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14. ABSTRACT

After traumatic brain injury (TBI), the human brain sometimes develops tau pathology partly resembling the hallmark neuropathological features of the tauopathy of Alzheimer's disease (AD). Although tau has been strongly linked to the pathogenesis of AD, its involvement in the pathophysiology of TBI and its influence on brain structural and functional outcomes are unclear. Here we used a novel mouse model of early stage AD-type tauopathy to critically evaluate three hypotheses: (i) tau exacerbates the neuronal damage and cognitive dysfunction after single and repetitive mild TBI in the acute and chronic post-injury periods; (ii) mild TBI promotes the severity and spread of tau pathology to contribute to development of a chronic neurodegenerative disorder; and (iii) novel biomarkers for neurodegeneration are non-invasive blood measures of brain dysfunction valuable for the diagnosis and prognosis of mild TBI-triggered chronic neurodegenerative disease.

Our data lead us to conclude that, in both the acute and chronic post-injury time periods, there is no interaction at the structural or functional level between hippocampal input-specific expression of pathological human tau and either single or repetitive mild TBI. Instead, long-term expression of pathological human tau in a specific mouse neural circuit leads to the onset of tau hyperphosphorylation, aggregation, and slowly progressive neurodegeneration, and this degenerative tauopathy occurs independent of single or repetitive mild TBI.

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Table of Contents

Introduction and Overall — Pages 4-5
Objectives Keywords — Page 5
Accomplishments — Pages 6-22

- Bulleted highlights — Page 6
- The Alzheimer tau model — Pages 6-8
- Study questions and the mouse cohorts evaluated — Pages 8-9
- Mild hippocampal damage in our mild TBI model is worse in the chronic post-injury period — Pages 9-11
- A control foreign protein does not endanger the perforant pathway chronically post-injury — Page 12
- Capability of detecting degeneration of perforant pathway neurons, axons, and synapses — Pages 12-13
- Pathological human tau does not enhance perforant pathway vulnerability to mild TBI — Pages 13-16
- Mild TBI does not influence tau pathology in the long term — Pages 16-18
- Slow progressive tau pathology triggers pathway degeneration, independent of mild TBI — Pages 18-20
- Tau and mild TBI do not interact to impair cognition chronically post-injury — Pages 20-22
- Serum biomarkers for chronic neurodegeneration triggered by mild TBI — Page 23
- Summary of major findings — Page 24

Impact — Page 25
Changes/Problems — Page 26
Products — Page 26
Participants — Pages 26-27
Changes in Other Support — Page 27-28
Special Reporting Requirements — Page 28
Appendix — Page 28

Introduction and Overall Objectives

After traumatic brain injury (TBI), the human brain sometimes develops tau pathology partly resembling the tauopathy that is well-established as a hallmark neuropathological feature of Alzheimer's disease (AD). In healthy brain cells, tau is a component of the microtubule network with vital roles in cytoskeletal structure and intracellular transport. However, within vulnerable neurons in signature regions of the AD brain, tau becomes hyperphosphorylated, dissociated from microtubules, aggregated, and mislocalized within cell bodies and proximal dendrites instead of axonal processes, abnormalities that collectively are referred to as tauopathy. There is considerable evidence in AD that tauopathy drives the loss of neurons and synapses underlying the onset and progression of regional brain atrophy and cognitive impairment. Given that AD is a slowly progressive neurodegenerative and cognitive disorder, and TBI induced by inertial forces, concussive blows, or blast will sometimes lead to chronic, progressive brain atrophy and cognitive decline, the question arises whether AD and TBI may share common underlying tau-dependent pathophysiology. At present, although tau is known to accumulate after TBI and become phosphorylated on multiple residues, its pathophysiological importance to brain damage and dysfunction during the acute and chronic post-injury time periods is unknown. From human TBI studies, it is difficult to determine the contribution of tau to progressive brain damage and dysfunction, owing to their dependence on non-invasive or post-mortem histopathological methods. Furthermore, there are currently no simple, validated blood tests for diagnosing at an early and potentially treatable stage the subset of TBI sufferers that go on to develop chronic progressive neurodegenerative disease.

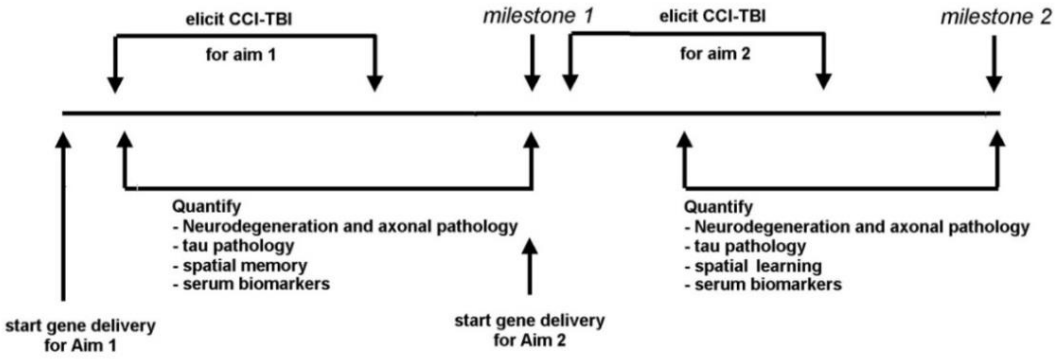
Previously, we established a new translational mouse model for studying pathogenic mechanisms of tau, using a viral vector to drive robust long-lasting expression of a pathological form of mutant human tau focally within a specific hippocampal input pathway that is both preferentially vulnerable in early-stage AD and critically important for long-term memory. The model confines expression of mutant human tau to the lateral perforant pathway, the projection from the lateral entorhinal cortex to the hippocampal dentate gyrus. This mouse model of early-stage AD tauopathy is characterized by rapid, dose-related, circumscribed human tau expression, tauopathy, trans-synaptic spread of human tau expression, and tau-dependent neurodegeneration. The model is exceptionally well suited for addressing whether human tau affects structure and function of the hippocampus after single or repetitive mild TBI, and whether mild TBI exacerbates ongoing tauopathy to promote a chronic neurodegenerative condition. In addition, over the past decade we have discovered and characterized new biofluid-based markers for neurodegeneration. Whereas these novel biomarkers have shown considerable promise as diagnostic and prognostic tools in acute trauma- and ischemia-induced brain injuries, they have never been evaluated as a potential blood diagnostic test for development of a TBI-associated chronic neurodegenerative condition.

<u>Objectives</u>

To study tau/TBI interactions and validate preclinically new biofluid-based diagnostic markers for chronic TBI-induced progressive brain atrophy, we collaborated with Dr. Douglas Smith, Director of the Penn Center for Brain Injury and Repair, who pioneered the development and characterization of a controlled cortical impact (CCI) model of TBI in the mouse, and our mutual colleague Dr. Victoria Johnson. We combined mild CCI with our novel mouse model of early-stage AD tauopathy to study three critical unanswered questions: (1) does tau exacerbate the acute and chronic effects of mild TBI on brain structure and function? (2) does mild TBI

worsen an ongoing tauopathy to promote development of a chronic neurodegenerative disorder? (3) do blood levels of biomarkers for neurodegeneration diagnose mild TBI cases developing progressive brain atrophy chronically after injury?

Our first two objectives addressed whether tau is an important target for therapeutic intervention after TBI. Our third objective was to generate pre-clinical evidence a blood test for improving the diagnosis of TBI-induced chronic neurodegenerative disease in the long-term post-injury time period. The time line and milestones for the 3 year project are illustrated below:



Keywords
Tauopathy; tau phosphorylation; traumatic brain injury; concussion; neurodegeneration; entorhinal cortex; perforant pathway; synapse loss; cognitive dysfunction; prognostic biomarker; diagnostic marker; brain atrophy; chronic traumatic encephalopathy; Alzheimer's disease.

Major Accomplishments

- Used a novel mouse model of early-stage Alzheimer-type tauopathy, with hippocampal input-specific expression of human tau P301L, or eGFP as a control foreign protein, to evaluate interactions between pathological human tau and controlled cortical impact mild TBI.
- Demonstrated that pathological human tau, expressed specifically in the lateral perforant pathway hippocampal input, does not endanger the neurons, axons, or synapses of the pathway to either single or repetitive mild TBI acutely (within 7 days) post-injury.
- Found that neither single nor repetitive mild TBI changes the expression level, distribution, or phosphorylation state of human tau acutely post-injury.
- Showed that both single and repetitive mild TBI impair hippocampus-dependent spatial learning acutely post-injury, and pathological human tau does not exacerbate this mTBI-induced acute cognitive dysfunction.
- Established that from 7 days to 4 months after single or double mild TBI, the cortical and hippocampal damage continues to worsen; this evolving brain atrophy occurs irrespective of the presence of mutant human tau in the perforant pathway.
- Found no appreciable interaction at the hippocampal structural or functional level between pathological human tau and either single or double mild TBI in the 4 month chronic post-injury period.
- Instead, the viral vector-based model of input-specific mutant human tau expression exhibits slowly progressive tau pathology and the onset of perforant pathway degeneration occurring independently of mild TBI.
- Tau phosphorylation at residues 212, 214, 217, and 262, aggregation, and packaging into granulovacuolar degeneration bodies are associated with the onset of perforant pathway degeneration, thereby identifying specific post-translational tau modifications and a cell biological process that are tied to slowly progressive tau neurotoxicity in chronic neurodegenerative disease.
- Generated evidence that at 7 days and 4 months after single or repetitive mild TBI, serum levels of SNTF, a mechanism-based biomarker for injury-induced axonal damage, are not changed appreciably, or affected by pathway-specific expression of pathological human tau.

Accomplishments and Major Findings

1. A novel mouse model of early-stage Alzheimer-type tauopathy

There is considerable evidence that dysfunction of the perforant pathway projection from entorhinal cortex (EC) to hippocampal dentate gyrus is an important contributor to the onset and progression of cognitive impairment in AD. This pathway is a major source for excitatory innervation of hippocampus, a structure vital for memory formation. Damage to the EC or perforant pathway projection in animals causes a rapid forgetting syndrome reminiscent of AD. The perforant pathway is especially vulnerable in AD. The entorhinal neurons of origin in layer II are among the first to develop aggregates of hyperphosphorylated tau in the form of neurofibrillary tangles (Braak stage I), and the terminal field in the dentate gyrus is a preferential early site for amyloid Aβ deposition. Recent evidence suggests that tauopathy initiating in the perforant pathway spreads over time through its afferent connections. Finally, the pathway dies beginning with the earliest signs of cognitive impairment, and the neuronal loss progresses coincident with cognitive decline, until more than 90% of the pathway has degenerated.

Consequently, we used an AAV vector approach to express pathological human mutant tauP301L linked genetically to human tauopathies, or an eGFP control, focally in the mouse

lateral perforant pathway. The vectors were microinjected by unilateral stereotaxic convection-enhanced delivery into the right lateral entorhinal cortex (LEC), and 4 weeks allowed for the foreign proteins to be expressed in the entorhinal layer II neurons of origin, the perforant pathway axons, and their synapses onto the distal dendrites of granule neurons in the hippocampal dentate gyrus outer molecular layer (OML). As schematized in Figure 1, in the mouse all of the synaptic inputs terminating in the dentate OML originate from the lateral EC and lateral perforant pathway afferent projection.

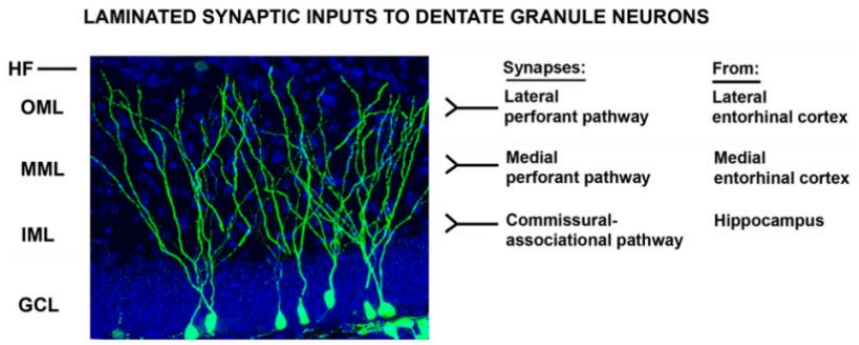


Figure 1 abbreviations used:
HF- hippocampal fissure
OML – outer molecular layer
MML – middle molecular layer
IML – inner molecular layer
GCL – granule cell layer
A subset of granule neurons and their dendrites are shown in green, nuclei are in blue.

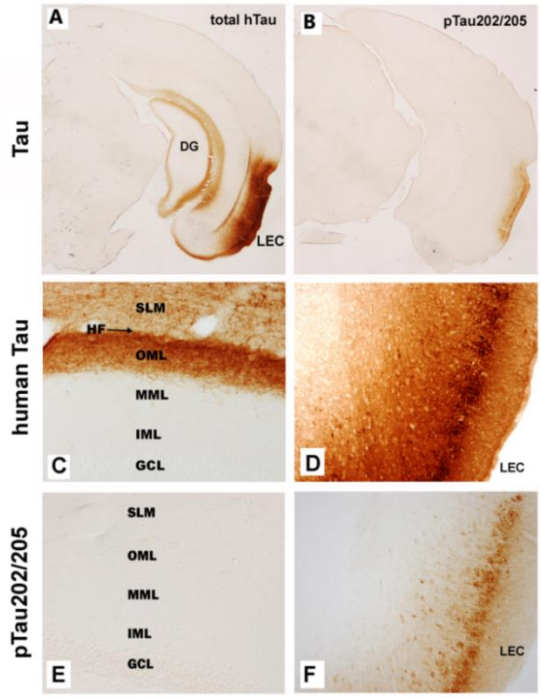


Figure 2. An AAV-based mouse model for Braak stage I Alzheimer-type tauopathy.
The AAV-hTau injected hemisphere 4 weeks after gene delivery, stained for total hTau (A,C,D) or pTau202/205 (B,E,F). Note that for the injected hemisphere human tau expression is confined to the lateral entorhinal cortex (LEC) layer II, the lateral perforant pathway axons in the hippocampal SLM and synaptic field in the dentate gyrus (DG) OML. There is no hTau expression in the contralateral hemisphere (not shown). In contrast to total hTau, pTau202/205 expression is restricted to the LEC layer II neuronal perikarya and is excluded from the perforant pathway axons and synapses. The distributions of pTau231 and pTau262 are identical to that of pTau202/205. This pattern of phosphotau accumulation mimics the earliest neuropathological stage of tauopathy in Alzheimer's disease. Modified from Siman et al., 2013.

Confirming earlier publications (Siman et al., 2013; 2015), delivery of AAV-hTauP301L to the right LEC led to robust human tau expression in the lateral peforant pathway LEC layer II neurons of origin and the entire projection as it traverses the stratum lacunosum-moleculare (SLM) of hippocampal CA1 sector before perforating the hippocampal fissure (HF) to terminate in the dentate OML (Figure 2). The human tau is distinguished from the widely distributed endogenous mouse protein by immunohistochemistry using the human specific monoclonal HT7. In contrast, tau phosphorylated on serine residues 202 and 205, labeled with the monoclonal AT8 and considered an early marker for hyperphosphorylation, is confined to layer II neurons of the

LEC, but does not undergo appreciable axonal transport and is undetectable in the perforant pathway axons or synapses. This distribution of pTau202/205 closely resembles the earliest neuropathological stage of Alzheimer tauopathy (Braak stage I), with hyperphosphorylated tau aggregates localized to the superficial trans-entorhinal region. An identical distribution pattern is observed for tau phosphorylated on Thr231 or Ser262 ranging from 4 weeks to 5 months after gene delivery. With AAV-eGFP as a control for vector-mediated foreign protein expression, the autofluorescent eGFP distributes similarly to human tau throughout the lateral perforant pathway neurons of origin, axons, and pre-synaptic terminals innervating the hippocampal dentate gyrus and terminating in the OML.

2. Acute and chronic interactions between mTBI and tauopathy: study design

Our project addressed three key questions in the search for tau/mTBI interactions in the acute and chronic post-injury periods:

(1) What are the effects of pathological hTau on the response to mTBI at 7 days and 4 months post-injury?
(2) Does mTBI exacerbate tau pathology or promote its anatomical spread either acutely or chronically post-injury?
(3) Is there a blood biomarker for neurodegeneration that in the long-term post-injury period serves as a surrogate marker for chronic mTBI-induced brain damage?

To identify mTBI-induced brain atrophy and any changes in tau pathology that may develop only in the long-term post-injury period, we compared genetically modified mice at 4 months post-injury (chronic phase) with mice at 7 days post-injury (acute phase). For the acute component of this study, we analyzed the following groups of mice at 7 days post-injury, 5 weeks after viral vector-driven transgene expression:

1) AAV-eGFP, sham injury (n=10)
2) AAV-hTau, sham injury (n=15)
3) AAV-eGFP, mild TBI (n=11)
4) AAV-hTau, mild TBI (n=9)
5) AAV-eGFP, double mild TBI (n=9)
6) AAV-hTau, double mild TBI (n=10)

For the chronic component of the study, comparably treated mice were analyzed at 4 months post-injury (5 months after viral vector-driven transgene expression):

1) AAV-eGFP, sham injury (n=7)
2) AAV-hTau, sham injury (n=13)
3) AAV-eGFP, mild TBI (n=8)
4) AAV-hTau, mild TBI (n=14)
5) AAV-eGFP, double mild TBI (n=10)
6) AAV-hTau, double mild TBI (n=11)

Our experimental design yielded sufficient numbers of cases in each experimental group for drawing definitive conclusions on the experimental questions posed above. Of the mice intended for the acute study component, 64 across the 6 treatment groups were confirmed by histological analysis blinded to the gene modification to exhibit strong and highly localized eGFP or hTau transgene expression throughout the entire rostro-caudal extent of the lateral perforant pathway. For the chronic study component, an additional 63 mice were confirmed by blinded histological analysis as maintaining strong and long-lasting transgene expression throughout the pathway.

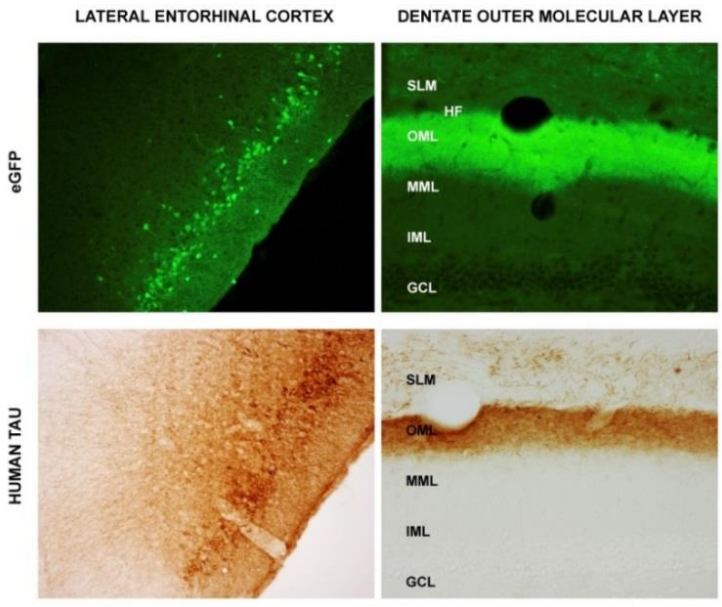
At 5 weeks or 5 months after gene delivery, and either 7 days or 4 months after injury, the mice were evaluated for the following hippocampal structural and functional endpoints:

(a) human tau expression (human-specific tau antibody HT7)
(b) tau phosphorylation (pTau202/205; pTau231; pTau262; pTau212/214/217)
(c) human tau trans-synaptic propagation (human-specific tau antibody HT7)
(d) perforant pathway neuronal survival (NeuN antibody)
(e) perforant pathway synapse and axonal integrity (synaptic zinc using Timm's stain)
(f) perforant pathway axonal pathology (APP and SNTF antibodies)
(g) hippocampus-dependent spatial learning (Morris Water Maze over 3 consecutive days)

In addition, serum samples were obtained at the time of sacrifice at 7 days or 4 months post-injury for quantification of the calpain-derived spectrin fragment SNTF (nonerythroid α-spectrin 1-1176) as a potential sustained blood biomarker for mTBI. For milestone I, we completed all short-term behavioral, histological (both qualitative and quantitative), and serum biomarker analyses for the mice with well-place vector injections and robust lateral perforant pathway transgene expression. This established the foundation for milestone II to search for progressive neurodegenerative changes or tauopathies that might develop only in the long-term period after mTBI. The following sections describe our findings and summarize conclusions on the influence of pathological hTau on the short- and long-term outcomes from single and double mTBI, along with the influence of single and double mTBI on the severity and distribution of tau abnormalities in our novel mouse model of early-stage Alzheimer tauopathy.

3. <u>A mild controlled cortical impact TBI in the mouse elicits subtle hippocampal structural damage on the impacted side that worsens over 4 months post-injury.</u>

As a prelude to examining the effect of long-term expression of pathological tau in the perforant pathway, we first evaluated eGFP and hTau expression for up to 5 months after gene delivery. As shown in Figure 3 below and later in Figure 8, robust and focal expression of both pathological human tau and eGFP persisted for at least 5 months throughout the lateral perforant pathway, originating in layer II of the LEC (left panels), projecting through the hippocampal SLM, and terminating in the dentate OML (right panels).



<u>Figure 3.</u> Strong transgene expression persists for at least 5 months in the lateral perforant pathway after AAV-mediated entorhinal gene delivery.
(Left panels) – Lateral entorhinal cortex. The lateral perforant pathway neurons in layer II continue to strongly express either eGFP or hTauP301L.
(Right panels) – Hippocampus. eGFP and hTau persist in the perforant pathway axons traversing the SLM in the CA1 sector, and in the synaptic field in the dentate outer molecular layer (OML). Other hippocampal regions are devoid of foreign protein expression.

One of our main objectives was to examine effects of mTBI on the structural and functional integrity of a major hippocampal input pathway when it expresses a pathological form of human tau. Consequently, it is vital for the study design that the method for inducing mTBI elicits discernable but minor hippocampal structural damage. To accomplish this, the magnitude of the controlled cortical impact was adjusted by varying the impounder velocity and cortical depth, so as to produce subtle but discernable hippocampal structural damage. Having examined 64 genetically modified sham-injured, single-injured and double-injured mice at 7 days after injury and another 63 mice at 4 months post-injury, we conclude that cortical lesions at the site of impact are larger at 4 months than at 7 days post-injury, and the distortions of hippocampal cytoarchitechture underlying the site of cortical impact are more prominent. For example, as shown in Figure 4, beneath the impacted lesioned parietal cortex, the hippocampus is mostly intact at 7 days post-injury, as revealed by immunostaining for the neuronal nuclear marker NeuN (compare the top left and bottom left panels). However, hippocampal structure changes subtly in two ways in the acute post-injury period. First, the hippocampus exhibits localized swelling and disrupted cellular organization in the CA1/CA3 sector border beneath the site of cortical impact compared with the uninjured contralateral side (arrows; compare the injured cases with the sham control at the top left). Second, the shape of the dentate gyrus granule cell layer (GC) is often distorted and the entirety of the dentate gyrus is frequently compacted.

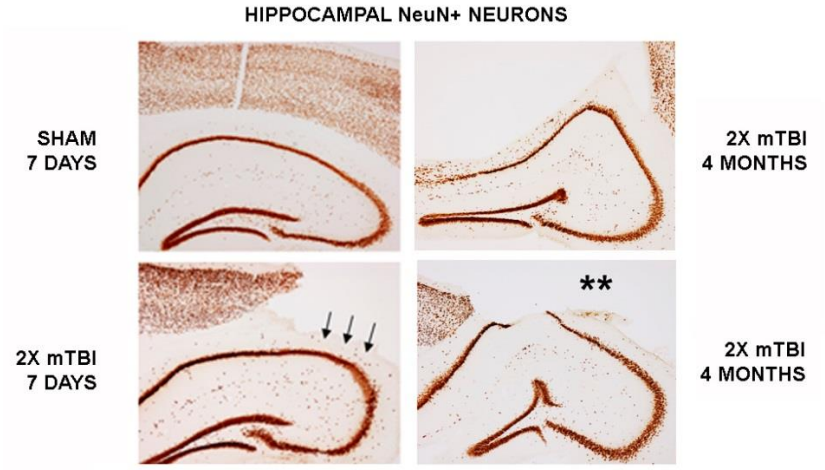


Figure 4. Hippocampal structural damage progressively worsens from 7 days to 4 months after mTBI.
Immunostaining for the neuronal marker NeuN in mouse brain shows the presence of a large focal lesion in parietal cortex at the site of impact (e.g., bottom right panel, double asterisks). In comparison with the sham-injured left hippocampus (top left panel), the CA1 sector is swollen and the cytoarchitecture of the pyramidal cell layer compressed and distorted (arrows) after a double mTBI (bottom left, at 7 days; top right, at 4 months). The same alterations occur after a single injury (shown in previous reports). In a subset of mice, the primary focal lesion extends into the dorsal CA1 sector at 4 months post-injury, and the organization of granule neurons in the dentate gyrus is both disrupted and compacted (right two panels). The hippocampal CA1 swelling and disruption in CA1 and dentate cytoarchitecture are consistently more pronounced at 4 months (right) than at 7 days (left) post-injury for both single and repetitive mTBI.

These structural abnormalities are much more pronounced at 4 months than at 7 days post-injury (Figure 4). Over time, the primary focal lesion at the cortical site of impact (asterisks) expands, sometimes including the dorsal part of the rostral hippocampus (lower right).

The swelling-induced distortion of the CA1/CA3 sector (top right), and compression and disrupted organization of the dentate granule cell layer (right panels) become more pronounced. The cortical and hippocampal structural damage is comparable between single and double mTBI, and does not differ depending on whether eGFP or hTauP301L is expressed in the lateral perforant pathway hippocampal afferent projection. These data confirm that the controlled cortical impact mTBI used herein spares the hippocampus from profound atrophy and degeneration, but distorts its structure, including altering the dentate gyrus that contains the lateral perforant pathway axons, synapses, and granule neuron targets of the pathway.

Immunostaining for SNTF, established by my laboratory over the past 25 years as a marker for necrotic neurodegeneration, supports the observations described above for the evolution of brain injury chronically post-injury. At 4 months after mTBI, occasional neurons are SNTF-immunopositive in the injured parietal cortex adjacent to the controlled cortical impact site, suggesting they are undergoing delayed neurodegeneration (Figure 5). Some of the neurons positive for SNTF have abnormal rounded perikaryal morphology and beaded dendritic varicosities, further indicating ongoing degeneration (right, arrow). On the other hand, despite showing disrupted cytoarchitechture, neither the hippocampal CA1 sector nor the dentate granule cell layer show any evidence for SNTF-positive actively degenerating neurons at 4 months post-injury. Similarly, the LEC containing the neurons of origin for the lateral perforant pathway in layer II is also devoid of SNTF-positive degenerating neurons.

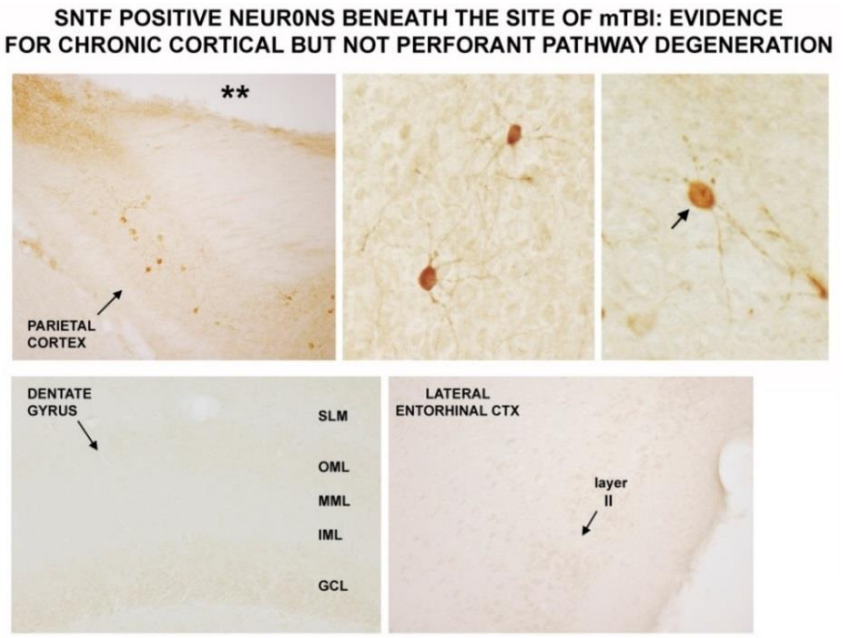


Figure 5. Evidence for ongoing cortical neuronal degeneration near the site of impact but not in the perforant pathway or dentate gyrus target neurons at 4 months after mTBI.

Immunostaining for SNTF, a marker for necrotic neurodegeneration, identifies scattered cortical neurons adjacent to the site of controlled cortical impact 4 months after mTBI. Top row: beneath the impact site (asterisks), scattered neurons are SNTF immunopositive, some of which also have abnormal morphology consistent with ongoing neurodegeneration (right, arrow). In contrast, neither the dentate gyrus (bottom left) or entorhinal cortex (bottom right) contain any acutely degenerating SNTF positive cells at 4 months post-injury, including the layer II perforant pathway neurons and their target neurons in the dentate granule cell layer.

4. Neither short- nor long-term expression of eGFP as a control foreign protein promotes degeneration of the perforant pathway after TBI.

The combination of AAV-driven expression of eGFP in the perforant pathway with single or double mTBI does not induce any loss of pathway neurons, axons, and synapses in either the acute or chronic post-injury period. In the hippocampus, staining for pre-synaptic terminal zinc is a pathway-specific method for analyzing the integrity of afferent inputs arising from multiple brain regions, including from the lateral entorhinal cortex via the lateral perforant pathway. For the eGFP expressing controls, zinc staining of lateral perforant pathway synapses in the dentate OML is unchanged at both 7 days and 4 months after mTBI, and this is true for both single and double injuries. The lack of toxicity of eGFP occurs despite strong expression of the protein in the lateral perforant pathway axons in the hippocampal SLM and synaptic field in the dentate OML. Similarly, at 4 months post-injury there is no overt loss of eGFP expressing lateral perforant pathway neurons of origin in layer II of the lateral entorhinal cortex, compared with the uninjured contralateral side and based on quantitative analysis of NeuN-immunopositive neuronal nuclei. These data establish our capacity to determine whether specific expression of human tauP301L in the lateral perforant pathway endangers these neurons and synapses to the acute and chronic effects of mTBI.

5. Our methods are readily capable of detecting perforant pathway neurodegeneration and tau propagation, if they are triggered by mTBI and exacerbated by pathological tau.

To confirm that our analytical methods are capable of detecting partial degeneration of the lateral perforant pathway, should it occur after mTBI combined with expression of hTauP301L, we evaluated the integrity of the pathway after administering a dose of AAV-hTau shown previously to induce partial degeneration even in the absence of TBI. All of the mTBI experiments in our mTBI study used intra-entorhinal delivery of 0.5 billion genome copies of the AAV-hTauP301L or AAV-eGFP vectors. This is the maximal dose of the hTau vector that drives robust human tau expression and hyperphosphorylation for up to 4 months without causing appreciable loss of perforant pathway neurons, axons or synapses.

Here we examined the effects of expressing a higher dose of pathological hTau (1.5 billion genome copies) on perforant pathway integrity and human tau distribution. After 3 weeks of expression driven by this gene dose, and exemplified in Figure 6 (left), human tau is present only in the perforant pathway axons of the hippocampal SLM and lateral perforant pathway pre-synaptic terminals of the dentate OML, but over the next several weeks its expression expands trans-synaptically to include scattered lateral perforant pathway target neurons, the dentate granule cells in the GCL (right panel). The trans-synaptic transfer of hTau expression in our AAV model occurs only in association with hTau-triggered partial degeneration of the lateral perforant pathway, and is mitigated by a pharmacotherapy that partially blocks the neurotoxicity of hTauP301L (Siman et al., PLoS One 10: e0142340 [2015]).

Degeneration of the lateral perforant pathway in response to the toxic dose of pathological human tau is illustrated in Figure 7, manifested as reduced density of synaptic zinc staining and thinning of the dentate OML (top panels), and also by the reduced number of NeuN-stained nuclei in layer II of the lateral EC (bottom panels). These results, coupled with our prior published findings (J Neuropathol Exp Neurol 72: 1062 [2013]), demonstrate that the zinc and NeuN labeling methods are capable of detecting partial degeneration of lateral perforant pathway axons, synapses, and neurons, should they be triggered by low dose hTauP301L expression either acutely or chronically after single or double mTBI.

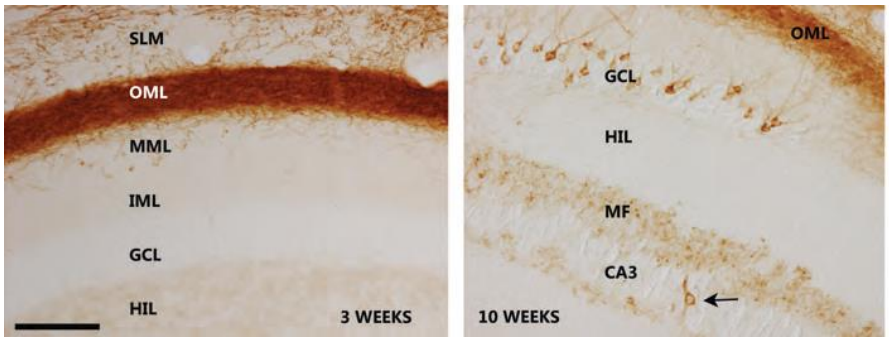


Figure 6. At a higher toxic gene dosage, human tau expression expands over time through polysynaptic afferent pathways. (A) At 3 weeks after intraentorhinal delivery of a higher, toxic dose of the AAV-tau vector, human tau expression in the hippocampus is confined to the perforant pathway afferents in the SLM and the lateral perforant pathway synaptic field in the dentate (OML). (B) By 10 weeks, human tau expression expands to include a subset of the granule neuron perforant pathway targets (GCL), the granule neuron mossy fiber projection (MF), and sparse numbers of mossy fiber target neurons in the pyramidal cell layer of the CA3 region (CA3).

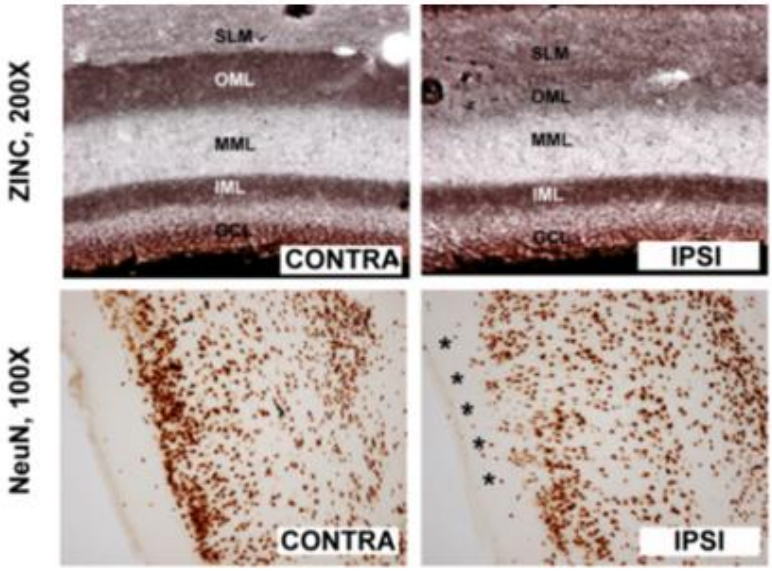


Figure 7. Detection of partial loss of perforant pathway neurons and synapses after delivery of a higher, toxic dose of AAV-hTauP301L. Top panels – Synaptic zinc staining reveals the hTau-induced partial loss of lateral perforant pathway synapses in the OML (compare the injected with the uninjected side). 200X mag.
Bottom panels - NeuN staining of the lateral EC reveals hTau-driven degeneration of layer II neurons of origin for the lateral perforant pathway (just right of the asterisks). 100X mag.

6. <u>Expression of pathological human tau does not enhance perforant pathway vulnerability to single or repetitive mTBI in the acute or chronic post-injury periods.</u>

We studied whether expression of pathological human tau in the lateral perforant pathway, at a level below the threshold for direct and rapid hTauP301L neurotoxicity, might sensitize the pathway to degeneration both acutely and chronically after single or double mTBI. To investigate these possibilities, we conducted analyses using NeuN for assessing the survival of lateral perforant pathway neurons, and zinc staining for evaluating the integrity of its synapses both in the absence and presence of pathological human tau. The survival of lateral perforant pathway neurons in layer II of entorhinal cortex was quantified from a total of 64 genetically modified mice at 7 days post-injury and another 63 at 4 months post-injury. The dose of hTau expression was titrated to 0.5 billion particles of AAV-hTauP301L, which in the absence of TBI does not cause any short-term degeneration of the pathway. To assess synapse integrity, zinc staining of lateral perforant pathway presynaptic terminals was used to demarcate the terminal

field in the dentate gyrus OML. Our qualitative findings demonstrate clearly the lack of appreciable neurodegeneration in the perforant pathway axonal projection and synaptic field both acutely and chronically after single or double mTBI (Figure 8). Neuronal integrity of the lateral perforant pathway at 4 months after single mTBI is illustrated in the bottom panels. For the majority of injured mice, with expression of either eGFP (bottom left) or hTauP301L (bottom right) there is no discernable difference in perforant pathway NeuN positive neuronal density between the injured and uninjured sides.

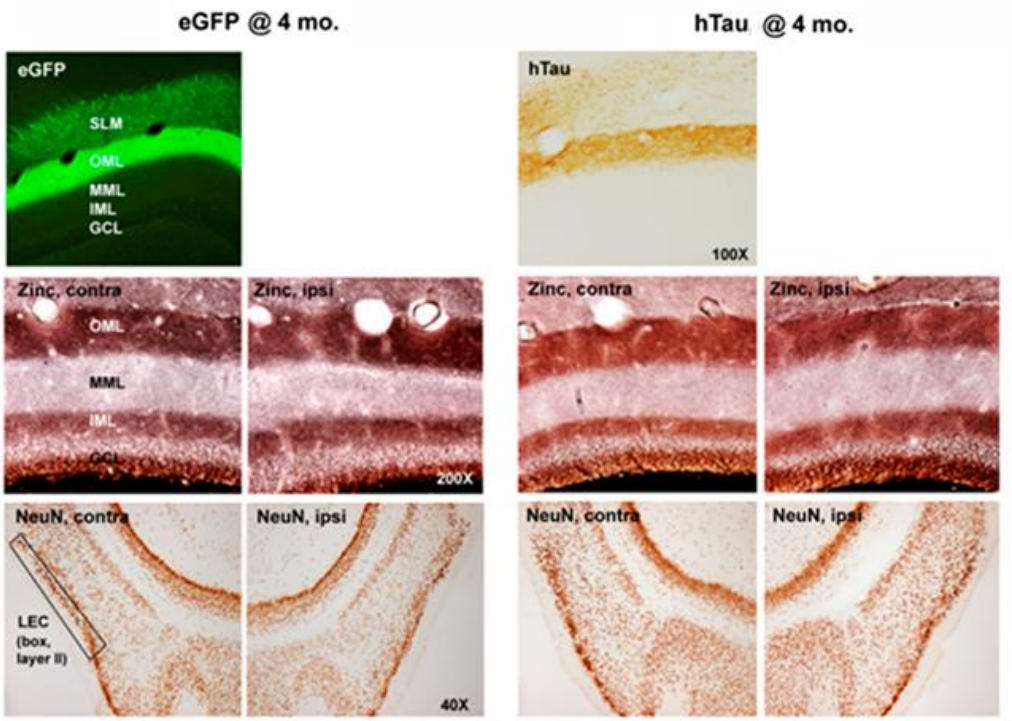


Figure 8. Evaluation of synapse and neuronal integrity at 5 months of eGFP and hTau expression and at 4 months after mTBI.
Top panels: Expression of eGFP and hTau persist in lateral perforant pathway axons and synapses in the dentate gyrus at 5 months after AAV-mediated gene delivery and 4 months after mTBI. Note the robust expression of both proteins in the lateral perforant pathway afferents in the hippocampal SLM and terminal field in the dentate OML.
Middle panels: Lateral perforant pathway synapses in the dentate OML are intact at 4 months after single mTBI coupled with eGFP or hTauP301L expression, as evidenced by the persistence of pre-synaptic terminal zinc staining.
Bottom panels: Lateral perforant pathway neurons in the entorhinal cortex are intact at 4 months after single mTBI, and after 5 months expression of either eGFP or hTauP301L, as evidenced by the persistence of NeuN-positive neuronal nuclei in layer II (denoted by the box).

These qualitative observations are substantiated by quantitative morphometric analysis of NeuN-positive neuronal density in lateral entorhinal cortex layer II at both 7 days and 4 months post-injury (Tables 1A and B). At the acute- and long-term time points, neither genotype shows robust neuronal loss in sham-, single-, or double-injured mice. Furthermore, there is no difference in survival of lateral perforant pathway neurons between mice expressing eGFP and pathological human tau in either the sham- or single mTBI injury groups. In mice with long-term expression of hTauP301L, there is a very small 11% decrease in lateral perforant pathway neuronal survival at 4 months after double mTBI.

Table 1. Quantification of the short-term (top) and long-term (bottom) effects of mTBI on survival of genetically modified lateral perforant pathway neurons. Lateral perforant pathway neuronal survival at 7 days (top) and 4 months after injury (bottom) are represented as the mean density of NeuN-positive layer II neurons on the injured LEC relative to the contralateral LEC. P value - unpaired t-test. NS – not significantly different from the corresponding control group.

Quantification of lateral perforant pathway neuronal survival after mTBI +/- hTauP301L

Acute Group	Injured (%contra +/- sem)	P value
eGFP sham	97 +/- 2	
eGFP mTBI	93 +/- 3	NS vs eGFP sham
eGFP mTBI x2	101 +/- 4	NS vs eGFP sham
hTau sham	103 +/- 5	NS vs eGFP sham
hTau mTBI	91+/- 7	NS vs tau sham; NS vs eGFP mTBI
hTau mTBI	94 +/- 4	NS vs tau sham; NS vs eGFP mTBI x2
Chronic group	Injured (%contra+/-sem)	P value
eGFP sham	98 +/- 3	
eGFP mTBI	104 +/- 5	NS
eGFP mTBI x2	104 +/- 4	NS
hTau sham	100 +/- 4	
hTau mTBI	105 +/- 5	NS
hTau mTBI x2	89 +/- 4	NS vs hTau sham; 0.02 vs. eGFP 2x mTBI

The lack of effect of mild TBI combined with pathological human tau on structural integrity of the lateral perforant pathway is confirmed by quantitative analysis of the density of its synapses. Staining for pre-synaptic terminal zinc was used as a pathway-specific method for analyzing the integrity of hippocampal afferent inputs arising from multiple brain regions, including from the lateral entorhinal cortex via the lateral perforant pathway. For each mouse, 3 sections were analyzed by comparing zinc staining intensity in the injured dentate outer molecular layer with the staining intensity of the uninjured contralateral synaptic field within each section. As shown in Figure 8 above (middle panels) and Tables 2 A and B, for mice expressing eGFP- or hTau in the right lateral perforant pathway, neither single nor double mTBI triggered a loss of pathway synapses at 7 days (Table 2A) or 4 months (Table 2B) post-injury. Our prior work established that the method is readily capable of detecting even minor loss (<30%) of lateral perforant pathway synapses (PLoS One 10: e0142340 [2015]; see also Section 6 that follows below). We conclude that neither single nor double controlled cortical impact mTBI damages the integrity of lateral perforant pathway axons or synapses, and expression of hTauP301L does not endanger the pathway either acutely or chronically to mTBI. This conclusion is further supported by immunostaining for axonal pathology after TBI (Johnson et al., Acta Neuropathol. *131*, 115 [2016]). Neither APP nor SNTF immunohistochemistry at 7 days and 4 months post-injury reveals axonal damage in the perforant pathway afferents in the SLM of the CA1 sector, or in the dentate gyrus OML. Instead, dysmorphic axons are confined to the corpus callosum just ventral to the site of impact, and occasionally to the alveus, the white matter tract lying along the dorsal surface of the rostral hippocampus.

Table 2: Lateral perforant pathway synapse integrity after mTBI +/- hTauP301L

Acute Group	Injured (%contra +/- sem)	P value (unpaired t-test)
eGFP sham	92.8 +/- 2.4	
eGFP mTBI	98.8 +/- 7.1	0.4 vs eGFP sham
eGFP mTBI x2	95.7 +/- 4.4	0.6 vs eGFP sham
hTau sham	91.2 +/- 3.6	0.7 vs eGFP sham
hTau mTBI	108.7 +/- 10.7	0.1 vs tau sham; 0.5 vs eGFP mTBI
hTau mTBI x2	94.4 +/- 3.6	0.6 vs tau sham; 0.8 vs eGFP mTBI x2

Chronic Group	Injured (%contra +/- s.e.m.)	P value
eGFP sham	92.8 +/- 2.4	
eGFP mTBI	98.8 +/- 7.1	0.42 vs sham
eGFP mTBI x2	95.7 +/- 4.4	0.57 vs sham
hTau sham	91.2 +/- 3.6	0.71 vs eGFP
hTau mTBI	108.7 +/- 10.7	0.10 vs sham
hTau mTBI x2	94.4 +/- 3.6	0.54 vs sham

We conclude that, in the controlled cortical impact mouse model, there is no appreciable interaction between pathological human tau and either single or repetitive mTBI to influence perforant pathway structure either acutely or chronically post-injury. The lack of a robust effect of controlled cortical impact mTBI on integrity of the lateral perforant pathway neurons, axons and synapses is not due to failure of the single and double injuries to reach the hippocampus and affect its structure. As described in section 3 earlier, there are clear abnormalities in the structure of the hippocampal formation and dentate gyrus at 4 months post-injury that are even more pronounced than at 7 days after mTBI, in the form of disrupted cytoarchitecture, compression and morphological distortion of the dentate gyrus, and in some cases circumscribed degeneration of hippocampal pyramidal neurons.

7. Mild TBI does not modify the expression level, subcellular distribution or synaptic spread of human tau or cause tau hyperphosphorylation either acutely or chronically post-injury.

With our AAV model for localizing human tau expression to the mouse lateral perforant pathway, we were positioned to determine whether mTBI exacerbates tau pathology chronically after injury. Using the HT7 antibody specific for human but not mouse tau, we demonstrated that neither single nor double mTBI change the expression level of human tau, or its cellular and subcellular distribution. After sham injury, single or double mTBI, human tau remained confined to the lateral perforant pathway neurons in entorhinal layer II and the lateral perforant pathway afferents to hippocampal dentate gyrus (e.g., Figure 9). At both 7 days and 4 months after single or repetitive mTBI, expression of total human tau remains restricted to the lateral perforant pathway neurons of origin in layer II of the LEC, the perforant pathway axons in the distal hippocampal CA1 sector, the SLM, and to their synaptic field in the dentate gyrus OML. Furthermore, most hTau-expressing mice had no detectable human tau in granule neurons at any rostro-caudal level, although in a few cases a tiny handful of granule neurons were hTau immunopositive. Quantitative analysis of human tau spread into the dentate granule neuron targets for the lateral perforant pathway confirms that the number of dentate granule neurons expressing human tau averaged fewer than two per brain section, and did not differ between sham-injury, single, or double mTBI.

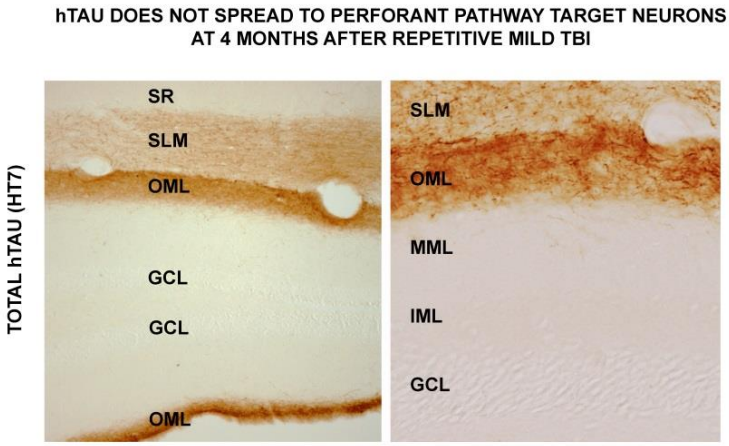


Figure 9. In the chronic post-mTBI time period, human tau expression remains confined to the lateral perforant pathway axons (SLM) and synaptic field (OML), and does not spread to the dentate granule target neurons (GCL) or any other hippocampal cell type. This is true for both blades of the dentate gyrus (left), and for all 3 injury groups.

An early step in tau hyperphosphorylation and aggregation is phosphorylation on serine residues 202 and 205, followed by phosphorylation of residues Thr231, and Ser262, measurable by reactivity with a panel of tau phospho-specific antibodies. In addition to the lack of synaptic spread of hTau after mTBI, there is no appreciable change in multiple tau phosphoforms post-injury, including pTau202/205, pTau231, and pTau262. In sham-injured controls these tau phosphoforms are restricted to the lateral perforant pathway neurons in layer II of the lateral EC, and their expression level and distribution do not change appreciably after single or repetitive mTBI at either post-injury time point (e.g., Figure 10). Furthermore, endogenous mouse tau does not undergo hyperphosphorylation chronically after single or double mTBI, as evidenced by the absence of pTau202/205, pTau231, and pTau262 in either entorhinal cortex or dentate gyrus at 4 months post-injury in mice expressing eGFP in the lateral perforant pathway. Taken together, these findings indicate that mTBI does not induce any discernable spread of pathological human tau from the perforant pathway or promote tau hyperphosphorylation in the controlled cortical impact mouse model either acutely or chronically after single or repetitive injuries.

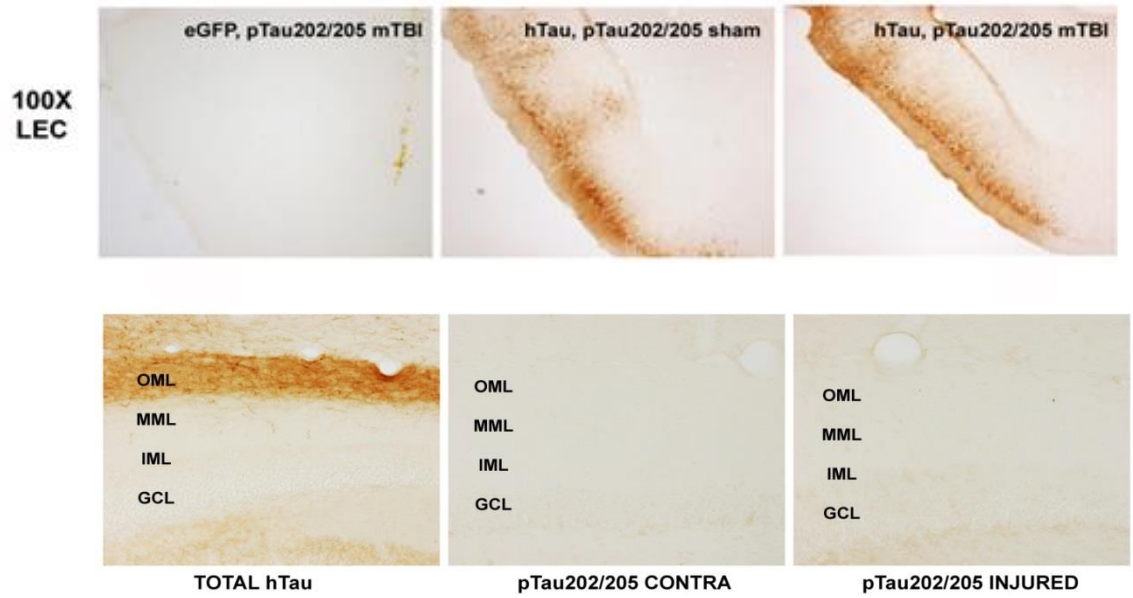


Figure 10. Single and double mTBI do not cause trans-synaptic spread or altered expression levels of total or phospho-tau via the perforant pathway or their redistribution within the pathway at 7 days post-injury.
Top panels – Entorhinal cortex: pTau202/205 is not induced in eGFP- or hTau-expressing mice by mTBI.
Bottom panels – Dentate gyrus: pTau202/205 remains excluded from lateral perforant pathway axons and synapses after mTBI.
Bottom left: total human tau is present in the lateral perforant pathway terminal field in the dentate outer molecular layer (OML), but not in perforant pathway target neurons, the dentate granule cells (GCL).

8. Evidence for slowly progressive tauopathy and tau-mediated perforant pathway neuronal degeneration in our mouse model, irrespective of mild traumatic brain injury.

8A. Localization of tau phosphoforms in perforant pathway neurons after 5 weeks of hTauP301L expression.

Using immunohistochemistry with antibodies specific for several phosphorylated forms of tau, including pTau202/205, pTau231, pTau212/214/217, and pTau262, we found that, at 5 weeks after AAV-hTauP301L delivery, phospho-tau was restricted to the neurons of origin for the lateral perforant pathway in layer II of the LEC. Each tau phosphoform showed diffuse and uniform distribution in the cytoplasm of neuronal perikarya (Figure 11, top panels). These phospho-tau positive neurons have the morphology of healthy pyramidal neurons. Phospho-tau localizations were not affected by either single or double mTBI at one week post-injury.

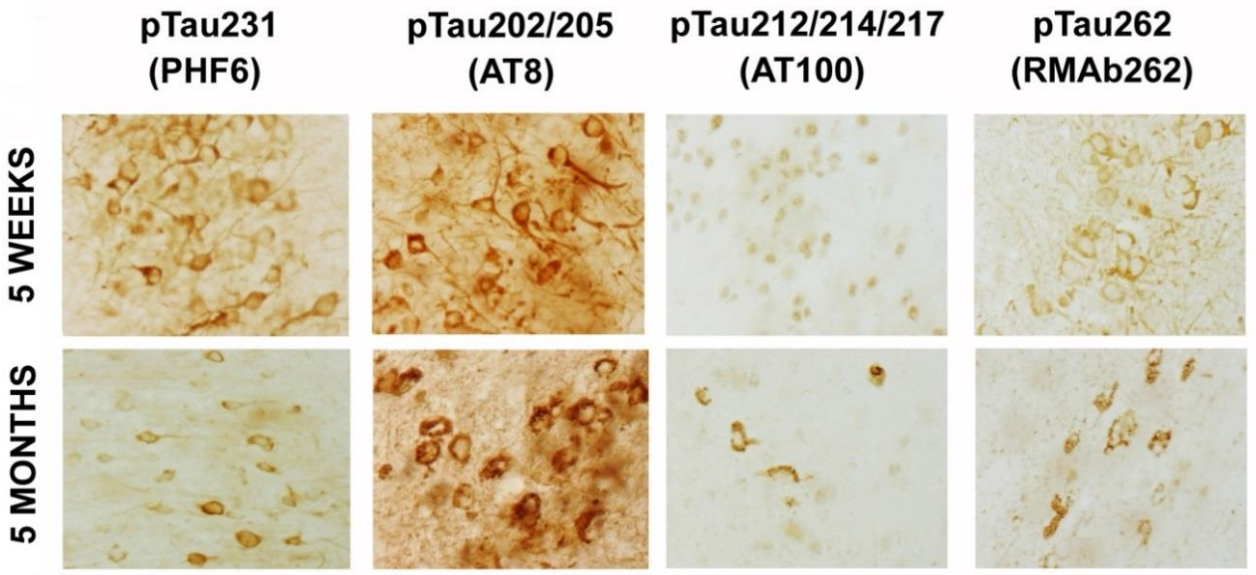


Figure 11. Evidence for slowly progressive focal accumulation of specific tau phosphoforms in scattered neurons of the lateral perforant pathway at 5 weeks and 5 months after hTauP301L gene delivery.
(LEFT 4 PANELS) – Acutely post-mTBI, both pTau231 and pTau202/205 are diffusely distributed throughout the cytoplasm of layer II entorhinal neurons. Distribution of the former is unchanged at 5 months, whereas some of the pTau202/205 accumulates within cytoplasmic puncta.
(RIGHT 4 PANELS) – There is little appreciable pTau212/214/217 whereas pTau262 shows diffuse cytoplasmic distribution at 5 weeks after gene delivery By 5 months, however, these two tau phosphoforms accumulate into cytoplasmic inclusions, often associated with vacuoles.

8B. Tau phosphoforms become mislocalized after 5 months of hTauP301L expression and are associated with degenerating perforant pathway neurons.

At 5 months after AAV-hTauP301L gene delivery, a subset of perforant pathway neurons with phospho-tau expression show profound morphological changes compared to the 5 week time point (Figure 11, bottom panels and Figure 12). Whereas pTau202/205 and pTau231 remain relatively uniformly distributed within most neurons with healthy morphology, sparse neurons exhibit intense pTau202/205 staining with abnormal round perikarya (Figure 12, left panel, arrow). The neurons with strong accumulation of pTau202/205 also contain this phospho-tau form in dendrites with beaded varicosities typical of ongoing dendritic degeneration (second from left, arrowheads). Phospho-tau accumulation within degenerating perforant pathway neurons is even more pronounced for the pTau262 phosphoform (Figure 12, right two panels) and for pTau212/214/217 (Figure 11). For example, intense pTau262 is found in neurons with abnormally shaped cell bodies (far right) and beaded dendritic varicosities (arrowheads). The long-term accumulation of this subset of tau phosphoforms in scattered, actively degenerating lateral perforant pathway neuronal perikarya and dendrites occurs independently of mTBI. As shown in Figure 12, at 5 months after gene delivery of pathological human tau, the p202/205 and p262 tau phosphoforms become mislocalized in perforant pathway neurons in layer II of the lateral entorhinal cortex even in sham-injured mice. Taken together, these data suggest that expression of a low dose of pathological human tau in the mouse lateral perforant pathway, below the threshold for eliciting rapid degeneration of the pathway, instead evokes a slowly progressive tau-mediated neurodegeneration. This proteotoxicity occurs independently of mTBI, and is preferentially associated with tau phosphorylation on residues 212, 214, 217, and 262.

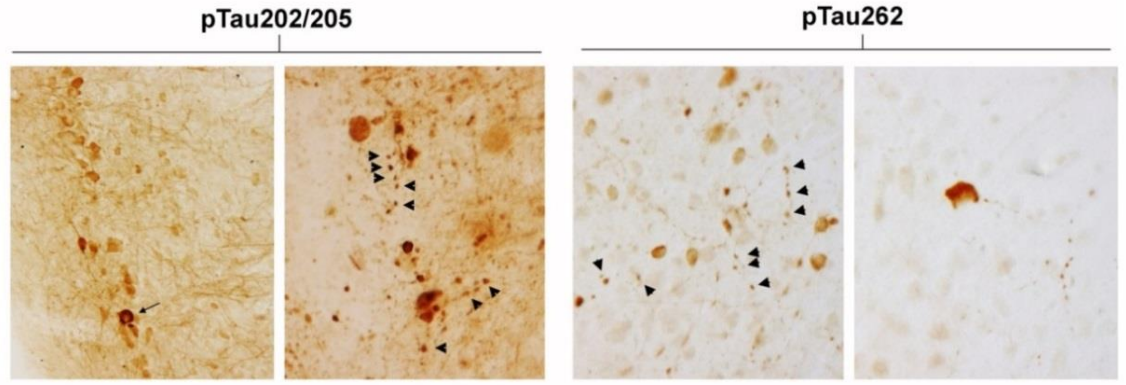


Figure 12. Tau phosphoforms p202/205 and p262accumulate in scattered perforant pathway neurons with degenerating processes after 5 months of pathological human tau expression in sham-injured mice.

A subset of lateral perforant pathway neurons express relatively high amounts of pTau202/205 (left panel, arrow) and pTau262 (far right panel) at 5 months after hTauP301L gene delivery. The neurons accumulating these two phospho-tau forms have rounded cell bodies and beaded dendrites (arrowheads), morphologies indicating active neurodegeneration. The onset of phospho-tau triggered degeneration is independent of mTBI, as it also occurs in sham-injured controls.

8C. Certain phospho-tau forms accumulate chronically in granulovacuolar degeneration bodies.

Another pathological feature of lateral perforant pathway neurons observed after 5 months but not 5 weeks of hTauP301L expression is a strong concentration of specific tau phosphoforms within cytoplasmic granules. The granules resemble pathological inclusions that have been termed granulovacuolar degeneration (GVD) bodies, which are known to accumulate intraneuronally in afflicted brain regions in Alzheimer's and other chronic neurodegenerative

diseases (reviewed by Kohler, 2016). After 5 months but not 5 weeks of hTauP301L expression, some pTau212/214/217 and pTau202/205 becomes concentrated in cytoplasmic inclusions resembling GVD bodies, and no longer shows uniform diffuse cytoplasmic distribution (Figure 13). Most strikingly, pTau262 is found in these scattered neurons exclusively in presumptive GVD bodies after 5 months of human tauP301L expression. This is the time when some perforant pathway neurons develop abnormal neurodegenerative morphologies (Figure 12), suggesting the pTau granule formation and neurotoxicity may be related processes. The histological similarity of focal granules of pTau262 to the GVD bodies in neurons of the Alzheimer's brain is illustrated by immunolabeling of GVDs in the bottom right panel for the marker casein kinase I (Kohler, 2016). The evidence linking the specific tau phosphoform pTau262 to GVD bodies and slowly progressive neurodegeneration has implications for understanding the molecular and cellular processes by which tau triggers neurotoxicity and drives regional brain atrophy in Alzheimer's disease.

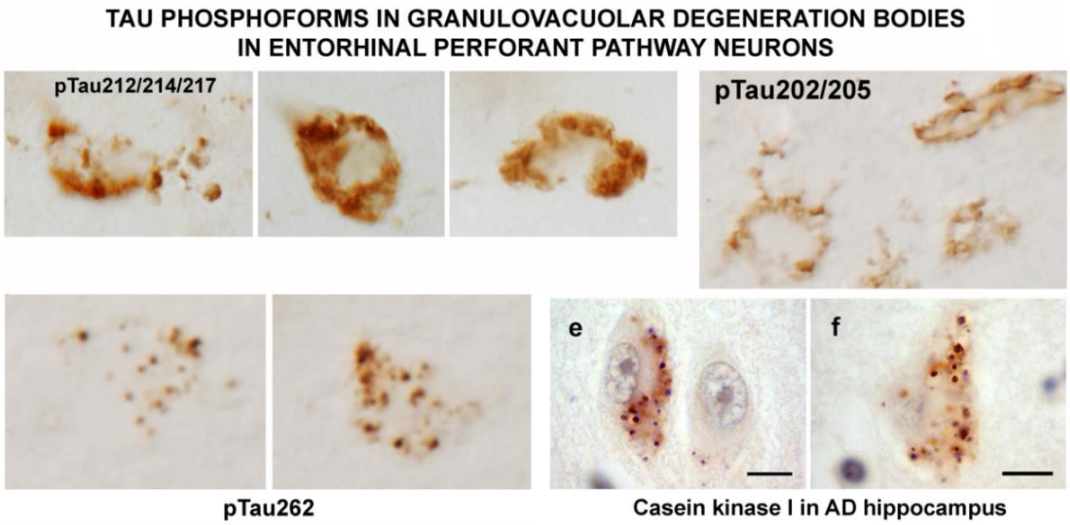


Figure 13. High power light microscopic assessment of cytoplasmic phospho-tau granules in mouse perforant pathway neurons developing 5 months after expression of hTauP301L.
Top left – pTau212/214/217 accumulates in cytoplasmic puncta surrounding the unstained nucleus.
Top right – pTau202/205. Like pTau212/214/217, this tau phosphoform remains diffuse in cytoplasm but mostly concentrates within granules.
Bottom left – Unlike other tau phosphoforms, pTau262 is localized exclusively to cytoplasmic inclusions with the appearance of GVD bodies.
Bottom right – From Kohler (2016). GVD bodies in neurons of the human AD brain immunostained for the marker casein kinase I highlights their similarity to the mouse brain granules with pTau262.

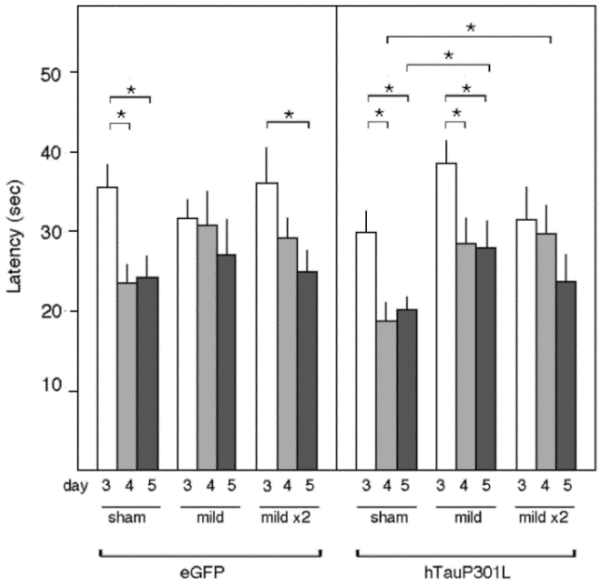
9. <u>mTBI impairs hippocampus-dependent spatial learning: no robust acute or chronic effect of pathological human tau.</u>

In addition to the array of histopathological methods for evaluating pathological tau and mTBI-induced long-term changes in hippocampal structure following TBI, we analyzed hippocampal function at both 3-5 days and 5 months post-injury. We used a Morris Water Maze to assess spatial learning over 3 consecutive days after single or double mTBI or sham injury (at 1 or 5 months after AAV-mediated gene delivery). Our cognitive test data indicate that both

single and double mTBI cause subtle impairments in spatial learning during the acute and chronic post-injury time periods, and unilateral human tau expression in the perforant pathway does not appreciably exacerbate the learning deficits when compared with eGFP expression as a control foreign protein. Unilateral human tauP301L by itself, in the absence of mTBI, shows a trend of impairing spatial learning after 5 months of transgene expression, but this effect does not reach statistical significance.

Figures 14 and 15 illustrate the effects of mTBI on swim latency of mice to find the hidden platform using visual cues as a measure of spatial learning. Figure 14 shows that sham-injured mice expressing either eGFP or hTauP301L learn to locate the hidden platform, based on statistically significant decreases in latency on days 4 and 5 (the second and third days of training) after sham surgery compared with the initial training on day 3. The two genotypes improve equally well. In contrast, eGFP expressing mice subjected to either single or double mTBI exhibited no significant change in swim latency on days 4 compared with day 3, indicating an impaired spatial learning ability. The hTau expressing mice also exhibited injury-induced learning impairment. After single mTBI, they showed longer latencies on days 4 and 5 than on day 3, and the difference at day 5 was statistically significant. Furthermore, after double mTBI, hTau mice showed no improvement in swim latency on days 4 or 5 compared with day 3. Most importantly, there were no significant differences between eGFP- and hTau-expressing mice across any treatment group on any day of training, indicating that unilateral expression of hTauP301L in the lateral perforant pathway does not worsen the mTBI-induced spatial learning deficit acutely post-injury, or cause an acute learning deficit on its own in the absence of TBI.

As shown in Figure 15, mice expressing either eGFP or hTauP301L and tested 4 months after sham surgery also learn with repeated training to locate the hidden platform, based on statistically significant decreases in latency on the third day of training compared with the initial training day. There is a non-significant trend for greater improvement with training in the eGFP sham-injured group than the hTauP301L mice. Chronically after single or double mTBI, eGFP expressing mice subjected to single mTBI exhibit longer latencies than sham-injured mice, a trend toward impaired spatial learning ability that did not reach statistical significance ($p<.06$). The sham-injured hTau expressing mice also exhibit spatial learning based on a significant decrease in swim latency from the first to the third day of training. Whereas no significant improvement with training was exhibited by the hTau single mTBI group, after double mTBI the hTau group showed significant improvement in swim latency on training day 3. Finally, there were no significant differences between eGFP- and hTau-expressing mice after either single or double mTBI on any day of training, indicating that unilateral expression of hTauP301L in the lateral perforant pathway does not appreciably worsen the mTBI-induced spatial learning deficit chronically post-injury.



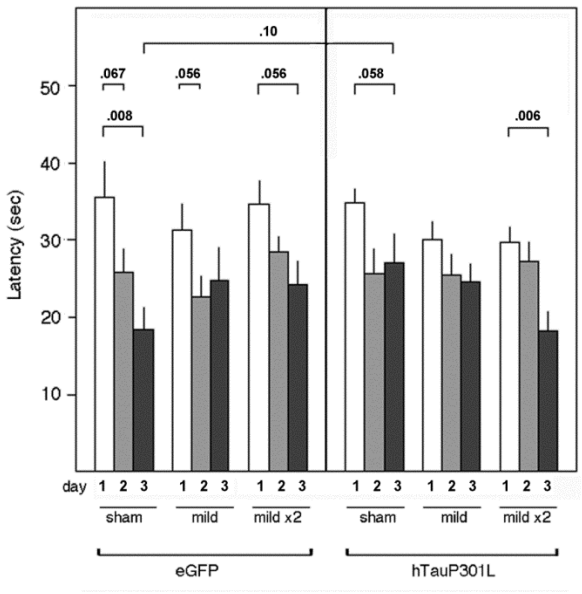


Figure 14 (LEFT). mTBI causes acute subtle impairment in spatial learning on the Morris Water Maze, an effect that is not exacerbated by perforant pathway hTauP301L expression. The mean swim time latency (in seconds+s.e.m.) is shown on three consecutive days of training (6 trials/day) at the indicated days after mice received either sham injury, mTBI, or double mTBI. Both single and double mTBI caused significant learning deficits for the eGFP-expressing mice on day 4, and hTau-expressing mice on day 5. It is noteworthy that there were no significant differences between groups expressing hTauP301L or eGFP in the lateral perforant pathway on any day of training or with any type of injury. *p<.05, unpaired t-test).

Figure 15 (RIGHT). mTBI subtly impairs spatial learning chronically on the Morris Water Maze, an effect of injury that is not exacerbated by perforant pathway hTauP301L expression. The mean swim time latency (in seconds+s.e.m.) is shown on consecutive training days starting 4 months after mice received sham injury, mTBI, or double mTBI. Both single and double mTBI caused learning deficits for the eGFP-expressing mice, and double injured hTau-expressing mice. It is noteworthy that there were no significant differences between groups expressing hTauP301L or eGFP in the lateral perforant pathway on any day of training or with any type of injury, although long-term expression of hTauP301L caused a non-significant learning impairment in the absence of mTBI. Shown along the top are P values (unpaired t-test).

From the collective data for both the acute and chronic post-injury phases, we conclude that (i) mice exhibit spatial learning with repeated training on consecutive days; (ii) both single and double mTBI cause subtle but discernable impairments in spatial learning both acutely and chronically post-injury; and (iii) unilateral expression of hTauP301L in the lateral perforant pathway does not worsen the deleterious effects of single or repetitive mTBI on spatial learning. Finally, our data suggest that long-term expression of pathological human tau in the lateral perforant pathway may subtly impair spatial learning in the absence of any brain injury, although this trend did not reach statistical significance. The collective behavioral data from the past 3 years indicate that pathological human tau has no worsening effect in the acute or chornic post-injury periods on hippocampus-dependent spatial learning, a conclusion fully consistent with the lack of overt effect of pathological human tau on perforant pathway structural integrity after single or double mTBI.

10. Blood-based biomarkers for mild TBI-induced brain damage: Serum SNTF at 7 days and 4 months post-injury.

We measured the serum level of a protein biomarker for TBI-induced axonal damage discovered and characterized previously by the Siman laboratory, the calpain-derived nonerythroid α-spectrin N-terminal fragment SNTF (αII-spectrin 1-1176). SNTF levels rise in the blood on the day of human concussion treated in the emergency room, and are elevated preferentially in those cases exhibiting white matter abnormalities detected by advanced neuroimaging, as well as persisting cognitive performance problems. In professional ice hockey players, serum SNTF increases after an in-game concussion preferentially in players that go on to develop persisting post-concussion symptoms delaying their return to play. Histopathological studies demonstrate that this calpain derivative normally is below the limit of detection in the brain, but accumulates within damaged axons after TBI in humans and mTBI in a large animal experimental model of concussion (Johnson et al., Acta Neuropathol. *131*, 115 [2016]). On these and other bases, SNTF has emerged as the lead blood biomarker for the prognosis of concussion, and its elevation is a biologically plausible surrogate marker for the diffuse axonal injury thought to underlie persisting brain performance deficits.

The current project determined that serum SNTF does not change appreciably at 7 days post-injury in the mouse. Compared with sham injured controls (mean SNTF = 61U +/- 17), neither single mTBI (SNTF = 62U +/- 15) nor double mTBI (SNTF = 59U +/- 19) altered serum SNTF. These data are consistent with our longitudinal study of serum SNTF in concussed ice hockey players, in which SNTF was elevated from 1-36 hours post-injury, and then declined to pre-season baseline thereafter (Siman et al., J. Neurotrauma *32*, 1294 [2015]). It is also consistent with our study of blood SNTF in concussion cases treated in the emergency room, in which plasma SNTF was elevated on the day of concussion in the subset of participants exhibiting persisting cognitive performance problems, but not at 4 days post-injury (Siman et al., Front. Neurol. *18*, doi: 10.3389/fneur.2013.00190 [2013]). However, the results from the mouse study described herein are confounded by our finding that occasional cases exhibit high serum SNTF levels 3 to 5 times above most others, and this occurs in sham-injured as well as the mTBI groups and in both eGFP hTauP301L expressers. This led to concern that the surgical procedure, involving separate craniectomies for intracranial gene delivery for the controlled cortical impact injury, might sometimes damage the cortical surface beneath, and in so doing induce an artefactual serum increase in SNTF. To test this concern directly, we also evaluated serum SNTF in mice 24 hours after receiving a single craniectomy for the TBI procedure, but no second surgery for gene modification. As shown in Figure 16, single TBI doubled serum SNTF at 24 hours post-injury compared to sham controls, confirming the protein as a blood biomarker of neuronal injury in the acute post-injury period in the mouse as well as in humans.

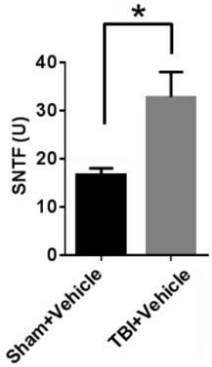


Figure 16. Serum SNTF in the wild type mouse 24 hours after sham-injury or controlled cortical impact TBI. N=6/group. SNTF rose 2-fold acutely post-injury, and the increase was statistically significant (unpaired t-test).

Similar findings were made with sera sampled at 4 months post-injury. The majority of cases had relatively low serum SNTF levels, no matter whether they were from sham-, single-, or double-injured mice, and irrespective of whether they expressed hTauP301L or eGFP as a control foreign protein. At this chronic post-injury time point, neither the mean nor the median serum SNTF levels were increased by either mTBI or pathological human tau expression, nor was the frequency of cases exhibiting much higher serum SNTF levels.

11. <u>Summary of major findings:</u>

Milestones I and II: (i) determine whether tau worsens hippocampal structure and function in the acute and long-term post-TBI time periods; (ii) determine whether mTBI worsens incipient tauopathy acutely and chronically after injury; (iii) determine whether blood levels of neurodegeneration biomarkers measured at 7 days and 4 months post-injury correlate with the severity of brain damage after mTBI.

Taken together, our results indicate that pathological human tau expressed in a major hippocampal input does not appreciably worsen TBI-induced changes in the structure of the pathway, or in pathway-dependent cognitive function in either the acute or chronic post-injury periods. This holds true for both single and double mild TBI. Our data also show that neither single nor double mTBI promote tauopathy, as they neither worsen tau hyperphosphorylation or promote its aggregation and anatomical spread at either 7 days or 4 months post-injury.

How do these conclusions, that no discernable interaction occurs in the mouse brain between pathological human tau and single or double mTBI in the acute and long-term post-injury periods, relate to current literature on the role of tau pathology in human TBI? They are largely consistent with recent reports. Although mouse lines engineered for deletion of brain tau expression reportedly show resistance to TBI-induced brain dysfunction, these approaches cannot distinguish effects of the tau deletion on altered sensitivity to the TBI itself from a pathomechanistic role after the injury. The histopathological study of post-mortem human brains obtained from a few days to one month after TBI reported no change in neuronal expression or phosphorylation of tau in the sub-acute post-injury time frame, quite similar to our observations in our mouse-based project. Whereas retrospective epidemiological studies initially suggested that TBI could increase the risk of developing Alzheimer's disease later in life, recent studies with improved designs have provided evidence that brain dysfunction developing long after mild TBI affects cognitive domains distinct from those especially impacted in Alzheimer's disease. Moreover, studies of elderly war veterans have failed to confirm an increased risk of Alzheimer's disease in the long-term period after mild TBI (Seal et al., 2016; Weiner et al., 2017). Coupling our findings reported herein using a well-controlled mouse experimental model with recent human mild TBI studies, there is no compelling evidence that tau pathology represents a key pathogenic event after mild TBI, and little support that tau pathology is a viable therapeutic target for mitigating the long-term effects of mild TBI.

Impact

Mild traumatic brain injury (mTBI) is the most common neurological injury in civilians, and affects over 1.5 million children and adults each year in the United States. Although mTBI is typically undetectable with computed tomography, it can elicit long-term and clinically significant brain dysfunction in ~25% of cases. At the present time, there are neither methods that can identify at an early and treatable stage the subset of mTBI sufferers who will go on to develop acute brain damage and long-term disability, nor clinically proven treatments for improving brain functional outcome. Consequently, new approaches are urgently needed for rapidly identifying mTBI patients in the acute post-injury period who are at risk of suffering persistent brain dysfunction, and for treating these at-risk cases to preserve brain structure and function. Furthermore, accumulating evidence suggests that both single and repetitive TBIs can lead in later life to a chronic, progressive Alzheimer's disease (AD)-like neurodegenerative disorder. New methods are needed to identify those individuals that are beginning to develop TBI-triggered chronic neurodegenerative disease, and new treatments urgently need to be developed for slowing their chronically progressive brain atrophy and cognitive decline.

In the long-term post-injury time period, mTBI shares neuropathological features with AD. Moreover, given that AD is a slowly progressive neurodegenerative and cognitive disorder, and TBI will sometimes lead to chronic progressive brain atrophy and cognitive decline, the question arises whether AD and TBI may share common underlying pathophysiology. One of the pathological hallmarks of AD is the aggregation of the protein tau into neurofibrillary tangles within vulnerable neurons in brain regions important for higher cognitive function. Considerable evidence implicates tau pathology as a key pathogenic driving force for the progressive brain atrophy and inexorable cognitive decline. On the other hand, whereas mTBI will also sometimes cause tau abnormalities that superficially resemble the tauopathy of AD, the pathophysiological roles for tau in the acute and chronic periods after TBI are unknown. Here, we examined directly and critically whether tauopathy plays important roles in the acute and chronic outcomes from single and repetitive mTBI. Our study determined in a well-controlled pre-clinical experimental model the interrelationships between TBI and subsequent AD, thereby fostering discovery of new therapeutic strategies for military personnel, veterans, and civilians exposed to single or repetitive mTBI. *Based on the histopathological and neurocognitive responses to single and double mTBI, our data indicate that the presence of a pathological form of human tau in the mouse perforant pathway does not render the neurons, axons, or synapses of this projection more vulnerable to single or double mTBI in either the acute or chronic post-injury periods. In addition, neither single nor double mTBI exacerbate ongoing tauopathy either acutely or chronically after mTBI. Our data do not support aspects of tauopathy as being viable therapeutic targets for mitigating the acute or chronic effects of mild TBI.*

Finally, our study has been evaluating pre-clinically new diagnostic and prognostic blood tests for identifying at early and treatable stages the subsets of mTBI cases at risk of developing brain damage and long-term dysfunction. Simple blood tests for TBI induced brain damage are vitally needed, and would have major applications for both military and civilian sufferers of mTBI. *We report here that levels of the neurodegeneration biomarker SNTF are not appreciably elevated at either 7 days or 4 months post-TBI, although this study component is confounded by the sporadic effects of the two surgical craniectomy procedures on the blood levels of this biomarker for neuronal degeneration.*

Changes/Problems

No significant changes in study design were introduced during the conduct of the project. The data analysis for blood biomarker changes at 7 days and 4 months post-injury was complicated by the sporadic effect of the two intracranial neurosurgical procedures on basal serum SNTF levels even in the absence of any mTBI.

Products

Nothing to report. Two manuscripts are in preparation detailing (i) the lack of interaction between tauopathy and mild TBI in the acute and chronic post-injury periods, and (ii) the development of slowly progressive neurodegeneration potentially driven by specific tau phosphoforms and cell biological processes in a neural circuit that is especially vulnerable at an early stage in Alzheimer's disease.

Participants and other support

1. Dr. Robert Siman
   Role –Principal Investigator
   Effort – 2.4 person months/year 3
   Contribution – Dr. Siman directed every aspect of the project. He formulated the experimental strategies. He trained personnel on the requisite methods of stereotaxic neurosurgical viral vector-based gene delivery, animal husbandry, histology, immunohistochemistry, microscopy, quantitative morphometry, and serum preparation. He assisted with histological assessments of perforant pathway neuronal, axonal, and synaptic integrity after traumatic brain injury. He performed photomicroscopic documentation of the research findings thus far, and prepared all quarterly and annual reports. He validated the immunoassays for neurodegeneration biomarkers using new equipment purchased through this award.

2. Dr. Victoria Johnson
   Role – Co-Investigator
   Effort – 1.2 person months/year 3
   Contribution – Dr. Johnson performed the controlled cortical impact traumatic brain injuries and sham injuries, and introduced methodological improvements to enhance the precision and consistency with which the injury device elicits mild TBI. She ensured personnel were trained thoroughly on the evaluation of spatial learning using the Morris Water Maze task. She assisted with histological study of mTBI-induced axonal pathology.

3. Ms. Hongmei Cui
   Role – Research Specialist, Siman laboratory
   Effort – 4.8 person months/years 1 and 2; reduced to 3 person months/year 3
   Contribution – Ms. Cui performed all of the neurosurgical gene delivery, animal husbandry, and histology, and most of the immunohistochemistry evaluating short-term responses to mild TBI. Upon completion of milestone 1, she performed all of the neurosurgical, animal husbandry, and histological methods conducted thus far on the long-term (4 month) outcomes from single and double mTBI. In the coming year, Ms. Cui will take on additional responsibility for

conducting immunoassays to measure serum changes in neurodegeneration biomarkers in the long-term post-injury period. She will perform fewer of the histopathological qualitative and quantitative analyses, as some of these responsibilities will be assumed by Mr. Feintech, a recent addition to the Siman laboratory.

4. Mr. Samuel Feintech
   Role – Research Specialist, Siman laboratory
   Effort – 6 person months/year 3
   In year 3, Mr. Feintech conducted the qualitative and quantitative histopathological evaluations of tau expression and pathology, neuronal and synapse integrity, and axonal vulnerability of the genetically modified perforant pathway at 4 months after single or repetitive mTBI. In April, 2017, he received training in the Morris Water Maze task of spatial learning, and completed the neurobehavioral evaluations of the final batches of mice in the long-term post-injury period.

Change in other support during the conduct of this project

"Investigating the neurologic effects of training associated blast"
DARPA award NEU-92-2913
Joshua Duckworth, Principal Investigator
Robert Siman, Co-Investigator (1.8 calendar months effort)
Period: 7/1/16 – 3/31/19
Administration: Henry M. Jackson Foundation
Subaward Specialist: Alison Dineen, adineen@hjf.org
Annual direct cost (Siman sub-contract):
Overlap: None. This award funds a human research study into the effects of heavy weapons training in the military on brain injury and functional status. Dr. Siman is assessing a set of blood biomarkers for neurodegeneration as potential surrogate markers for training-induced brain injury. Longitudinal serum and plasma levels of SNTF and hypophosphorylated Neurofilament H are being compared with neuroradiological and neurobehavioral evaluations of training-induced brain structural and functional changes, and with cumulative blast exposures. Now on a six month no cost extension.

"Diagnosis and mechanisms of mild traumatic brain injury"
NIH R01NS092398-01
Douglas Smith, Principal Investigator
Robert Siman, Co-Investigator (0.6 calendar months effort)
Period: 4/1/15 – 3/31/20
Administration: National Institute of Neurologic Disorders and Stroke
Annual Direct Cost (Siman laboratory):
Overlap: None. The project is focused on mechanisms and biomarkers for diffuse axonal injury in a large animal model of concussion.

"Diffuse and focal brain injury in a large animal model of post-traumatic epilepsy: Mechanisms underlying epileptogenesis"
Source: Department of Defense award W81XWH-15-ERP-IDA
John A Wolf, Principal Investigator

Robert Siman, Co-Investigator (0.6 months calendar effort)
Period: 9/30/2016 – 9/29/2019
Administration: Department of Defense
Award Specialist:
Annual direct cost (Siman laboratory):
Overlap: None. The project studies the comparative contributions of diffuse and focal brain injury to the development of epileptogenesis in two large animal models of traumatic brain injury, and seeks discovery of a blood biomarker predictive of post-traumatic epilepsy.

Special Reporting Requirements

Not applicable.

Appendix

None to report.