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PRINCIPAL INVESTIGATOR: Dr. Robert Siman

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, PA 19104

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6. AUTHOR(S)

Robert Siman, PhD

E-Mail: siman@mail.med.upenn.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Department of Neurosurgery
Perelman School of Medicine
University of Pennsylvania
502 Stemmler Hall
36th and Hamilton Walk
Philadelphia, PA 19104

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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 are critically evaluating three hypotheses: (i) tau exacerbates the neuronal damage and cognitive dysfunction after single and repetitive TBI in the acute and chronic post-injury periods; (ii) 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 damage and dysfunction valuable for the diagnosis, prognosis, and theragnosis of TBI-triggered brain damage and chronic neurodegenerative disease.

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Introduction and Overall Objectives

After traumatic brain injury (TBI), the human brain sometimes develops tau pathology partly resembling the tauopathy that is a well-established 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 patients developing chronic neurodegenerative disease and progressive cognitive impairment.

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 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 TBI, and whether 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 TBI-triggered chronic neurodegenerative disease.

<u>Objectives</u>

To study tau/TBI interactions and validate preclinically new biofluid-based diagnostic markers for chronic TBI-induced progressive brain atrophy and cognitive decline, we are collaborating 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 are combining 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 TBI on brain structure and function? (2) does TBI worsen an ongoing tauopathy to promote development of a chronic

neurodegenerative disorder? (3) do blood levels of biomarkers for neurodegeneration diagnose the subset of TBI cases developing acute brain damage, or chronic progressive neurodegeneration and behavioral dysfunction?

Our first two objectives address whether tau is an important target for therapeutic intervention in TBI. Our third objective is to discover and validate preclinically a blood test for improving the prognosis of mild TBI in the acute post-injury time period, as well as the diagnosis of TBI-induced chronic neurodegenerative disease in the long-term post-injury time period.

Keywords
Tauopathy; tau phosphorylation; traumatic brain injury; concussion; neurodegeneration; entorhinal cortex; perforant pathway; synapse loss; cognitive dysfunction; prognostic biomarker; surrogate marker for brain injury.

Accomplishments

Highlights

- Used a novel mouse model of early-stage Alzheimer-type tauopathy, with hippocampal input-specific expression of human tau P301L, to evaluate interactions between pathological human tau and controlled cortical impact mild TBI (all 3 objectives)
- Generated evidence that, compared with eGFP as a control foreign protein, pathological human tau does not appreciably change the vulnerability of neurons, axons, and synapses of this hippocampal input to mild TBI in the acute post-injury period (objective 1)
- Generated evidence that mild TBI does not appreciably change the expression level, distribution, or phosphorylation of human tau at 7 days post-injury (objective 2)
- Generated evidence that mild TBI impairs hippocampus-dependent spatial learning acutely post-injury, and pathological human tau does not exacerbate this cognitive dysfunction (objective 1)
- Developed a serum sample bank from mice that were subjected to either sham injury, mild TBI, or moderate TBI, and were characterized for cognitive and histopathological changes acutely post-injury, for pending analysis of candidate diagnostic and prognostic surrogate markers for TBI-induced brain damage and dysfunction (objective 3)

1. The 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 are microinjected by unilateral stereotaxic convection-enhanced delivery into the right lateral EC, and four weeks are 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 dentate gyrus outer molecular layer (OML). As shown schematically in Figure 1A, in the mouse all of the synaptic inputs terminating in the OML originate from the lateral EC and lateral perforant pathway projection.

As shown in Figure 1B and confirming our earlier publication (J Neuropathol Exp Neurol 72: 1062-1071 [2013]), delivery of AAV-hTauP301L to the right lateral EC leads within 3-4 weeks to robust human tau expression in the lateral peforant pathway layer II neurons of origin and the entire lateral perforant pathway projection as it traverses the stratum lacunosum-moleculare (SLM) of hippocampal CA1 sector before perforating the hippocampal fissure (HF) to terminate in the dentate gyrus OML (panels A,C,D). The human tau is distinguished from the widely distributed endogenous mouse protein by immunohistochemistry using the human specific

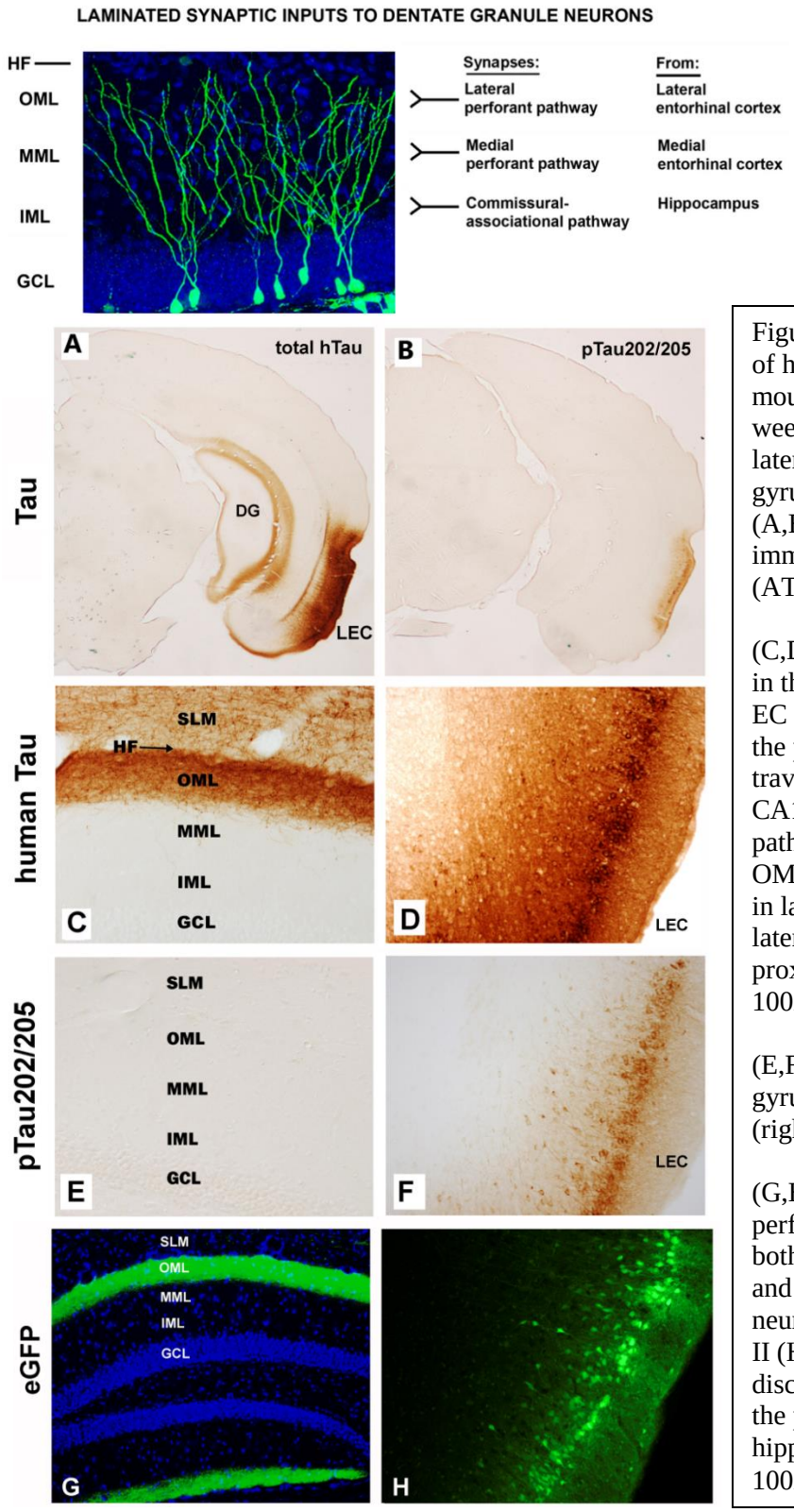

Figure 1A abbreviations used:
HF- hippocampal fissure
OML – outer molecular layer
MML – middle molecular layer
IML – inner molecular layer
GCL – granule cell layer
A subset of granule neurons and their dendrites are shown in green, nuclei are in blue.

Figure 1B. AAV-driven expression of human TauP301L or eGFP in the mouse lateral perforant pathway 4 weeks after gene delivery to the right lateral EC (LEC). DG – dentate gyrus.
(A,B) Total human tau (A; HT7 immunostaining) and pTau202/205 (AT8 immunostaining). 10X mag.

(C,D) Higher power views of hTau in the dentate gyrus (C) and lateral EC (D). Note that hTau is present in the perforant pathway axons traversing the SLM of hippocampal CA1 sector and the lateral perforant pathway synaptic zone in the dentate OML. In EC, hTau is concentrated in layer II neurons of origin for the lateral perforant pathway and their proximal dendrites. 200X (right), 100X (left) mag.

(E,F) pTau202/205 in the dentate gyrus (E) and lateral EC (F). 200X (right), 100X (left) mag.

(G,H) eGFP expression in the perforant pathway synaptic zone in both blades of the dentate gyrus (G) and in the lateral perforant pathway neurons of origin in lateral EC layer II (H). Lighter but readily discernable eGFP is also present in the perforant pathway axons in the hippocampal SLM (not shown). 100X mag.

monoclonal HT7. In contrast, tau phosphorylated on serine residues 202 and 205, considered an early marker for hyperphosphorylation and aggregation and labeled with the monoclonal AT8, is

confined to layer II neurons of the lateral EC, but does not undergo appreciable axonal transport and is below the limit of detection in the perforant pathway axons or synapses (panels B,E,F). This distribution pattern of phosphorylated human tau closely resembles the earliest neuropathological stage of Alzheimer-type tauopathy (Braak stage I) with hyperphosphorylated, aggregated tau localized to the superficial trans-entorhinal region. An identical distribution pattern is observed for tau phosphorylated on either Thr231 or Ser262 (data not shown). After delivery of AAV-eGFP as a control (bottom panels), the autofluorescent eGFP protein distributes the same as pathological human tau (hTau) throughout the lateral perforant pathway neurons of origin, axons, and pre-synaptic terminals innervating the hippocampal dentate gyrus and terminating in the OML.

2. Controlled cortical impact TBI in mice with lateral perforant pathway expression of either pathological hTau or eGFP

At 4 weeks after gene delivery, mice receive a craniectomy over the right parietal cortex, and either no injury (sham injury), mild TBI (mTBI), moderate TBI (modTBI), or double mTBI. The first part of our two-part study is focused on the acute responses to TBI. Mice are evaluated for hippocampus-dependent spatial learning on day 3, 4 and 5 post-injury, and for histopathology and serum biomarkers of neuronal damage on day 7 post-injury. The TBI is produced by a controlled cortical impact device, in which the velocity of the impounder and depth of its penetration into the neocortex together determine the initial severity of TBI. As shown in Figure 2, the magnitude and scope of histological changes in the hippocampus depend on the initial severity of the TBI. Compared with sham injured cases or the uninjured left hemisphere, single mTBI causes necrosis of the overlying parietal cortex, swelling of the underlying hippocampus at the CA1/ CA3 sector border (arrows), and dorso-ventral compression of the dentate gyrus. The latter is manifested by the decreased distance between the two blades of granule neurons, and shrinkage of the molecular layer. All of these changes are more pronounced at 7 days after modTBI, which additionally causes partial lesions of the CA3 sector (asterisks), along with tissue cracking and upward displacement of the lateral hippocampus. In the limited number of double mTBI cases analyzed so far, the pathology resembles that resulting from single mTBI.

Two changes were introduced during the conduct of the project, one to the experimental design and another to the injury protocol. First, the moderate injuries were deemed too damaging to hippocampal structure to be considered useful for evaluating whether pathological human tau expression in the lateral perforant pathway could exacerbate the short-term outcomes from the TBI. This concern is especially problematic for the second part of our study, examining the chronic responses to TBI, given the expectation that any acute hippocampal structural damage will worsen further chronically post-injury. Consequently, we made the strategic decision to no longer pursue modTBI and instead focus our analyses on the single and double mTBIs in comparison with the sham injuries. Second, the controlled cortical impact injury device was optimized for consistent delivery of mTBI eliciting mild structural changes to the underlying hippocampus at 7 days post-injury. The device was upgraded to incorporate a new digital and automatic Rod Speed Measurement Unit. The accessory device utilizes the voltage changes determined by the impactor motion to generate a waveform that permits highly accurate digital measurements of impactor velocity. This precise approach reduces variability between individual animal experiments, and improves the discriminative power of the experimental design going forward.

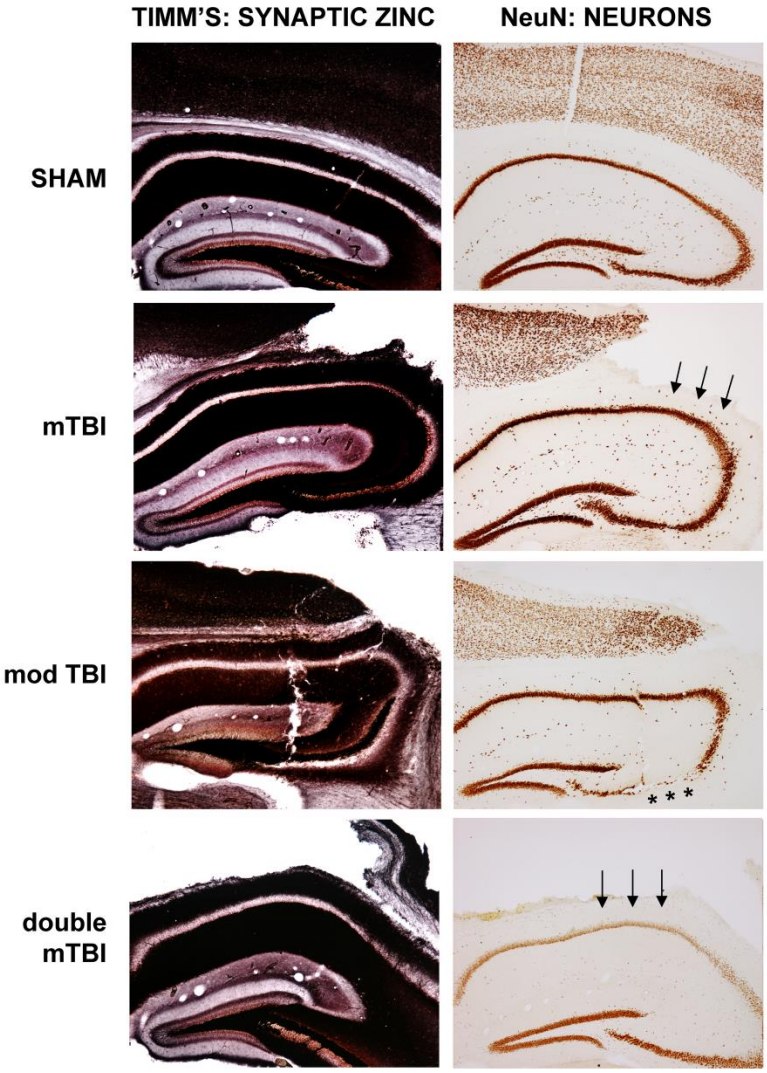

Figure 2. Controlled cortical impact TBI in the mouse: effects on hippocampus and dentate gyrus. Representative examples are shown of hippocampal changes at 7 days after sham injury, single mild TBI (mTBI), moderate TBI (modTBI), or double mTBI. LEFT – synaptic zinc staining. RIGHT – Neuronal nuclear staining for the marker NeuN. Note that all 3 TBIs cause damage to the parietal cortex overlying the rostral hippocampus. Single mTBI also distorts the normal cytoarchitecture of the hippocampus, inducing swelling in the CA3 sector (arrow) and compression of the two blades of the dentate gyrus. Mod TBI causes more extensive hippocampal structural damage, including vertical cracks and partial CA3 lesions (asterisks). In the limited number of cases evaluated so far, double mTBI causes minor hippocampal structural change similar to single mTBI in the CA1 sector (arrows) and dentate gyrus.

3. <u>Study questions, and numbers of mice evaluated so far</u>
   In its first year, our project has addressed three key questions:
   (1) What is the effect of pathological hTau on the acute response to mTBI?
   (2) Does mTBI exacerbate tau pathology or promote its anatomical spread acutely post-injury?
   (3) Is there a biomarker for neurodegeneration measurable in the blood that serves as a surrogate marker for the acute TBI-induced brain damage and dysfunction?

The original work plan proposed analyzing the following groups of mice:

1) AAV-eGFP, sham injured
2) AAV-hTau, sham injured
3) AAV-eGFP, mild TBI
4) AAV-hTau, mild TBI
5) AAV-eGFP, moderate TBI
6) AAV-hTau, moderate TBI
7) AAV-eGFP, repetitive mild TBI
8) AAV-hTau, repetitive mild TBI

Our work plan in the funded application was to perform 15 AAV microinjections per group, with the expectation that at least 70% of the injections would be successfully placed in the right lateral entorhinal cortex and drive robust foreign protein expression throughout the entire extent of the lateral perforant pathway. This design was expected to yield 10-12 mice per treatment group for all subsequent analyses, group numbers projected to be sufficient for drawing definitive conclusions on all the research questions under evaluation. Given the severity of the hippocampal structural changes occurring within a week after modTBI, and as described in the section above, we elected to no longer pursue groups 5 and 6, and instead focus our attention on the remaining groups 1-4 and 7-8 of sham injuries and mTBIs. Thus far, a total of 63 mice distributed among these treatment groups received AAV vector microinjections, then a month later were injured and evaluated within 7 days post-injury period for hippocampus-dependent learning and histopathological analyses of perforant pathway neuronal and synaptic integrity, hippocampal structure, human tau expression and distribution, and pTau202/205 expression and distribution. Serum samples were prepared from all of these mice for subsequent quantitative analysis of surrogate biomarkers for mTBI. An additional 9 genetically-modified and behaviorally-analyzed mice are currently undergoing histopathological evaluations, and a further group of 12 has already received their AAV injections and is scheduled for injuries and subsequent full behavioral and histological analyses starting the end of October. Our initial focus has been on single mTBI, in order to establish a method with the injury device and the CCI parameters eliciting consistent mild hippocampal damage at 7 days post-injury. Now that this has been accomplished, we have recently begun evaluating double injuries as well, and in order to derive sufficient numbers of these cases, are now delivering more double mTBIs than single or sham injuries. This effort will continue over the next six months as the project concludes part one, evaluating interactions between tau and TBI acutely post-injury.

For the 6 experimental groups that are the current focus, we have completed all behavioral and qualitative histological analyses for 32 mice that received well-place vector injections and exhibited robust transgene expression throughout the lateral perforant pathway. An additional 12 mice with well-placed AAV injections and robust transgene expression were subjected to modTBI, but as described in Section 2 above, the injuries produced acute hippocampal structural damage even in the eGFP control group too strong to allow reliable determination whether perforant pathway hTau expression worsened the short-term outcome. Similarly, the hippocampus-dependent spatial learning deficit produced by modTBI was too powerful in the control (eGFP) group to discern whether hTau exacerbated cognitive dysfunction acutely post-injury (see Section 9). Table/Figure 3 below shows the breakout of the completed cognitive and histological analyses among each experimental group receiving either sham injury or mTBI:

1) AAV-eGFP, sham injured (n=7)
2) AAV-hTau, sham injured (n=12)
3) AAV-eGFP, single mTBI (n=6)
4) AAV-hTau, single mTBI (n=5)
5) *AAV-eGFP, single mod TBI (n=6) - abandoned*
6) *AAV-hTau, single mod TBI (n=6) - abandoned*
7) AAV-eGFP, double mTBI (n=1)
8) AAV-hTau, double mTBI (n=1)

The following sections summarize our findings to date on the influence of pathological hTau on the short-term outcomes from mTBI, and the influence of mTBI in the short-term on the severity and distribution of tau abnormalities.

4. Acute effects of mTBI on lateral perforant pathway neurons and synapses in eGFP expressing mice

In mice expressing eGFP in the right lateral perforant pathway as a control foreign protein, single mTBI does not cause any appreciable damage to the lateral perforant pathway neurons of origin in layer II of the lateral EC, or to the lateral perforant pathway axons and synapses innervating the hippocampal dentate gyrus. Immunostaining for the neuronal nuclear marker NeuN is being used to evaluate any injury-induced neuronal loss in the hippocampus and lateral entorhinal cortex, while staining for pre-synaptic zinc (which is concentrated within synaptic vesicles) reveals the afferent lamination in the dentate gyrus and distinguishes the relatively zinc rich nerve terminals of the lateral perforant pathway in the OML from neighboring synapses containing lower amounts of zinc and arriving from other brain regions. As shown in Figure 4 (top panels), mTBI did not cause appreciable hippocampal neuronal loss at 7 days post-injury. In this example, as well as the case shown in Figure 2, the mTBI is sufficient to change hippocampal structure at 7 days post-injury, causing localized swelling at the CA1/CA3 border (arrows) and compression of the dentate gyrus, manifested in the top panels as a reduced separation between the two blades of dentate granule neurons. Compression of the dentate gyrus was also evident from staining for synaptic zinc, which highlights shrinkage of the molecular layer extending from the granule cell layer to the hippocampal fissure (middle panels).

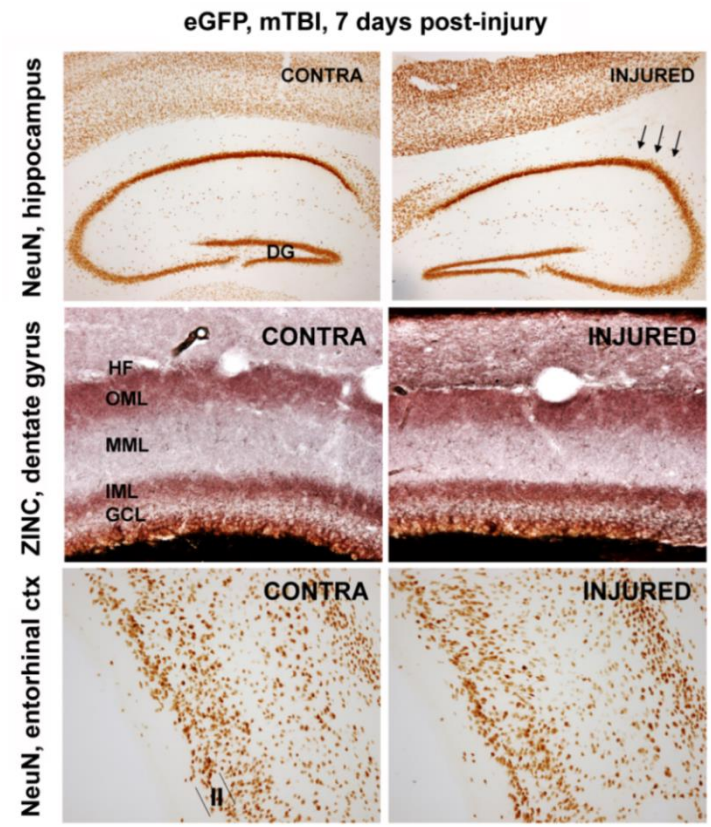

Figure 4. mTBI elicits no damage to lateral perforant pathway neurons expressing eGFP at 7 days post-injury, or their axons and synapses.
Representative case with robust eGFP expression in the right lateral EC layer II and the perforant pathway projection after single mTBI. As evidenced by immunostaining for NeuN (top), the mild injury caused hippocampal structural changes at the CA1/CA3 border (arrows) along with compression of the dentate gyrus (40X mag). Nevertheless, as shown by staining for synaptic zinc, the injury did not appreciably alter the density of lateral perforant pathway synapses in the dentate OML at 7 days post-injury (200X mag). Note also the compression of the dentate molecular layer caused by the mTBI. Similar to its lack of effect on axons and synapses, the mTBI did not trigger loss of lateral perforant pathway neurons of origin in layer II of the lateral EC, as evidenced from immunostaining for NeuN (bottom, 100X mag).

Despite these mTBI-induced hippocampal structural changes, there was no discernable loss of lateral perforant pathway synapses or axons at 7 days, as evidenced by the preservation of zinc in the pre-synaptic vesicles of the terminal field in the dentate OML. Similarly, mTBI did not affect survival of the eGFP-expressing neurons in layer II of the lateral EC at 7 days (bottom).

Collectively, our data provide evidence that an mTBI sufficient to cause subtle but discernable hippocampal structural alterations in the acute post-injury period, when coupled with eGFP expression, does not cause loss of neurons, axons, or synapses in the lateral perforant pathway.

5. Acute effects of mTBI on lateral perforant pathway neurons and synapses in hTau expressing mice

Given the evidence that the lateral perforant pathway expressing eGFP as a control foreign protein does not undergo acute degeneration despite the overt hippocampal structural changes caused by single mTBI, our study is well positioned to address whether pathological hTau affects the vulnerability of this pathway to mTBI. As shown in Figure 5, expression of hTauP301L in the lateral perforant pathway has little influence on neuronal, axonal, and synaptic survival in the acute post-injury period. Mice expressing hTau and receiving mTBI exhibited profound necrosis of the parietal neocortex around the site of impact, and minor distortion of hippocampal structure. The lateral perforant pathway still retained human tau in the SLM and OML (left). Staining for synaptic zinc revealed little to no loss of perforant pathway synapses on the injured dentate in comparison to the uninjured side (middle panels). In addition, there was no appreciable loss of lateral perforant pathway neurons in layer II of the lateral EC (bottom panels). Based on qualitative examination conducted thus far of the entire hTau mTBI group, and in comparison to the short-term histopathological outcome in mice expressing eGFP, hTauP301L appears to have little effect on the vulnerability of lateral perforant pathway neurons, axons, and synapses to single mTBI. Quantitative analysis of neuronal and synaptic densities coupled with the addition of more cases to the eGFP-mTBI and hTau-mTBI groups will be required to determine definitively whether pathological human tau fails to endanger perforant pathway neurons and synapses acutely after single and double mTBI.

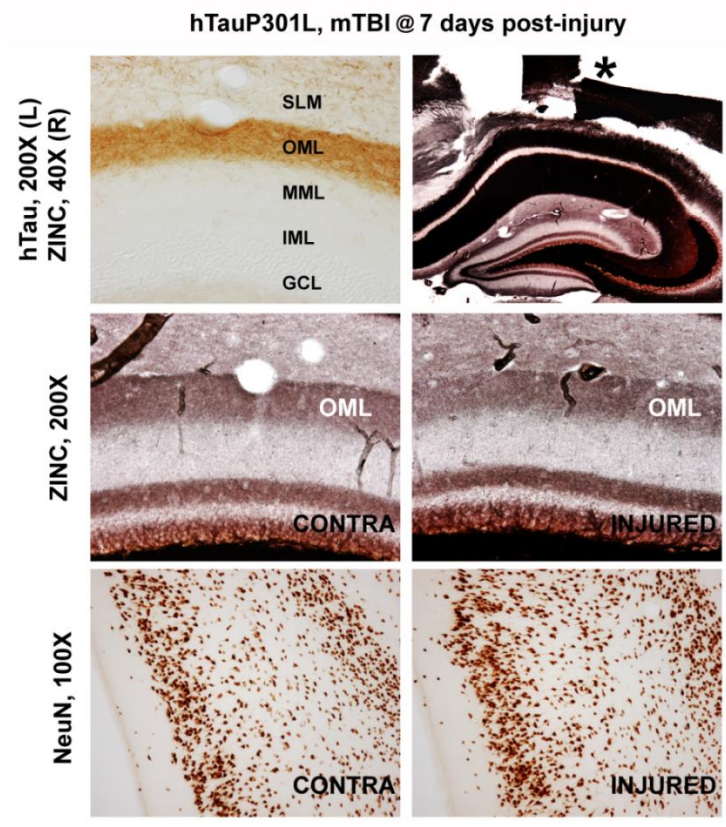

Figure 5. mTBI elicits no damage to lateral perforant pathway neurons expressing pathological hTau at 7 days post-injury, and has at most a very minor effect on survival of axons and synapses.
Top left- hTau expression persists in the lateral perforant pathway synapses in the dentate OML at 7 days post-injury. (200X mag) Top right – the mTBI had little effect on hippocampal structure. (40X mag)

Middle panels – Staining for synaptic zinc in the lateral perforant pathway synaptic zone in the OML. Note that there is little appreciable difference in synaptic density between the uninjured side and the hTau-expressing injured side. (200X mag).

Bottom – Neuronal staining with NeuN shows that mTBI coupled with hTau expression does not cause acute degeneration of lateral perforant pathway neurons originating in layer II of the lateral EC. (100X mag).

6. Our methods are capable of detecting perforant pathway neurodegeneration and synapse loss.

To confirm that our analytical methods are capable of detecting partial lateral perforant pathway degeneration, should it occur after mTBI combined with expression of pathological hTau, we evaluated the integrity of the pathway after administering a dose of AAV-hTau shown previously to induce the pathway to partially degenerate even in the absence of TBI. All of the experiments described above used entorhinal delivery of 0.5 billion genome copies of the AAV-hTauP301L or AAV-eGFP vectors. Based on our prior experience, and consistent with current observations from sham-injured mice, this is the maximal dose of the hTau vector that drives robust human tau expression and tau hyperphosphorylation without causing any loss of perforant pathway neurons or degeneration of axons or synapses. Figure 6 shows an example of the effects of expressing a higher dose of pathological hTau (0.75 billion genome copies) on perforant pathway integrity and human tau distribution. At this dose, hTau is found not only in the perforant pathway axons of the hippocampal SLM and lateral perforant pathway pre-synaptic terminals of the dentate OML, but its expression expands trans-synaptically to include scattered lateral perforant pathway target neurons, the dentate granule cells in the GCL. 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 reduces the neurotoxicity of hTau (Siman et al., in press).

A neurotoxic level of hTauP301L expression also triggers loss of lateral perforant pathway synapses, as evidenced by the reduced density of zinc staining in the OML, and of lateral perforant pathway neurons, as evidenced by the markedly reduced number of NeuN-stained nuclei in layer II of the lateral EC. These results demonstrate the effectiveness of our zinc and NeuN labeling methods for detecting loss of lateral perforant pathway synapses and neurons.

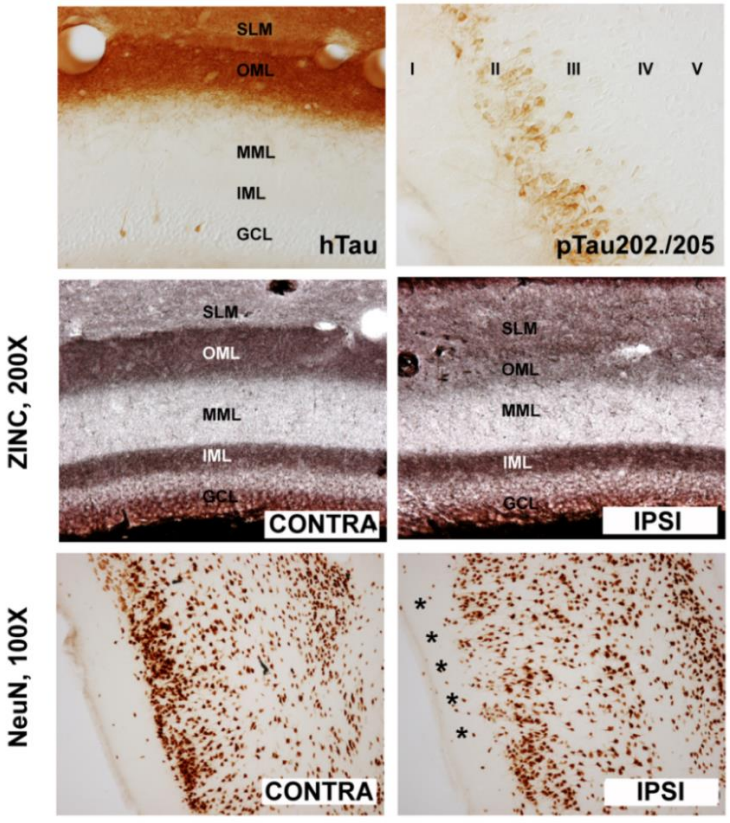

Figure 6. Detection of perforant pathway neuronal and synapse partial loss along with trans-synaptic spread of human tau after delivery of a toxic dose of AAV-hTauP301L.

Top panels – Human tau in dentate gyrus and pTau202/205 in lateral EC layer II 5 weeks after delivery of 0.75 billion genome copies. 200X mag.

Middle 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 (asterisks). 100X mag.

7. Acute effect of mTBI on human tau expression and hyperphosphorylation

The experiments described above address the question of whether human tau endangers a neural circuit especially vulnerable early in AD to mTBI-induced structural damage acutely after injury. We also examined whether mTBI might worsen tau pathology acutely by analyzing the expression level and distribution of human tau and pTau202/205 at 7 days post-injury. As shown in the left panels of Figure 7, single mTBI coupled with eGFP expression in the lateral perforant pathway distorts hippocampal structure as revealed by NeuN staining (swelling at the CA1/CA3 border and compression of the dentate gyrus) at 7 days. To examine if mTBI triggered tau phosphorylation acutely, we stained for pTau202/205. In no case was pTau202/205 detectible in any brain region at 7 days post-injury, providing evidence that mTBI does not acutely stimulate phosphorylation of endogenous mouse tau.

We also examined pTau202/205 at 7 days post-injury in mice with lateral perforant pathway expression of hTau (Figure 7A middle, right panels). In comparison with sham-injury (middle panels), single mTBI did not consistently alter the expression level or anatomical distribution of phosphorylated tau (right panels). In both sham-injured and mTBI cases, hTau was found in the perforant pathway axons of the hippocampal SLM and synaptic field in the dentate OML. There was no trans-synaptic transfer of hTau expression to dentate granule neurons. After both types of injury, pTau202/205 was restricted to the lateral EC layer II neurons of origin, and there was no consistent change in levels of this phosphoform indicative of early tau pathology. These qualitative data collected thus far suggest that, in the acute post-injury period, a single mTBI does not appreciably alter the expression, phosphorylation, or distribution of tau.

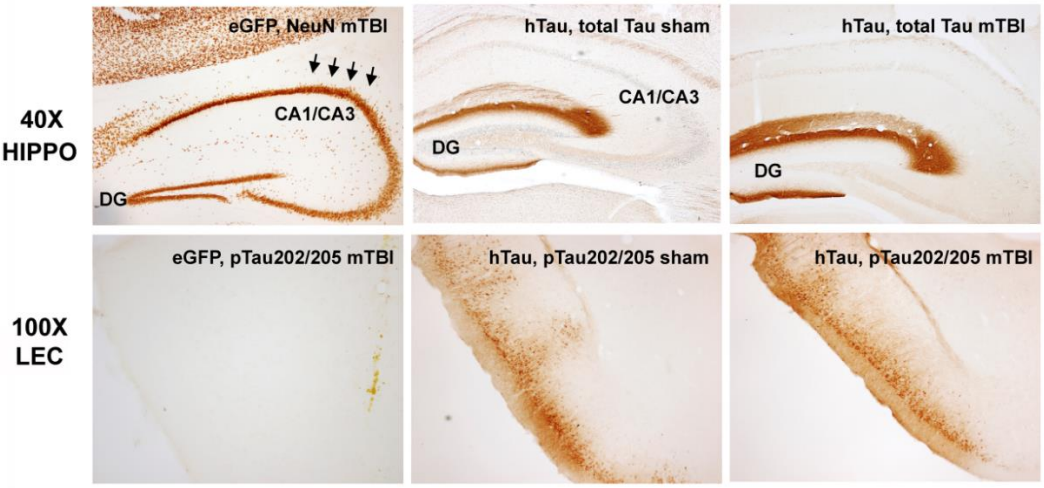

Figure 7A. Single mild TBI does not induce hyperphosphorylation of either human or endogenous mouse tau acutely post-injury, and does not trigger trans-synaptic expansion of human tau expression.

Left panels – NeuN labeling shows distortion of hippocampal structure 7 days after single mTBI coupled with lateral perforant pathway expression of eGFP (top). At this time post-injury, the lateral EC was devoid of pTau202/205 (bottom).

Middle panels – Human tau in hippocampus (top) and pTau202/205 in lateral entorhinal cortex (bottom) at 7 days following sham injury.

Right panels – Human tau in hippocampus (top) and pTau202/205 in lateral entorhinal cortex (bottom) at 7 days following single mTBI.

Having established a method for inducing controlled cortical impact TBI of mild severity with improved consistency, we have begun analyzing the effects of repetitive mTBI on perforant pathway integrity and tau localization. As shown in Figure 7B, preliminary analysis at 7 days after a second injury shows that double mTBI in a mouse with hTauP301L expression in the lateral perforant pathway resembles single mTBI, and triggers neither trans-synaptic expansion of human tau expression in the hippocampus, nor mislocalization of pTau202/205 to include perforant pathway axons or synapses. To determine definitively whether repetitive mTBI might change the expression, distribution, or phosphorylation of tau acutely post-injury, we plan to examine many more cases of double mTBI in the coming months.

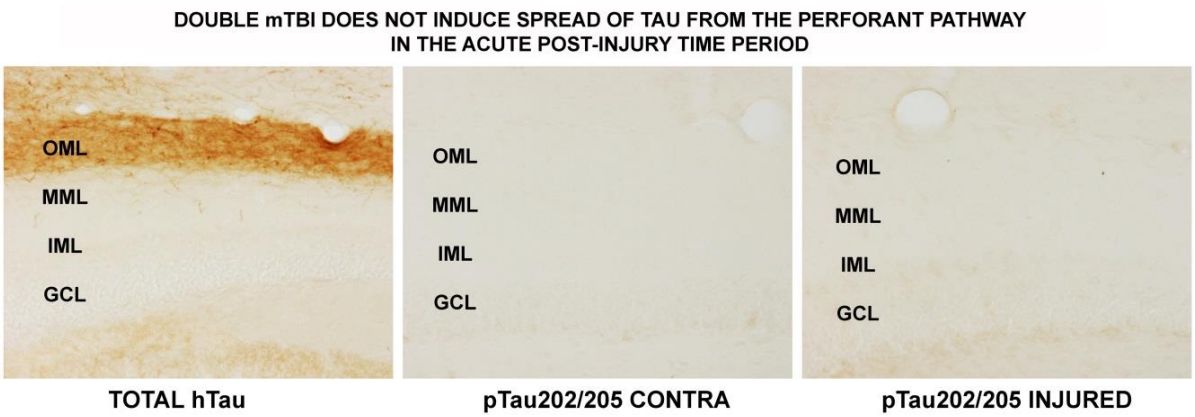

Figure 7B. Double mTBI does not induce the spread of human tau or mislocalization of phosphorylated tau in the acute post-injury period.

All panels show the dentate gyrus at 7 days post-injury (200X mag). As in sham-injured mice, human tau in the dentate is confined to the lateral perforant pathway synaptic field in the OML, but is absent from the granule neuron targets of the pathway (right). Also as in sham-injured mice, pTau202/205 is not present outside of the lateral entorhinal cortex, and is not detectible in lateral perforant pathway axons or synapses after double mTBI.

8. Acute effect of double mTBI on perforant pathway structural integrity

To assess neuronal degeneration in the hippocampus and entorhinal cortex acutely after double mTBI combined with lateral perforant pathway expression of pathological hTau, we labeled neurons for NeuN and compared the uninjured and injured hemispheres. In the example shown in Figure 8 of a mouse expressing hTau and receiving injuries 24 hours apart, double mTBI caused not only focal necrosis of the parietal cortex around the site of impact, but also induced hippocampal structural changes. These included distortion of hippocampal cytoarchitecture as was typically seen after single injuries, and in addition induced partial and focal loss of CA1 pyramidal neurons (arrow). There was no appreciable degeneration of the lateral perforant pathway, as evidenced by qualitative analysis of NeuN-positive surviving neurons in layer II of the lateral EC (bottom panels) as well as little discernable change in the density of synaptic zinc staining in the dentate OML (data not shown). Once again, we plan to analyze many more cases of double mTBI and conduct quantitative analyses of perforant pathway neurons and synapses before drawing firm conclusions on the effects of pathological human tau on the acute response to repetitive mTBI.

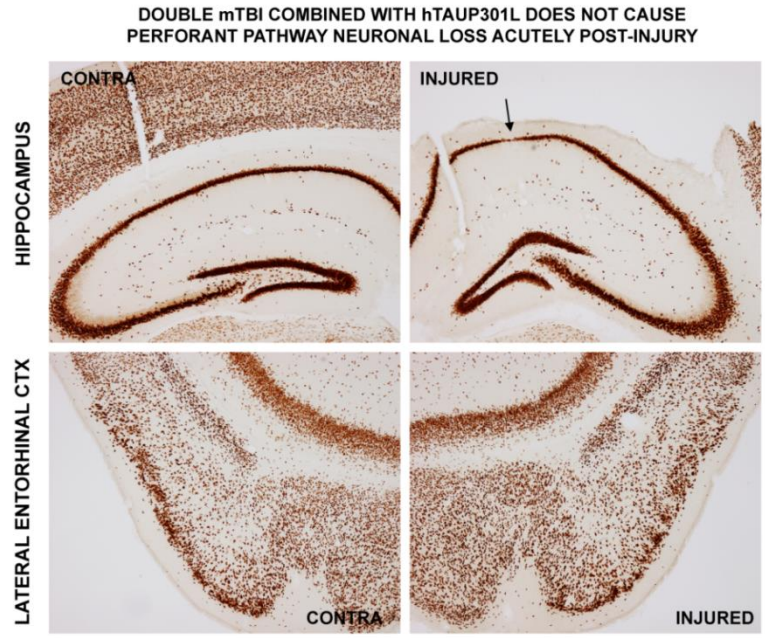

Figure 8. Double mTBI coupled with expression of hTauP301L does not appreciably affect survival of lateral perforant pathway neurons.

Top – Hippocampus (40X mag). Note that double mTBI distorted the normal architecture and caused localized loss of CA1 neurons (arrow).

Bottom – Lateral EC (40X). The number of surviving layer II neurons is not altered appreciably.

9. Acute effects of mild TBI alone and combined with pathological hTau on spatial learning

We evaluated the acute effects of controlled cortical impact mTBI and of pathological human tau expression in the lateral perforant pathway on hippocampal function using the Morris water maze, a spatial learning task known to be dependent on hippocampal integrity. An acute impairment in spatial learning in the task is manifested in two ways: (1) as an increase in the swim time latency to find the hidden platform during 6 trials conducted daily on days 3,4, and 5 after sham injury or mTBI, and (2) as a decrease in the proportionate time spent in the quadrant that once contained the platform during a platform removal trial on day 5. We assessed the effects of mTBI by comparing these measures between sham-injured and mTBI cases, whereas the effects of hTau were determined by comparison with eGFP expressing controls after both sham injury and mTBI. Thus far, we have completed behavioral and histopathological evaluations of 19 sham injuries, 11 single mTBIs, 2 double mTBIs, and 12 modTBIs with robust transgene expression localized to the entorhinal neurons of origin for the lateral perforant pathway and the lateral perforant pathway projection to the hippocampal dentate gyrus.

As summarized in Figure 9, mTBI impaired both measures of hippocampus-dependent spatial learning at 4 and 5 days post-injury. With eGFP expression in the lateral perforant pathway, the mice demonstrated spatial learning by finding the hidden platform more quickly on days 4 and 5 than on day 3. Confirming the learning of the platform location, in the platform removal trial the mice spent 42% of their swim time in the correct quadrant (exceeding chance performance of 25%). The eGFP-expressing mice with sham injuries had shorter latencies and spent more time in the correct quadrant than those with single mTBI, and the latter difference was statistically significant by t-test. These data provide evidence that CCI mTBI in the mouse causes an acute deficit in hippocampus-dependent spatial learning. The modTBI group also exhibited an acute spatial learning deficit, with longer latencies and reduced time spent in the correct quadrant compared with sham-injured controls (data not shown).

The data collected thus far suggest that, in the acute post-injury time period, pathological hTau expression in the lateral perforant pathway does not by itself impair spatial learning, and does not exacerbate the spatial learning deficit caused by single mTBI. As was true for eGFP-

expressing controls, the hTau expressing sham-injured mice showed evidence of spatial learning by having shorter swim time latencies on days 4 and 5 than on day 3, and by spending 46% of their swim time in the correct quadrant. Also similar to the eGFP expressing controls, mTBI coupled with hTau expression led to longer latencies on days 4 and 5 compared to sham controls and reduced to 36% the swim time spent in the correct quadrant. The magnitude of the effects of mTBI on the latencies and the time spent in the correct quadrant were nearly identical between the eGFP and hTau groups, and there were no significant differences in any measure of spatial learning as a function of foreign protein expression on any day of training. The lack of appreciable effect of perforant pathway hTau expression on hippocampus-dependent learning acutely post-injury, both alone and in combination with single mTBI, is consistent with the histological observations made to date and described in detail above, in which hTau has not shown evidence of endangering the lateral perforant pathway to mTBI, as well as the lack of effect of mTBI on the expression, cellular and subcellular distribution, or phosphorylation of hTau.

Figure 9. Hippocampus-dependent spatial learning after mTBI or sham injury: Lack of effect of pathological human tau

Latency (seconds) to find hidden platform

Transgene	Injury/days post	mean +/- s.e.m.	P value
eGFP	sham/day 4	24.4 +/- 3.9	0.09 vs day 3
eGFP	**mTBI/day 4**	**39.5 +/- 4.6***	**0.03 vs sham**
eGFP	sham/day 5	25.3 +/- 3.8	0.10 vs day 3
eGFP	mTBI/day 5	35.3 +/- 5.6	
hTau	sham/day 4	17.6 +/- 2.3**	0.005 vs day 3
hTau	**mTBI/day 4**	**30.0 +/- 5.2***	**0.02 vs sham**
hTau	sham/day 5	18.7 +/- 1.8**	0.001 vs day 3
hTau	mTBI/day 5	24.4 +/- 3.4**	0.01 vs day 3

% Time in correct quadrant (platform removal trial)

Transgene	Injury	% +/- s.e.m	P value
eGFP (n=7)	sham/day 5	42 +/- 3	
eGFP (n=6)	**mTBI/day 5**	**32 +/- 3***	0.05 vs sham
hTau (n=12)	sham/day 5	46 +/- 4	
hTau (n=5)	mTBI/day 5	36 +/- 4	0.12 vs. sham
			>0.4 vs eGFP mTBI

10. Status of progress toward milestone I in year 2:

By month 18 (i) determine whether tau worsens hippocampal structure and function in the acute post-TBI time period; (ii) determine whether TBI worsens incipient tauopathy acutely after injury; (iii) identify neurodegeneration biomarkers whose blood levels correlate with the severities of brain damage and cognitive dysfunction acutely after TBI.

During the first year, the project has made major strides toward reaching this milestone. The data collected thus far suggest that pathological human tau does not appreciably worsen TBI-induced changes in hippocampal structure and cognitive function acutely post-injury. The

data to date also suggest that single mTBI does not worsen the hyperphosphorylation or distribution of hTau at 7 days post-injury. To determine whether these preliminary conclusions on acute interactions between pathological hTau and mTBI are definitive, over the next 6 months we aim to increase the number of mice in every comparative group. Having established an improved method for reliable delivery of mild CCI, we also plan to evaluate large numbers of double mTBIs in the coming months, in order to discern whether there may be interactions between hTau and repetitive mTBI that do not occur after a single injury. Finally, we aim to perform quantitative morphometric analyses of lateral perforant pathway neuronal survival in the entorhinal cortex and synaptic integrity in the dentate terminal field. The project is on target to meet milestone I at the 18 month time point. The first year of the project has been conducted under budget, enabling us to increase the percent effort on the project of a Research Specialist, thereby ensuring that quantitative analyses of neuronal and synaptic integrity in the presence and absence of pathological hTau will be completed to meet milestone I.

Should the observations continue to hold that pathological hTau does not appreciably affect the acute responses to mTBI, and that mTBI does not acutely alter tau hyperphosphorylation and spread, the project will be well positioned to address in its second part whether long-term interactions might occur between tau and mTBI that take time to develop chronically post-injury. The aggregate data collected from the acute post-injury time period will serve as a baseline for evaluating whether hTau and TBI influence one another chronically after brain injury in a manner not observed acutely after brain injury.

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 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 are examining directly and critically whether tauopathy plays important roles in the acute and chronic outcomes from single and repetitive mTBI. Our study evaluates 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 mTBI observed thus far, our data suggest that the presence of a pathological form of human tau in the mouse perforant pathway projection does not render the neurons, axons, or synapses of the pathway more vulnerable to single mTBI in the acute post-injury period. In addition, a single mTBI does not appear to exacerbate features of tauopathy acutely after mTBI. The second year of the project will complete the study of acute interactions between tau and mTBI, and begin analyses of the potential chronic interactions between the two in the long-term post-injury period after both single and repetitive mild injuries.*

Finally, the ongoing study is evaluating preclinically 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 urgently needed and would have major applications for both military and civilian sufferers of mTBI. *Thus far, a serum sample bank has been prepared at 7 days post-injury from mice well characterized for their acute histopathological and cognitive responses to TBI or sham injury. During the second year of the project, sera will be analyzed for a set of biomarkers for neuronal degeneration, in order to identify a marker whose blood levels are a surrogate measure for mTBI-induced brain structural damage and dysfunction.*

Changes/Problems

Two changes were introduced during the conduct of the first year of the project, one to the work plan and another to the injury protocol. First, the moderate injuries were deemed too damaging to hippocampal structure to be considered useful for evaluating whether pathological human tau expression in the lateral perforant pathway could exacerbate the short-term outcomes from the TBI. This concern is even more problematic for the second part of our study, examining the chronic responses to TBI, given our expectation that any acute hippocampal structural damage will further evolve over the long-term post-injury period. Consequently, we made the strategic decision to drop moderate TBI groups and instead to focus all our analyses on the single and double mild TBIs in comparison with the sham injuries. Second, the controlled cortical impact injury device was optimized for consistent delivery of a mild TBI that elicits subtle but readily discernable structural change to the underlying hippocampus acutely post-injury period. The device was upgraded to incorporate a new digital and automatic Rod Speed Measurement Unit (Amscien Instruments). This accessory device utilizes the voltage changes determined by the impactor motion to generate a waveform that permits highly accurate digital measurements of impactor velocity. This precise approach reduces variability between individual animal experiments, and improves the discriminative power of the experimental design going forward.

Products

Nothing to report.

Participants and other support

1. Dr. Robert Siman
   Role –Principal Investigator
   Effort – 2.4 person months/year
   Contribution – Dr. Siman has directed every aspect of the project. 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 the neurodegeneration biomarkers under study using new equipment purchased through this award.

2. Dr. Victoria Johnson
   Role – Co-Investigator
   Effort – 1.2 person months/year
   Contribution – Dr. Johnson has performed the controlled cortical impact traumatic brain injuries and sham injuries, and has introduced methodological improvements to enhance the precision and consistency with which the injury device elicits mild TBI. She ensured personnel were trained on the evaluations of spatial learning using the Morris Water

Maze task. She assisted with histopathological evaluations of axonal pathology at 7 days post-injury.

3. Mr. Ryan Cocca
   Role – Research Specialist, Siman laboratory
   Effort – 4.8 person months/year
   Contribution – For the first ten months of the year, Mr. Cocca performed all of the neurosurgical gene delivery, animal husbandry, and histology, and nearly all of the immunohistochemistry evaluating short-term responses to TBI. Mr. Cocca left the laboratory 7/15/15 to attend medical school. Before his departure, he trained his replacement Ms. Cui on all of the requisite stereotaxic neurosurgical and histological methods as well as methods for serum preparation and animal husbandry.

4. Ms. Hongmei Cui
   Role – Research Specialist, Siman laboratory
   Effort – 1.5 person months/year
   Contribution – For the last three months of the year, Ms. Cui worked with Mr. Cocca and Dr. Siman to learn all of the neurosurgical gene delivery, animal husbandry, histology, and immunohistochemical methods evaluating the effects of pathological human tau on the acute response to TBI, and the effects of TBI on Alzheimer-type tau pathology. She has performed all of the neurosurgical, histological, and microscopic methods since Mr. Cocca left the laboratory.

5. Ms. Maura Weber
   Role – Research Specialist, Johnson laboratory
   Effort – 4.8 person months/year
   Contribution - Ms. Weber performed the neurobehavioral assessment of hippocampus-dependent spatial learning, using the Morris Water Maze task.

Other support – There has been no change in Dr. Siman's other support over the past year.

Special Reporting Requirements

Not applicable.

Appendix

None to report.