samples were centrifuged at 14000rpm for 20 minutes at 4°C to pellet out debris. To ensure that the same amount of protein in every sample was being analyzed through immunoblotting, the total amount of protein was determined using the Bicinchoninic acid assay. Five micrograms of extracted protein were mixed with a denaturing buffer containing β-mercaptoethanol and boiled at 95°C for 10 minutes. Samples were then loaded on 4-15% SDS-PAGE gels for protein separation. Next, proteins were transferred onto PVDF membranes overnight. Transferred membranes were blocked with 5% non-fat milk in 0.05% TBST for 1 hour at room temperature. Subsequently, membranes were incubated with the primary antibodies (see Table 4.1 for antibody list) at 4°C overnight. Membranes were washed in TBST 3 times (5 minutes each) prior to the exposure of horseradish peroxidase-conjugated secondary antibodies (see Table 1) for 1 hour at room temperature. Membranes were washed in TBST and deionized water and developed by using a chemiluminescent system (ECL or FEMTO) (Thermofisher, USA). Imaging of the revealed markers was done using a ChemiDoc MP imager. Subsequently, densitometry analysis of each marker was done employing Image Lab software. B-actin protein was used to normalize the data.

4.2.8 Tau concentration in ACM

The protein concentration of human tau in astrocyte-conditioned media (ACM) samples from our three mouse models (WT, GFAP\textsuperscript{P301L} and CaMKI\textsuperscript{α}P301L sham and TBI) was quantified using ELISAs. ACM total tau was quantified using Human Tau (total) ELISA Kit (#KHB0041, Invitrogen) according to the manufacturer’s protocol. Briefly, ACM samples were diluted in a diluent buffer prior to being incubated in capture antibody-coated wells for 2 h at room temperature. Wells were washed several times before being incubated in detection antibody for 1 h at room temperature.
Wells were washed again before being incubated with horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. Wells were then washed again before being incubated with stabilized chromogen for 30 min at room temperature. After this incubation, stop solution was added to each well and the plate was read at 450 nm. A set of standards of known (p)Tau concentration [0, 10, 75, 125, 250, 500, 1000, 2000 pg/ml for total tau, for quantification of tau content in ACM samples from the standard curve. For this analysis, we utilized ACM collected from astrocytes used in section 4.2.1

4.2.9 Cytokine quantification in microglial secretome after ACM exposure.

Cytokine levels in microglia-derived media after ACM were analyzed using a Meso Scale Discovery 96-well MULTI-SPOT® Ultra-sensitive V-PLEX proinflammatory Panel 1 mouse kit (K15048D-1) according to the manufacturer’s instructions. Briefly, 50μl of microglia-derived media was added to the plate and incubated at room temperature for 2 hours. Wells were washed to remove unbound analyte; secondary antibody was then added, and the samples were left to incubate at room temperature for one hour and quantified using the MESO QuickPlex SQ120 plate reader. Following quantification, all data were normalized to the protein content of corresponding microglial lysates, determined by Bicinchoninic acid protein assay (BCA) (ThermoFisher). Cytokine data are presented as mean ±SEM pg/mg protein. This assay measures ten cytokines: IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, KCGRO, IL-10, IL-12p70, and TNF. The assay was performed according to the manufacturer’s instructions.

4.2.10 Intracerebral injection of ACM

Every mouse undergoing this procedure was deeply anesthetized with 3% isoflurane and 1.5% oxygen for 3 min. Before the mouse was placed in the stereotaxic frame, its head was shaved and it was administered a subcutaneous injection of pre-analgesic Ketoprofen (5mg/kg). Before starting the surgery, we ensured that the mouse lacked reflexes. Then we made a midline incision on the head using a sterile scalpel to expose the skull. With the help of a digital stereotaxic arm we located the injection site (−1.5 AP; −1.5 ML relative to Bregma) and drilled a hole on the skull. Subsequently, we performed the intra-cerebral injections were done using a Hamilton syringe; the coordinates for the cortical injections were −1.5 AP; −1.5 ML relative to Bregma −1 DV from
the dural surface. A volume of 8 µL of serum-free ACM was injected at a rate of 0.2 µL/min. The syringe was removed slowly after 10 min following delivery of ACM to avoid leakage of the inoculum. Then, the head incision was closed using blue glue. Following surgery, the animals were kept in a warm blanket and monitored until they recovered from the anesthesia. Before the mouse was returned to the cage, we administered Ethique® (3.25mg/kg) for post-surgery pain management. Injected animals were returned to their original cages that belong to the same experiment (i.e., other inoculated mice).

4.2.11 Immunohistochemistry

After transcardiac perfusion, mice were decapitated. The brains of each mouse were collected in 4% PFA. After 24-48h, brains were dehydrated and paraffin-embedded using the Tissue-Tek VIP and Tissue-Tek TEC, respectively. Brains were coronally sectioned at 6µm using a Leica RM2235 microtome and mounted on slides. Sections were deparaffinized using HistoClear® and rehydrated in a decreasing gradient of ethanol before the immunohistochemistry procedures. Following rehydration, slides were submerged in PBS for 5 minutes to wash the excess of ethanol. Then slides were immersed in hydrogen peroxide for 15 minutes to deactivate endogenous peroxidases. Next, antigen retrieval was performed using an acidic buffer (pH 6). Sections were incubated with 2.5% normal goat serum at room temperature for 1 hour and subsequently incubated with primary antibody at 4°C overnight (table 4.1). The next day, slides were rinsed in PBS and incubated with horse-radish-peroxidase labeled secondary antibody at room temperature for 1 hour. The staining was developed using 3,3’-Diaminobenzidine (DAB). Finally, after slight counterstaining with hematoxylin, tissue was dehydrated in an increasing gradient of ethanol. Next, slides were mounted using toluene. Images of sections were collected using an Olympus DP72 microscope.
<table>
<thead>
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<th>Dilution primary antibody</th>
<th>Host species</th>
<th>Vendor</th>
<th>Cat. Number</th>
<th>Dilution secondary antibody</th>
<th>Protocol</th>
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<tr>
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<td>Mouse</td>
<td>Dr. Peter Davis</td>
<td></td>
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<td>WB</td>
</tr>
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**Table 4.1. Antibodies used.** Abbreviations: Iba1, Ionized calcium-binding adaptor molecule; GFAP, Glial Fibrillary Acidic Protein; p/tNFκB, phosphorylated/total Nuclear Factor κappa B; p/tSTAT3, phosphorylated/total Signal Transducers and Activators of Transcription 3; IHC, Immunohistochemistry; WB, Western blotting.

### 4.2.12 Bright-field microscopy and image analysis

Multiple regions of interest in the cortex and corpus callosum (CC) were analyzed in a standardized fashion for each marker. For each mouse (n=6), a minimum of 4 microscopic fields imaged by 10x objective lens were analyzed per region. The immunoreactivity of cell markers was measured by quantitative image analysis performed blindly by the investigator using ImageJ software. Before quantitative analysis, color deconvolution was applied on bright-field images to separate the DAB staining from the hematoxylin staining. The percentage area analysis was performed using the “DAB” set of images.
4.2.13 Statistical analyses

The statistical analysis of all presented data was performed using GraphPad Prism Version 9. Data were tested for normality using the Shapiro-Wilk test. Then statistical analysis was obtained using Two-way or Three-way ANOVA accompanied by the Benjamini, Krieger, and Yekuteli test to correct multiple comparisons. In the case that data was not normally distributed, we performed log transformation data. If after transformation, data remained with a non-normal distribution, Kruskal-Wallis non-parametric test was performed. Data are presented as mean ±SEM. P values <0.05 were considered statistically significant. Graphs in the Results Section show asterisks representing different p-value ranges: < 0.05 = *; <0.01 = ** and <0.001 = ***.

4.3 RESULTS

4.3.1 Astrocyte reactive state under normal conditions (baseline) and after LPS exposure, ex-vivo.

To investigate the astrocyte reactivity at baseline and after LPS exposure, we collected primary astrocytes from the brain of our three mouse models at the age of 7 months or 3mpi. A factorial three-way ANOVA was conducted to compare the main effects of genotype, injury, and LPS exposure as well as their interaction effects on astroglial cellular activity (pNFκB and pSTAT3 levels). At baseline (i.e., no LPS treatment), pNFκB levels remained unchanged in the r-sham and r-mTBI groups across the three models (Fig. 4.2A-B). After LPS exposure, WT TBI astrocytes did not exhibit an increase in pNFκB levels compared to WT sham astrocytes. As to GFAP<sup>P301L</sup> astrocytes, LPS elicited a significantly increased pNFκB response in TBI astrocytes compared to their sham counterparts. In the case of CaMKIIα<sup>P301L</sup> astrocytes, there was no difference in pNFκB levels in TBI and Sham astrocytes after LPS exposure (three-way ANOVA genotype effect [F(2,24)=7.38, p=0.0032]; injury effect [F(1,24)=4.54, p=0.044]; LPS treatment [F(1,24)=33.43, p=<0.0001]; genotype*injury [F(2,24)=2.10, p=0.1559]; genotype*LPS treatment [F(2,24)=2.28, p=0.123]; injury*LPS treatment [F(1,24)=2.749, p=0.11]; and genotype*injury*LPS treatment [F(2,24)=0.307, p=0.737]). Next, in order to determine how different sham or TBI astrocytes responded to LPS compared to untreated counterparts, we compared sham (LPS treated)-to-sham (untreated) and TBI (LPS treated)-to-TBI (untreated). We found that pNFκB levels in both
sham and TBI WT astrocytes remained unchanged compared to their untreated counterparts. As to GFAP\textsuperscript{P301L} astrocytes, LPS-treated TBI astrocytes manifested pNFκB changes that were significantly higher compared to untreated GFAP\textsuperscript{P301L} TBI astrocytes. No difference between LPS-treated GFAP\textsuperscript{P301L} sham astrocytes and untreated GFAP\textsuperscript{P301L} sham astrocytes was observed. In the case of CaMKII\textalpha\textsuperscript{P301L} astrocytes, there was a significant increase in pNFκB in LPS-treated TBI astrocytes compared to untreated TBI counterparts. Lastly, pNFκB response in CaMKII\textalpha\textsuperscript{P301L} sham astrocytes after LPS exposure was significantly higher compared to LPS-treated WT sham astrocytes. Besides, LPS-treated GFAP\textsuperscript{P301L} TBI astrocytes showed a significantly increased pNFκB-mediated cellular response compared to LPS-treated WT TBI astrocytes.

In contrast to pNFκB levels, pSTAT3 levels varied across genotypes at baseline conditions. Untreated GFAP\textsuperscript{P301L} and CaMKII\textalpha\textsuperscript{P301L} Sham astrocytes revealed a significant 2 and \sim\textasciitilde3-fold increase in pSTAT3-mediated cellular response compared to untreated WT sham astrocytes, respectively (three-way ANOVA genotype effect [F(2,23)=16.97, p=<0.0001]; injury effect [F(1,23)=11.12, p=0.0029]; LPS treatment [F(1,23)=59.80, p=<0.0001]; genotype\*injury [F(2,23)=2.02, p=0.155]; genotype\*LPS treatment [F(2,23)=2.90, p=0.075]; injury\*LPS treatment [F(1,23)=1.87, p=0.184]; and genotype\*injury\*LPS treatment [F(2,23)=1.36, p=0.276]) (Fig. 4.2C). Untreated CaMKII\textalpha\textsuperscript{P301L} TBI astrocytes also showed a significant \sim\textasciitilde2-fold increase in pSTAT3 levels compared to untreated WT TBI astrocytes. In the case of untreated GFAP\textsuperscript{P301L} TBI astrocytes, we could not perform statistical comparisons with any other group because the group only had n=2 (the third value was an outlier; therefore, it was excluded). As to pSTAT3 astroglial levels in response to LPS exposure, we did not observe differences between TBI and Sham astroglial response in any of the three genotypes. However, we observed that in contrast to LPS-treated CaMKII\textalpha\textsuperscript{P301L} TBI astrocytes, LPS-treated GFAP\textsuperscript{P301L} TBI astrocytes exhibited a significant \sim\textasciitilde2-fold increase in pSTAT3 levels compared to LPS-treated WT TBI astrocytes. Additionally, when we compared LPS-treated astrocytes irrespective of injury to untreated astrocytes we observed that, overall, primary astrocytes exposed to LPS show reduced levels of pSTAT3. LPS-treated WT TBI astrocytes showed a significant \sim\textasciitilde2-fold decrease in pSTAT3 compared to its untreated WT sham counterpart. LPS-treated GFAP\textsuperscript{P301L} Sham astrocytes also showed a significant \sim\textasciitilde2-fold decrease in pSTAT3 levels compared to untreated GFAP\textsuperscript{P301L} sham astrocytes. As to CaMKII\textalpha\textsuperscript{P301L}
astrocytes, LPS-treated sham and TBI astrocytes manifested a significant 3- and ~2-fold decrease compared to untreated CaMKIIαP301L sham and TBI astrocytes, respectively.

In summary, under no treatment conditions, there was no difference in pNFκB levels among astrocytes harvested from our three mouse models irrespective of genotype or injury. However, following 8h of LPS exposure, GFAPP301L TBI astrocytes revealed a significant increase in pNFκB levels not only compared to their treated sham counterparts but also to LPS-treated WT TBI astrocytes. Untreated GFAPP301L and CaMKIIαP301L sham astrocytes exhibited a significant increase in pSTAT3 levels compared to untreated WT sham astrocytes at baseline. However, after 8h of LPS exposure, pSTAT3 levels appeared to diminish compared to their untreated counterparts irrespective of the genotype or injury.
Figure 4.2: Response of primary cultured astrocyte isolated from WT, GFAP<sup>P301L</sup> and CaMKII<sup>α</sup>P301L r-mTBI vs sham mice at baseline and following exposure to 50ng/ml LPS for 8 hours. Qualitative (A) and quantitative immunoblotting levels of pNFkB/total-NFkB (B) and pSTAT3/total-STAT3 (C) in astrocyte cell lysates. Data were analyzed by Three-Way ANOVA followed by the Benjamini, Krieger, and Yekutiel test. Table under the graph details injury, genotype and treatment effects and their interactions after Three-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses.

Note: Primary astrocytes were prepared from mice 3-month post-last injury.
4.3.2 Secretome-to-transcriptome analysis of astrocytes after r-mTBI.

We performed a secretome-to-transcriptome analysis using our 3mpi RNA data from Chapter 3 and an online database of astroglial secretome profile after IL-1β, TNF and IFNγ stimulation. The reason why we selected the above-mentioned secretome profile was that it was the more relevant published secretome data that could, to some extent, relate to the astrocytic secretome profile following TBI. By comparing the WT TBI vs Sham RNA analysis with the online secretome database, we found the upregulation of Thbs1 and the downregulation of Lyz2 and complement system proteins: C1qc and C1qb. As to GFAP<sup>P301L</sup> TBI vs Sham transcriptional changes and secretome database comparison, we found that 16 genes of astroglial secreted proteins were dysregulated (1 downregulated and 15 upregulated) including upregulation of C1qc, H2D1, and Serpina3n. Regarding CaMKIIα<sup>P301L</sup> TBI vs Sham RNA analysis compared to the secretome database, we found increased expression of Fbln5, Ecm1, and Lgals3. Thus, GFAP<sup>P301L</sup> TBI astrocytes manifest an increase in the abundance of transcripts related to secreted proteins compared to WT and CaMKIIα<sup>P301L</sup> TBI astrocytes (Table 4.2).
4.3.3 Presence of tau in GFAP\textsuperscript{P301L} primary astrocytes and their secretome

Next, we sought to investigate the presence of tau in the cell lysates as well as the astrocyte-conditioned media harvested from primary astrocytes collected from our three mouse models. A factorial three-way ANOVA was conducted to determine the amount of tau protein in the cell lysate from our three mouse models in r-sham/r-mTBI environments at baseline and after LPS exposure. We found that there was a genotype effect where GFAP\textsuperscript{P301L} astrocytes contain a

| Table 4.2: Gene expression levels of candidate (reactive) astrocyte secretomic proteins identified by RNaseq of ACSA2+ primary astrocytes obtained from WT, GFAP\textsuperscript{P301L} and CaMKII\textsuperscript{αP301L}-mTBI vs sham mice at 3 months post-last injury. Top panel (blue) represents data from WT r-mTBI vs sham astrocytes. LogFC reflects log-fold change values between r-mTBI vs sham groups per genotype. Middle panel (green) represents data from GFAP\textsuperscript{P301L} r-mTBI vs sham astrocytes. Bottom panel (purple) represents data from CaMKII\textsuperscript{αP301L} r-mTBI vs sham astrocytes. |
higher presence of tau compared to WT and CaMKIIαP301L astrocytes (three-way ANOVA genotype effect [F(2,24)=8.54, p=0.0016]; injury effect [F(1,24)=1.62, p=0.214]; LPS treatment [F(1,24)=0.78, p=0.383]; genotype*injury [F(2,24)=1.95, p=0.164]; genotype*LPS treatment [F(2,24)=0.94, p=0.40]; injury*LPS treatment [F(1,24)=2.83, p=0.10]; and genotype*injury*LPS treatment [F(2,24)=2.64, p=0.0916]) (Fig. 4.3A). Additionally, at baseline, GFAPP301L astrocytes manifested a TBI effect represented by the significant 6-fold increase in tau levels compared to sham GFAPP301L astrocytes. After LPS exposure, total tau levels (DA9) did not increase in the GFAPP301L sham astrocytes compared to their GFAPP301L non-LPS sham counterpart. Tau levels in the GFAPP301L TBI astrocytes after LPS significantly decreased by a 4-fold factor compared to GFAPP301L baseline TBI counterparts.

Additionally, through ELISA, we measured the amount of tau in secretome collected from primary astrocytes after 30DIV. We observed that, as expected, WT astrocytes did not secrete human tau while GFAPP301L astrocytes secrete around five times more hTau compared to CaMKIIαP301L at baseline (two-way ANOVA genotype effect [F(2,12)=28.26, p=<0.001]; injury effect [F(1,12)=0.88, p=0.366] with no factorial interaction) (Fig. 4.3B). There was no TBI effect on tau concentration in the ACM across genotypes.
4.3.4 Microglial response to astrocyte conditioned media (ACM), *in-vitro*.

Then, we exposed immortalized microglia (IMG cell line) to astrocyte-conditioned media (ACM) obtained from primary astrocytes harvested from our three mouse models. We assessed the levels of microglial reactivity in response to 30-minute exposure to ACM. We evaluated the phosphorylation of NFκB as well as STAT3. Immortalized microglia did not manifest TBI- or genotype-mediated changes in pNFκB-mediated reactivity in response to ACM exposure (Fig. 4.4A-C). The two-way ANOVA showed an injury and a genotype effect in pSTAT3 levels, driven by the significant drop of pSTAT3 signal between microglia exposed to GFAP<sup>P301L</sup> TBI ACM and...
CaMKIIα<sup>P301L</sup> Sham ACM (two-way ANOVA: genotype effect [F(2,28)=4.31, p=0.023]; injury effect [F(1,28)=5.22, p=0.030)].

4.3.5 Analysis of microglial inflammatory cytokine secretion after ACM exposure.

We analyzed the media harvested from IMG after ACM exposure using a multiplex antibody-based assay capable of detecting 10 different inflammatory cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL10, IL-12p70, KCGRO, IFNγ, and TNF). In our samples, all cytokines were detected within the standard range, however, not all of them showed significant changes. There were no changes in IL-1β, IL-2, IL-5, IL-10, KCGRO, IFNγ, and TNF and IL-6 in the media of IMG exposed to GFAP<sup>P301L</sup>.
TBI ACM compared to GFAP\textsuperscript{P301L} Sham ACM (Fig. 4.5A-H). However, IL-4 and IL-12p70 levels in media collected from IMG exposed to GFAP\textsuperscript{P301L} TBI ACM were significantly higher compared to GFAP\textsuperscript{P301L} Sham ACM (two-way ANOVA IL-4: genotype effect [F(2,12)=3.49, p=0.064]; injury effect [F(1,12)=5.52, p=0.037]; interaction [F(2,12)=4.32, p=0.039]), and (two-way ANOVA IL-12p70: genotype effect [F(2,12)=3.51, p=0.063]; injury effect [F(1,12)=6.64, p=0.026]; interaction [F(2,12)=2.96, p=0.090]) (Fig. 4.5I-J). IL-4 and IL-12p70 in the media of IMG exposed to WT and CaMKII\textsubscript{a}\textsuperscript{P301L} TBI ACM remained unchanged compared to their respective sham ACM.
Figure 4.5: Cytokine profiles of microglial cell lines 30 minutes following exposure to astrocyte conditioned media (ACM) obtained from primary cultured astrocytes of WT, GFAP$^{P301L}$ and CaMKIIα$^{P301L}$ r-mTBI vs sham mice at 3-months post-last injury. A-J shows ELISA multiplex concentration levels of 10 cytokines in the media of ACM exposed microglial cell lines. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interactions after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses.
4.3.6 Microglial and astroglial response to intracerebral injection of ACM.

Next, we inoculated the cortex of naïve mice with 8μl of serum-free ACM collected from astrocytes harvested from WT and GFAP\textsuperscript{P301L} mice (Sham/TBI). One-month post-intracerebral injection we collected the tissue and analyzed changes in microglial reactivity (IBA1 immunoreactivity) or astrocyte reactivity (GFAP immunoreactivity) in the ipsilateral cortex and corpus callosum (CC) (Fig. 4.6B-E). However, the two-way ANOVA showed a significant genotype effect in the iba1 immunoreactivity in the cortex of the GFAP\textsuperscript{P301L} compared to WT (two-way ANOVA: genotype effect [F(1,16)=4.64, p=0.047]; injury effect [F(1,16)=0.01, p=0.906]; interaction [F(1,16)=0.16, p=0.691]) (Fig. 4.6B). Herein, it is important to mention that we did not inject naïve mice with ACM media collected from astrocytes of CaMKII\textalpha\textsuperscript{P301L} mice (Sham/TBI) because as observed in Fig. 4.5, CaMKII\textalpha\textsuperscript{P301L} astrocytes failed to show any TBI or genotype effect on microglial secretome.
Figure 4.6: Astrocyte and microglial reactivity in naïve (isogenic) WT recipient mice following intracerebral (IC) injection of 8μl concentrated astrocyte conditioned media (ACM) obtained from WT and GFAP<sup>P301L</sup> r-mTBI/sham primary cultured astrocytes. Top-two (cortex) and Bottom-two (corpus callosum) panels depicts IBA1 and GFAP staining in naïve recipient WT mice injected with WT or GFAP<sup>P301L</sup> TBI-ACM or sham-ACM (A). Images were captured at x10 magnification. Percentage area of IBA1 (B, C) and GFAP (D, E) in the cortex and corpus callosum tissue, respectively. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses. Note: ACM were obtained from primary astrocytes prepared from mice 3-month post-last injury. ACM was concentrated using a 3KDa filter and injected into the cortex at a rate of 0.2μl/min. Scale bar 50μm.
4.4 DISCUSSION

The lack of understanding of TBI pathophysiology has likely been a key impedance to the development of therapeutics. For instance, it remains unstudied what the role of non-neuronal cells, such as astrocytes, or tau astrogliopathy in the TBI-related pathophysiology is. In the current thesis, in Chapters 2 and 3 we focused on understanding the contribution of astrocytes to TBI-related pathology, but we acknowledge that astrocytes do not work in isolation and the understanding of cell-cell interaction with other brain cells is crucial for the development of promising treatment strategies. Given that our transcriptional data in Chapter 3 showed that GFAP<sup>P301L</sup> astrocytes manifest an immunosuppressed state, we were prompted to investigate the astrocyte-microglia interaction in vitro and in vivo in r-mTBI conditions.

Before looking at the astrocyte-microglia interaction, we evaluated the reactive state (NFκB and STAT3 activation) of Sham and TBI primary astrocytes (WT, GFAP<sup>P301L</sup>, and CaMKIIα<sup>P301L</sup>) at baseline (i.e., untreated) and after the exposure to an inflammatory stimulus, LPS. After 30 days in vitro (DIV), we had an unexpected increase in pSTAT3 activation and unchanged NFκB activation in untreated sham GFAP<sup>P301L</sup> astrocytes, which did not match what we observed in our transcriptomic analysis (Chapter 3) where GFAP<sup>P301L</sup> sham astrocytes had a significantly downregulated STAT3 and NFκB signaling compared to WT. This could be because astrocytes might not have retained their initial phenotype after 30 DIV. However, when astrocytes were primed with LPS, we observed that overexpression of tau within astrocytes caused an exacerbated increase in pNFκB levels in TBI GFAP<sup>P301L</sup> astrocytes in response to LPS which might show that despite the fact that astrocytes were cultured for a long period they retained epigenetic changes that made TBI GFAP<sup>P301L</sup> astrocytes respond significantly differently compared to their sham counterparts and TBI WT astrocytes after a secondary inflammatory stimulus. In regard to STAT3 activation, we did not observe increased levels of STAT3 activation in response to LPS in the r-sham or r-mTBI groups which might mean that we lost the peak of STAT3 activation response that is known to fluctuate with time.

Next, we were curious to see if TBI astrocytes have a more reactive secretome profile based on the RNA levels of astrocyte secreted protein in the GFAP<sup>P301L</sup> TBI astrocytes compared to WT and CaMKIIα<sup>P301L</sup>-TBI astrocytes could potentially alter microglial reactivity. We acknowledge that an
unbiased proteomic analysis of the ACM would be a better approach to assessing the sub-chronic changes in the secretome profile of astrocytes three months after r-mTBI, however, with our secretome-to-transcriptome analysis, we were able to obtain some insights into possible changes in the secretome of TBI astrocytes (three mouse models) based on the changes in the transcription of genes of secreted proteins. WT TBI astrocytes, compared to their sham counterparts, appear to have an upregulation in thrombospondin 1, an inflammatory agent that has been reported to inhibit angiogenesis and modulate endothelial cell survival and function (Guo et al., 1997). CaMKIIα<sup>P301L</sup> TBI astrocytes appear to upregulate the expression of secreted molecules that are involved in the modulation of microglial response to inflammation (<em>Lgals3, Ecm1</em>), extracellular matrix regulation (<em>Ecm1, Fbln5</em>) (Tan et al., 2021; Williamson et al., 2021; Yanagisawa et al., 2009; Zhang et al., 2020). GFAP<sup>P301L</sup> TBI astrocytes appear to release more inflammatory messengers (e.g. upregulation of <em>C1qc, Csf1, H2-D1, C4b, Fmod, Aebp1</em>) and mutant tau that could trigger microglial reactivity and release of cytokines (Dejanovic et al., 2022; Liu et al., 2023; Mangold et al., 2017; Rapino et al., 2023; Shijo et al., 2018; Suzumura et al., 1990; Zhao et al., 2023). Next, we evaluated microglia response to ACM collected from Sham and TBI astrocytes from WT, GFAP<sup>P301L</sup>, and CaMKIIα<sup>P301L</sup> mice. Although we did not observe different microglial NFκB- or STAT3-based reactivity 30 minutes after exposure to the ACM (TBI vs sham) in any of our mouse models, microglia exposed to TBI GFAP<sup>P301L</sup> ACM released significantly more IL-4 and IL-12p70 compared to the other groups. IL-4 is a cytokine reported to be implicated in regulating pro-inflammatory cytokines, upregulating anti-inflammatory cytokines production, favoring microglial anti-inflammatory phenotype, inhibiting astrocyte reactivity, and attenuating neuronal apoptosis (Brodie et al., 1998; Radpour et al., 2023; J. Wang et al., 2023; Yi et al., 2020). With regard to IL-12p70 or IL-12, it is a pro-inflammatory cytokine that activates natural killer and T cells which induce the production of IFNγ, proliferation and enhances cytotoxic activity (Stahel et al., 1998; Trinchieri, 1998). While there is limited contribution of IL-12p70 to TBI-elicited pathophysiological changes, IL-4 has been reported to be implicated in microglial-dependent synaptic pruning and tissue remodeling in repair, a possible neuroprotective astrocyte-dependent mechanism at a sub-chronic timepoint following r-mTBI (Guedes et al., 2023; Ma, et al., 2021; Zheng, et al., 2021). In future studies, we will seek to perform transcriptional analysis
on IMG exposed to ACM to understand what pathways are dysregulated as a consequence of tau astrogliopathy and its interaction with r-mTBI. Human CTE astrocytes might have a secretome profile that shares commonalities with the secretome of GFAP\textsuperscript{P301L} and CaMKII\textsubscript{a}P301L TBI astrocytes because as mentioned in our previous chapters, in CTE, astrocytes contained tau and are also exposed to immunopanning with reactive microglia and tau-containing neurons.

Moreover, given that GFAP\textsuperscript{P301L} astrocytes secrete mutant tau, it is of high interest for us to assess the participation of astrocytes in the propagation of pathological tau. Thus, subsequent experiments should be executed to determine if the secreted tau is able to serve as a seed for tau accumulation in other brain cells such as neurons, in a prion-like manner. It would also be interesting to quantify the concentration of tau in the ACM media after LPS exposure, perhaps the reduced levels of tau in the cell lysates after LPS exposure might mean that there is more secretion of tau. Additionally, the unexpected release of tau from CaMKII\textsubscript{a}P301L astrocytes led us to think that they might have internalized tau from their surroundings when they were in vivo enabling them to release tau into the media during their in-vitro life. Looking in our transcriptional analysis “CaMKII\textsubscript{a}P301L vs WT astrocytes” we did not observe dysregulation of pathways involved in tau internalization such as LRP1-mediated uptake, endocytosis, and phagocytosis (J. Zhao et al., 2021); or protein clearance such as the proteasome system, the autophagy-lysosome pathway, and the endolysosomal system (Tang et al., 2019).

In vivo exposure to ACM did not elicit significant changes in astrocyte and microglial reactivity, and tau pathology, RZ3, (data not shown) in the cortex or CC one-month post-ACM injection which could be the result of a low concentration of secreted proteins known to evoke a significant microglial or astroglial reaction or a timing issue. We wanted to evaluate the response of microglia and astrocytes at a sub-acute timepoint (one month post-injection) but probably the time selection was not appropriate. Perhaps an earlier timepoint could have revealed more information as to glial responses to ACM in vivo. Additionally, we only evaluated GFAP and IBA1 immunoreactivity, which does not fully capture the heterogeneous nature of astrocyte and microglial reactivity. In the future, to analyze glial response to ACM injection in more detail, we plan to interrogate other markers such as CD68 or CD45 to investigate the phagocytic and
reactive state of microglia as well as the presence of astrocyte reactivity such as SERPINA3N, LCN2 reported to be present in reactive astrocytes (Matusova et al., 2023; Zamanian et al., 2012).

4.5 LIMITATIONS OF THE STUDY

Despite that the available literature reports that harvested astroglial cultures maintain the morphological phenotype up until 35DIV (Bertrand et al., 2011), we consider that 30DIV might be a long period for sustaining the baseline molecular phenotype, especially the TBI phenotype given that once outside the brain and upon cellular division the astrocytes, no longer, found themselves in a TBI environment. Thus, it is very likely that we might have lost the TBI phenotype of the astrocytes. However, the fact that primary astrocytes manifest quite varied reactive levels at baseline and after LPS exposure might be because astrocytes have not lost their phenotype completely and that there are epigenetic changes in sequences governing immune response to a second inflammatory stimulus. Additionally, although the majority of ex vivo experiments with astrocytes are performed using pup astrocytes that have a fast division rate, because of our study design, we had to use adult astrocytes with a lower division rate which meant that to have a sufficient amount of astrocytes to perform our experiments (baseline vs LPS exposure) we had to let the astrocytes grow for 30 days ex-vivo. At 30DIV our astrocytes did not show evidence of cellular beading, a marker of senescence. However, we did not evaluate the presence of other senescence markers such as iNOS, β-galactosidase, and Laminin B1 p16 (Matias et al., 2022), thus, we cannot discard the possibility that astrocytes used in this chapter could have altered physiology due to ex-vivo aging or senescence. We also acknowledge that astrocytes living outside their in vivo environment have altered phenotypes because they no longer receive important cues from neurons (Hasel et al., 2017), microglia and endothelial cells. Furthermore, it is known that separating microglia from the astrocyte bed by shaking, may not deplete all microglia, thus a small percentage of microglia are also part of the “enriched astroglial pellet” which might contribute to the astrocyte reactivity readouts (pNFκB and pSTAT3) we present in this chapter. In more recent years, the immunopanning technique has been used to obtain “purified astroglial pellets” for cell culturing where a smaller amount of non-astrocytic cells is carried over. In future studies, we will utilize immunopanning techniques to increase the purity of our astroglial cultures in addition to increasing the number of adult brains from which we
harvest the adult astrocytes. Thus, we will be able to reduce the number of days that astrocytes have to live ex-vivo which will increase our ability to sustain the phenotype of both sham and TBI astrocytes. Additionally, it is important to mention that harvesting the astrocytes from the entire brain means that we are working with different populations of astrocytes that may respond differently to the studied stimuli (TBI and LPS exposure). Therefore, astrocyte reactivity readouts might be masked by the utilization of a heterogenous population. Further investigation is warranted to identify the different populations of astrocytes in a TBI brain, to know how faithfully those astrocyte populations can be maintained in vitro and to know how different astrocyte populations respond to TBI (distal vs proximal areas to the impact) and LPS exposure.

In regards to astroglial response to LPS, there is opposing evidence. Despite the fact that some studies show that astrocytes do not respond to LPS, several more studies have demonstrated that murine astrocytes respond to LPS (Acaz-Fonseca et al., 2019; Ling et al., 2021; Tarassishin et al., 2014). This is in line with our preliminary work on immortalized murine astrocytes, C8D1A, and the ex-vivo data presented here on primary murine astrocytes. However, we understand that the response of primary astrocytes to LPS may be debatable given that the technique utilized in the present study (and others) to isolate astrocytes (i.e., shaking) carry over a very small population of microglia that grew intermingled in the astrocyte branches (Milner et al., 2022). The fact that microglia are more active in responding to cytokine and LPS compared to astrocytes (Sheng et al., 2011) could lead to skepticism in the field. Thus, the reported changes in astrocyte cellular response to LPS may be contaminated with microglial response to an unknown extent.

Moreover, we should be aware that astroglial responses might be influenced by the conditions in which astrocytes are cultured, as well as the techniques used for astroglial collection. For instance, there are differences in astrocytes grown in media containing fetal bovine serum or in serum-free media (Jia et al., 2018; Prah et al., 2019). Isolating astrocytes by magnetic isolation or immunopanning might manifest different cellular responses compared to those harvested through shaking. Additionally, cells in vitro have been shown to express genes that are not observed in their in vivo counterparts (Pennypacker et al., 1996). We advise, caution when comparing in vitro and ex-vivo experiments to in vivo experiments.
Regarding our secretome-to-transcriptome analysis, although the approach we utilized has been employed by other authors (Ankney et al., 2019), we acknowledge that this approach is limited and biased. To perform our secretome-to-transcriptome analysis we utilized a published secretome profile from immortalized astrocytes (C8D1A) stimulated with IL-1, IFNγ, and TNF. Although those three pro-inflammatory cytokines have been shown to be increased after r-mTBI, other molecules influence astrocyte response after TBI. Thus, at the moment, we are currently working in collaboration with Dr. Jon Reed at Boehringer Ingelheim to obtain an unbiased proteomic analysis of ACM collected from TBI/Sham astrocytes from our three mouse models to obtain a more accurate proteomic profile of astroglial secretome at chronic timepoint after r-mTBI. Additionally, we should keep in mind that primary astrocytes might react differently to any stimulus compared to immortalized astrocytes.

Finally, due to breeding constraints with GFAP\textsuperscript{P301L} and CaMKIIα\textsuperscript{P301L} mice, we only had an n=3 per group for experiments in (Fig.4.1) (astrocyte reactivity at baseline and after LPS exposure) & (Fig 4.3-4.4) (microglial response to ACM and cytokine concentration in media collected from microglia after ACM exposure). The reduced sample size and reduced statistical power might have contributed to the lack of significant TBI-related effects and the influence of LPS exposure. Therefore, in future studies, we will aim for a minimum n=6 to strengthen the reliability of the obtained results.

4.6 SUMMARY

In summary, the results provided in this section of the thesis show that our hypotheses are partially upheld. First, Tau\textsuperscript{P301L}-bearing astrocytes from sham GFAP\textsuperscript{P301L} mice do not manifest reduced levels of pNFκB or pSTAT3-mediated cell reactivity compared to sham WT and CaMKIIα\textsuperscript{P301L} astrocytes. We did not observe increased astrocyte reactivity in response to r-mTBI in any of our mouse models. Moreover, when exposed to a secondary inflammatory stimulus (LPS) for 8h, tau overexpression in TBI GFAP\textsuperscript{P301L} astrocytes leads to an increased pNFκB-mediated reactivity compared to TBI WT astrocytes but not TBI CaMKIIα\textsuperscript{P301L} astrocytes. Besides, after 8h of LPS exposure, pSTAT3-mediated astrocyte reactivity appears to be diminished. These findings
do not fully support our first hypothesis. Second, the transcriptional changes of tau-bearing astrocytes in response to r-mTBI led to the secretion of ~15 and ~5 times more inflammatory molecules compared to TBI WT and TBI CaMKIIαP301L astrocytes, respectively. Thus, our second hypothesis is upheld. Third, although microglial exposure to ACM from our mouse models (TBI or Sham) did not manifest differences in cellular reactivity, the cytokine analysis of the media collected from microglia exposed to ACM from astrocytes harvested from our three mouse models shows that sham GFAPP301L ACM does not elicit exacerbated release of inflammatory cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL10, IL-12p70, KCGRO, IFNγ, and TNF) while TBI GFAPP301L ACM does trigger a significant increase in the release of pro-inflammatory IL-4 and IL-12p70 compared to sham GFAPP301L ACM, and TBI WT/CaMKIIαP301L ACM. Thus, our third hypothesis is partially upheld. Finally, injection of ACM collected from TBI tau-bearing astrocytes (GFAPP301L astrocytes) did not elicit increased astrocyte and microglial reactivity in the cortex of recipient mice compared to sham GFAPP301L astrocytes or TBI WT astrocytes. Therefore, our fourth hypothesis is rejected.
CHAPTER 5 – SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY OF FINDINGS

In Chapter 2, we found that tau astrogliopathy progressed with age and that TBI did not accelerate or exacerbate tau accumulation within astrocytes. We also found that, in contrast to overexpression of mutant tau in neurons, overexpression of mutant tau within astrocytes did not elicit increased astrocyte and microglial reactivity compared to WT; furthermore, r-mTBI did not exacerbate astrocyte and microglial reactivity in our GFAP\textsuperscript{P301L} mice compared to WT. Additionally, the presence of mutant tau either in astrocytes or neurons elicited temporal hippocampal-specific downregulation of astrocyte homeostatic markers important for optimal neuronal transmission and water transport (GLT1, synapsin1; and AQP4, respectively); r-mTBI did not influence further dysregulation of these other markers.

In Chapter 3, we found that tau-bearing astrocytes underwent more changes in their transcriptome profile compared to astrocytes from WT and CaMKIIα\textsuperscript{P301L} mice exposed to robust neuronal tau pathology and microgliarial reactivity. Tau-bearing murine astrocytes exhibit an immunosuppressed state and a compromised bioenergetic system which might interrupt their physiological roles, such as astrocyte-microglia bidirectional communication, neuronal trophic and metabolic support, and ion homeostasis. Three months post-last injury (3mpi), tau-bearing astrocytes showed transcriptional dysregulation compared to WT and CaMKIIα\textsuperscript{P301L} astrocytes. However, such changes were not sustained to the more chronic timepoint (6mpi); possibly because the pathology in tau-bearing astrocytes in these more aged mice (10 months) might have masked the effect of r-mTBI. Moreover, comparison of our mouse transcriptomic data with transcriptomic analysis of human hippocampal astrocytes from CTE cases showed that the latest stage of CTE (CTE-IV), appeared to develop an immunosuppressed phenotype comparable to the effects of tau overexpression in murine astrocytes from our GFAP\textsuperscript{P301L} mouse model. It is important to mention that the hippocampal CTE-derived astrocytes used in our analysis do not show mitochondrial dysfunction like tau-bearing murine astrocytes harvested from
hippocampus-depleted brains. This could either be because human astrocytes in different brain regions respond differently or because the origin of tau (internalization vs intracellular overexpression) triggers different molecular changes.

The immunosuppressed state of tau-bearing astrocytes and its commonality with CTE-IV-derived astrocytes prompted us to further investigate the effects of the astroglial immunosuppressed phenotype on baseline astrocyte state/profile, astroglial response to inflammatory agents, and the bidirectional molecular communication with microglia, in-vitro. In Chapter 4, we found that tau-bearing primary astrocytes from sham GFAP\textsuperscript{P301L} mice did not manifest reduced levels of pNFκB or pSTAT3-mediated cell reactivity at rest compared to sham WT and CaMKII\textgreek{a}\textsuperscript{P301L} primary astrocytes as we expected based on our astrocyte-specific transcriptional analyses. Furthermore, r-mTBI did not increase astrocyte reactivity in any of our mouse models. However, when exposed to a secondary inflammatory stimulus (in our case, LPS), tau overexpression in TBI GFAP\textsuperscript{P301L} primary astrocytes resulted in an exacerbated pNFkB-mediated reactivity compared to its sham counterpart and TBI WT primary astrocytes exposed to LPS. However, pSTAT3-mediated astrocyte reactivity appeared to be diminished after LPS irrespective of injury. Moreover, astrocyte-conditioned media (ACM) collected from unprimed WT, GFAP\textsuperscript{P301L}, and CaMKII\textgreek{a}\textsuperscript{P301L} primary astrocytes did not elicit differences in early transcriptional cellular reactivity, however, the cytokine analysis of the media collected from microglia exposed to ACM showed that TBI GFAP\textsuperscript{P301L} ACM triggered a significant increase in the release of pro-inflammatory IL-4 and IL-12p70 compared to sham GFAP\textsuperscript{P301L} ACM, and WT and CaMKII\textgreek{a}\textsuperscript{P301L} (sham and TBI) ACM. By performing secretome-to-transcriptome analysis using our astrocyte-specific RNA datasets and an online astrocytic secretome database we were able to identify that TBI GFAP astrocytes (3mpi) show greater upregulation of transcripts that code for secreted proteins involved in inflammatory processes (complement proteins, H2-D1, tau, among others). (see Fig. 5.1.1-5.1.3)
Figure 5.1.1 Proposed impact of r-mTBI on astroglial homeostasis of WT astrocytes based on changes observed at three months post-last injury which corresponds to the age of 7 months. Changes in the cortex.
Figure 5.1.2 Proposed impact of tau astrogliopathy on astroglial homeostasis under normal circumstances (Sham conditions) and r-mTBI conditions based on changes observed at three months post-last injury which corresponds to the age of 7 months. * changes in the hippocampus; † changes in the cortex.
Figure 5.1.3 Proposed impact of robust neuronal tau on astroglial homeostasis under normal circumstances (Sham conditions) and r-mTBI conditions based on changes observed at three months post-last injury which corresponds to the age of 7 months. * changes in the hippocampus; ^ changes in the cortex.

5.2 CONCLUSIONS

There is controversy around the contribution of tau astrogliopathy to the progression of the disease. Dr. Ann McKee and her team have suggested that tau astrogliopathy in CTE is a mere byproduct of age, similar to what is believed to happen in ARTAG (Butler et al., 2022; A. McKee et al., 2016). In contrast, Dr. William Stewart and colleagues paradoxically suggest an opposing theory where tau astrogliopathy emerges from repeated head trauma encounters, and is principally involved in the pathophysiological changes of CTE(Ameen-Ali et al., 2022; Lee et al., 2019). However, our studies appear to support the notion that accumulation of pathogenic tau within astrocytes over
time (with aging) ONLY occurs when there is an alteration in the murine (astrocyte-specific) genome since we only saw tau accumulation in GFAP<sup>P301L</sup> mice but not in WT or CaMKII<sup>α</sup><sub>P301L</sub> mice harboring wild-type murine tau and neuronal specific P301L-tau mutation, respectively.

Our GFAP<sup>P301L</sup> model shows an increased presence of tau astrogliopathy by the age of 10 months, with no obvious TBI effect on tau astrogliopathy. Despite more investigation being warranted to determine the origin of tau astrogliopathy in the human TBI, we also cannot overlook the negative impacts that tau astrogliopathy has on astroglial physiology. It is particularly intriguing that despite the dramatic increase in mutant tau levels in the brains of CaMKII<sup>α</sup><sub>P301L</sub> mice (up to 16-fold) compared to GFAP<sup>P301L</sup> mice, astrocyte-derived tau caused the same profound changes in astrocyte specific markers known to participate in the homeostasis of glutamate-mediated synaptic transmission, water transport, and clearance system compared to neuron-derived tau. Additionally, astrocyte-derived tau was able to cause an increased astroglial response and a pro-inflammatory bidirectional communication with microglia after a second encounter with inflammatory stimuli. Therefore, our studies shed some light on the possible consequences of tau astrogliopathy in astroglial pathobiology not only in CTE but other diseases such as PSP and CBD where tau astrogliopathy is a hallmark feature. However, further investigation is needed to validate and determine the mechanism by which astrocytes contribute to ongoing neurodegeneration and neurobehavioral abnormalities. Understanding the contribution of tau astrogliopathy in the neurodegenerative sequelae of r-mTBI will be important for developing novel therapeutic approaches or diagnostic markers that can reveal the extent of astrocyte damage.

**5.3 FUTURE DIRECTIONS**

As mentioned in the limitation section of Chapter 2 (2.5), besides the origin of aggregated tau in astrocytes, the difference between tau-bearing human astrocytes and tau-bearing murine astrocytes (GFAP<sup>P301L</sup> astrocytes) is the time that pathological tau resides inside astrocytes. The fact that GFAP<sup>P301L</sup> astrocytes have been producing tau since they were differentiated into GFAP<sup>+</sup> cells (embryonic day 14) (Brenner & Messing, 2021) results in prolonged exposure to a
pathological agent (mutant tau) which might change astroglial response. To overcome this issue, we designed a study where using intracerebral injection of a viral vector we specifically transfected astrocytes to make them produce mutant tau under the GFAP promoter (Fig. 5.2A). We were aiming to investigate the effects of “late-onset” tau astrogliopathy on astrocyte and microglial reactivity, and tau pathology (brain-wide and astrocyte-specific), however, this experiment (being conducted in the final months of the thesis research) had to be discontinued. We managed to gather qualitative data of in vivo AAV transfection shown in Fig 5.2B. Preliminary data on GFAP immunoreactivity in the cortex of naïve mice injected with the AAV-Tau vector revealed that transfected mice showed a significantly increased astrocyte reactivity compared to their sham counterparts (Fig. 5.2C). We still need to compare these findings with the cortical GFAP immunoreactivity of mice that was injected with the control vector. In future experiments, either as an extension of the AAV experiment or through the utilization of a CRE- model that will also allow us to study the effects of late-onset tau astrogliopathy and its consequences, we plan to analyze microglial reactivity, astrocyte homeostatic markers and tau pathology (tau phosphorylation, tau spreading and tau abundance) in this cohort and compare it to findings in our GFAPP301L model.
Additionally, because our GFAP\textsuperscript{P301L} and CaMKII\textalpha\textsuperscript{P301L} mice are doxycycline-dependent conditional models, we took advantage of this to design a study where the production of tau,
either in astrocytes or neurons, was interrupted by administration of doxycycline chow for three consecutive months post-last injury (Fig. 5.3A). Our goal was to investigate the temporal effects of tau suppression on astrocyte and microglial reactivity, and tau pathology as well as the abundance of astrocyte homeostatic markers and synaptic markers. So far, we have performed some histopathological analyses on GFAP$^{P301L}$-treated mice and compared them to the untreated GFAP$^{P301L}$ cohort used in Chapter 2 Fig. 5.3B-D&5.4. Because the doxycycline treatment suppresses the expression of the promoter protein and the linked protein (Das et al., 2016), in our case GFAP and tau protein, we were not surprised to find that 3 months of doxycycline treatment led to a significant decrease of TBI-related astrocyte reactivity (GFAP immunoreactivity) in the cortical area underneath the impact both 3mpi and 6mpi (Fig. 5.3B). As to tau, the presence of phosphorylated tau (RZ3) in the cortical area underneath the hit was significantly reduced at 3 months but appeared to increase after doxycycline treatment withdrawal (Fig. 5.3C). In terms of microglial reactivity (IBA1 immunoreactivity), we found that compared to untreated GFAP$^{P301L}$ mice Fig. 2.7, doxycycline-treated GFAP$^{P301L}$ mice failed to show a TBI-dependent increase in IBA1 immunoreactivity (Fig. 5.3D). This finding might suggest that TBI-related microglial reactivity in GFAP$^{P301L}$ mice is linked to the presence of tau in astrocytes. Histopathological analyses of doxycycline treated CaMKII$^{α}$P301L mice are ongoing. In the near future, we plan to perform biochemical analysis on doxycycline-treated transgenic mice to learn if the repression of pathogenic tau has interfered with the tau-dependent decrease in cortical synapsin 1 level as well as hippocampal AQ4, GLT1, and synapsin1 levels. It would also be interesting to reveal the transcriptional changes following astroglial and neuronal tau suppression. Nonetheless, caution should be applied when interpreting the results obtained from this experiment given that it has been reported in GFAP KO mice that interrupting the normal production and abundance of GFAP protein might have negative consequences on astroglial physiology and cell-to-cell interaction that might mask the effects of suppressing tau expression (Davila et al., 2013). In the future, we also plan to explore other mouse models where astrocytic tau production is not controlled by GFAP promoter, which we know changes in response to TBI. For instance, we could use the Aldehyde dehydrogenase 1 family, member L1 (ALDH1) or
Connexin 30 (Cx30) promoter that seemed to also increase astrocyte specificity (Srinivasan et al., 2016).

Figure 5.3: Conditional and reversible depletion of Tau from GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice following r-mTBI/sham injuries. (A) Schematic representation of doxycycline treatment paradigm in GFAP<sup>P301L</sup> and CaMKIIα<sup>P301L</sup> mice. At 3 months of age, animals will be exposed to their injuries for 1-month and subsequently receive doxycycline treatment for 3 months beginning 24hrs after the last injury. Histopathological analyses will be performed on the brain at 3 months and at 6 months after injury (in the latter paradigm doxycycline will be withdrawn for 3 months prior to euthanasia). GFAP (B), RZ3 pTau T231 (C) and IBA1 (D) immunoreactive percent area in the cortex at 3-months and 6-months post-last injury following 3-months of doxycycline treatment regimen described above. Data were analyzed by Two-Way ANOVA followed by the Benjamini, Krieger, and Yekuteli test. Table under the graph details injury and genotype effects and their interaction after Two-way ANOVA. Asterisks denote: *P<0.05; **P<0.01 and ***P<0.001 for post-hoc analyses.
Figure 5.4: Qualitative images of pTau and astrocyte/microglial activation markers in the cortex of doxycycline treated GFAP\textsuperscript{P301L} mice at 3- and 6-months after r-mTBI/sham injury. (A) Left and middle panels depict GFAP and RZ3 (pTau T231) immunostaining, respectively. Each representative staining is from 3- and 6-months post-last injury in r-mTBI GFAP\textsuperscript{P301L} mice treated with/without doxycycline for 3 months. (B) Right panel depict IBA1 immunostaining taken at 3- and 6-months post-last injury from r-mTBI/sham GFAP\textsuperscript{P301L} mice treated with doxycycline for 3 months. Images were captured at x20 magnification. Scale bar 50μm.
The significant dysregulation of hippocampal and cortical AQP4 in transgenic models compared to WT at 7 and 10 months of age, respectively, prompted us to evaluate AQP4 vascular polarization in the cortex and hippocampus of our mouse models. Therefore, we calculated the percentage area of colocalization between AQP4 immunoreactivity and Lectin (blood vessel marker) immunoreactivity using ImageJ. We observed that there were no changes in the astrocytic blood vessel coverage in the cortex at 7 and 10 months of age irrespective of injury or genotype (Fig. 5.5A-B). AQP4 polarization analysis in the hippocampus is still ongoing.

Moreover, as mentioned in Chapters 2 and 3, we are interested in evaluating the age-dependent effects on tau astrogliopathy in our GFAPP301L model and its interaction with r-mTBI. Thus, we induced r-mTBI (20hit model) on an aged cohort (12 months old) and analyzed the astrocyte-specific transcriptional changes 3mpi and 6mpi that correspond to 16 and 19 months of age, respectively. As of now, we have only gathered information about 1) genotype-dependent transcriptional changes in sham GFAPP301L astrocytes compared to sham WT astrocytes harvested
from the hippocampus-depleted brains at 16 and 19 months of age (Fig. 5.6), and 2) TBI-dependent transcriptional changes in aged TBI GFAP\textsuperscript{P301L} astrocytes compared to their sham counterparts 3 mpi and 6mpi (Fig. 5.7). We were able to observe that GFAP\textsuperscript{P301L} astrocytes still have an immunosuppressed phenotype with mitochondrial dysfunction and an increased calcium signaling when compared to WT sham astrocytes at both time points. In terms of TBI-related changes in astroglial transcriptome, we found that 6mpi aged GFAP\textsuperscript{P301L} astrocytes undergo 4 times more transcriptional changes than aged GFAP\textsuperscript{P301L} astrocytes at 3mpi. Additionally, aged GFAP TBI \textsuperscript{P301L} astrocytes (vs Sham counterparts) at 3mpi and 6mpi exhibit a greater number of DEGs compared to young GFAP TBI \textsuperscript{P301L} astrocytes (vs Sham counterparts) 6mpi but less than at 3mpi. We hypothesize that this effect could be attributed to the temporal fluctuation in astroglial response(s) to r-mTBI. In the future, we plan to keep performing bioinformatic analyses with our data to increase our understanding of astroglial changes in response to progressive tau accumulation from 7 months through 19 months of age. Additionally, we aim to complement this analysis with the assessment of histopathological and biochemical changes in the cortex and hippocampi of the aged cohorts, human-murine comparison using our astrocyte-specific transcriptional data from CTE-IV (vs NDC) and aged GFAP\textsuperscript{P301L} cohorts (vs WT), plus behavioral analysis.
Figure 5.6: Astrocyte specific pathways that are dysregulated in aged GFAP$^{P301L}$ mice compared to Wild-type mice at 16 and 19-month-old. Venn diagram of differentially expressed genes (DEGs) between GFAP$^{P301L}$ vs WT mice are shown in A. Histogram in B and C depicts results of IPA pathway analyses after analyzing the entire DEG list between aged GFAP$^{P301L}$ vs WT mice at 16 and 19 months of age, respectively. Upregulated and downregulated pathways in B-C are depicted in red and blue, respectively. Heat-bar in B-C represents -log 10 of the P value (yellow – Topmost significant; sky blue – least significant). Threshold for obtaining the DEGs: adj. p-value $\geq 0.05$ with its respective -log value $\geq 1.3$. N=6 mice/group (N=3 technical replicates – i.e., two pooled hemispheres).

Note: The 16- and 19-month-old mice above correspond with 3- and 6-month post-last injury sham mice from our aged GFAP$^{P301L}$ study.
Figure 5.7: Astrocyte specific pathways that are dysregulated in aged GFAP^{P301L} mice at 3- and 6-month post-last injury. Venn diagram of injury dependent differentially expressed genes (DEGs) in GFAP^{P301L} r-mTBI vs sham mice are shown in A. Volcano plot of injury dependent DEGs are shown in B (3-month post-last injury) and D (6-month post-last injury). Top 10 DEGs are highlighted on the volcano plots. Upregulated DEGs are in red, Downregulated DEGs are in blue. Histogram in C (3-month post-last injury) and E (6-month post-last injury) depicts results of IPA pathway analyses after analyzing the entire DEG list between GFAP^{P301L} r-mTBI vs sham groups. Upregulated and downregulated pathways in C, E are depicted in red and sky blue, respectively. Heat-bar in C, E represents -log 10 of the P value (yellow – Topmost significant; sky blue – least significant). Threshold for obtaining the DEGs: adj. p-value≥0.05 with its respective –log value ≥1.3.

N=6 mice/group (N=3 technical replicates – i.e., two pooled hemispheres). Note: GFAP^{P301L} mice were exposed to their 1-month long r-mTBI/sham injury paradigm at 12 months of age.
In regards to our research on the bidirectional interaction between astrocytes and microglia, we could further characterize our astrocytes in vivo or in-vitro by for instance assessing astrogial markers that have been reported to be increased in white matter astrocytes of CTE cases such as CD44, BCL6, and SERPINA3 (Chancellor et al., 2021) or other markers reported to be increased in reactive astrocytes such as A1/A2 markers (Liddelow et al., 2017). It is important to mention that we acknowledge that astrocyte reactivity is context-dependent and the difference in the inflammatory stimulus is likely to elicit different astrogial responses, however, looking at A1/A2 profile can serve as guidance. Nonetheless, the major limitation we faced with the in-vitro experiments was the possible loss of phenotype through time, thus before designing further experiments we should first solve this issue. This could be done by performing our studies acutely (within hours) after primary cells have been isolated from the brain which will require more mice (and is the main limiting factor). Alternatively, we could explore in-vitro TBI-like models (mono- or co-cultures, 3D-cultures/organoids) where simulated injuries can be administered directly to cell in culture or in-vitro models can be exposed to CSF fluid collected from an injured brain.

5.4 CLOSING REMARKS

In conclusion, the findings of this thesis underscore the significance of unraveling the enigma surrounding tau astrogliopathy and illustrate its role in the astrogial immune state and their reaction to a second encounter with inflammatory stimuli (very common in the brain parenchyma following r-mTBI) which alters the astrocyte-microglia bidirectional molecular communication triggering a pro-inflammatory state in microglia. The studies performed in this thesis represent the establishment of a valid platform for future research focused on identifying astrocyte-specific molecular mechanisms of tau astrogliopathy. The identification of these mechanisms holds the potential for targeted interventions and the development of effective disease-modifying therapeutics for r-mTBI.
REFERENCES


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