HHS Public Access

Author manuscript

Mol Cell Neurosci. Author manuscript; available in PMC 2016 May 01.

Published in final edited form as:

Mol Cell Neurosci. 2015 May ; 66(0 0): 91–98. doi:10.1016/j.mcn.2015.02.005.

The Pathophysiology of Repetitive Concussive Traumatic Brain Injury in Experimental Models; New Developments and Open Questions

David L Brody, MD PhD[1,*], **Joseph Benetatos**[1], **Rachel E Bennett, PhD**[1,2], **Kristen C Klemenhagen, PhD**[1,3], and **Christine L Mac Donald, PhD**[1,4]

[1] Department of Neurology, Washington University School of Medicine and Hope Center for Neurological Disorders, St Louis, Missouri, USA

Abstract

In recent years, there has been an increasing interest in the pathophysiology of repetitive concussive traumatic brain injury (rcTBI) in large part due to the association with dramatic cases of progressive neurological deterioration in professional athletes, military personnel, and others. However, our understanding of the pathophysiology of rcTBI is less advanced than for more severe brain injuries. Most prominently, the mechanisms underlying traumatic axonal injury, microglial activation, amyloid-beta accumulation, and progressive tau pathology are not yet known. In addition, the role of injury to dendritic spine cytoskeletal structures, vascular reactivity impairments, and microthrombi are intriguing and subjects of ongoing inquiry. Methods for quantitative analysis of axonal injury, dendritic injury, and synaptic loss need to be refined for the field to move forward in a rigorous fashion. We and others are attempting to develop translational approaches to assess these specific pathophysiological events in both animals and humans to facilitate clinically relevant pharmacodynamic assessments of candidate therapeutics. In this article, we review and discuss several of the recent experimental results from our lab and others. We include new initial data describing the difficulty in modeling progressive tau pathology in experimental rcTBI, and results demonstrating that sertraline can alleviate social interaction deficits and depressive-like behaviors following experimental rcTBI plus foot shock stress. Furthermore, we propose a discrete set of open, experimentally tractable questions that may serve as a framework for future investigations. In addition, we also raise several important questions that are less experimentally tractable at this time, in hopes that they may stimulate future methodological developments to address them.

[*]to whom correspondence should be addressed at brodyd@neuro.wustl.edu.

[2]Current address: Department of Neurology, Massachusetts General Hospital, Boston, MA, USA

[3]Current address: Department of Psychiatry, Columbia University and Research Foundation for Mental Health, New York State Psychiatric Institute, New York, NY, USA

[4]Current address: Department of Neurosurgery, University of Washington, Seattle, WA, USA

Traumatic Axonal Injury

Based on human and experimental animal data, it has been hypothesized that axons are fundamentally the most vulnerable structures in the brain to relatively mild, concussive traumatic brain injury. Specifically, in a series of autopsy cases in which the victims had recent concussive TBI and then died of other causes, Blumbergs et al reported that traumatic axonal injury was found in 6/6 cases. Other pathological signs of TBI such as hemorrhage, contusion, and skull fracture were absent in the concussion cases, though they were readily detected in more severe TBI cases. (Blumbergs et al., 1994, 1995). In a model of mouse repetitive concussive TBI, we reported electron microscopic evidence of axonal injury as well (Shitaka et al., 2011). However, most of the injured axons were not dilated, making them difficult to visualize using conventional immunohistochemistry. Thus, we reasoned that conventional light microscopic methods may substantially underestimate the extent of traumatic axonal injury. This line of reasoning was further supported by the findings that diffusion tensor imaging, an MRI method sensitive to white matter microstructure, revealed abnormal signals even in mice with controlled cortical impact injuries too mild to demonstrate substantial light microscopic immunohistochemical abnormalities (Fig. 1, adapted from Brody et al, “Current and Future Diagnostic Tools for Traumatic Brain Injury: CT, Conventional MRI, and Diffusion Tensor Imaging” Handbook of Neurology, 2015 *in press*). Silver staining, an indirect approach to assessing degenerating white matter, correlated only modestly with diffusion tensor abnormalities following experimental rcTBI (Bennett et al., 2012).

Thus, developing methods to efficiently assess non-dilated, injured axons following rcTBI became a top priority. We considered several approaches: (Table 1) and settled on array tomography-based immunofluorescent assessments as the most immediately promising. Array tomography involves cutting ribbons of serial ultrathin sections using an ultramicrotome, then performing immunofluorescent labeling on the ribbons (Micheva and Smith, 2007). The ultrathin sections provide exceptionally good signal to noise and spatial resolution, substantially better than optical (e.g. confocal) sections. The method has been used to quantify synaptic loss in both mouse models and human tissue (Koffie et al., 2012; Koffie et al., 2009). We adapted this technique to the study of injured white matter following repetitive concussive TBI by using the monoclonal antibody SMI32, which works well in resin-embedded sections and stains only injured axons in the mouse brain. Using largely automated image processing techniques, we determined that mouse corpus callosum contains approximately 2,000 SMI32- immunoreactive axons per cubic mm 7 days after repetitive concussive TBI (vs. essentially zero in control mice and in conventional thick sections stained with APP). Co-labeling with tubulin antibody in the same sections allowed us to determine that there were approximately 20,000 total axons per cubic mm in the region, and that the total number was not diminished by rcTBI at this time point (Bennett et al *submitted*).

Tau Pathology

Tau pathology is a prominent finding in post-mortem assessments of boxers, American football players, military personnel and others who have suffered repetitive concussive

traumatic brain injuries (Goldstein et al., 2012; McKee et al., 2009; McKee et al., 2012). However, the mechanisms underlying this tau pathology are not known. Our previous findings in a mouse model of more severe contusional TBI indicated that tau pathology induced by TBI may be mechanistically distinct from tau pathology due to age-related neurodegenerative diseases (Tran et al., 2011a; Tran et al., 2012; Tran et al., 2011b). Notably, much of the tau accumulation occurs in dilated axons via an amyloid-beta independent but cjun N-terminal kinase dependent mechanism.

To date, we have not detected any effect of rcTBI on immunohistochemically apparent tau pathology in either hTau or P301S tau transgenic mice 1 week, 1 month and 6 months using conventional light microscopic analyses. For example, we tested the effects of 4 closed skull concussive traumatic brain injuries spaced 24 hours apart alternating between right and left sides of the skull in 6-8 week old hTau mice (Andorfer et al., 2003). Littermates were randomly assigned to either 4 injuries or 4 sham procedures, then sacrificed 7 days later. The injuries produced substantial abnormal silver staining, indicative of injury (Fig 2A-B). However, there was no change in the extent of phospho-tau immunoreactivity using the CP13 antibody (Fig. 2C-D), which recognizes tau phosphorylated at serine 202 and has been commonly used to assess human CTE pathology (McKee et al., 2012). Tau knockout mice that were littermates of the hTau animals were also assessed as negative controls. There was no difference in the extent of silver staining between injured hTau mice and injured tau knockout mice (Fig 2B).

We have used 2 to 4 injuries 24 hours apart; these experimental parameters are arbitrary, and there is no consensus regarding the optimal rcTBI model. Several other groups have also developed rcTBI models (**Table 2**) with widely varying experimental parameters. Most models have resulted in acute to subacute behavioral deficits and relatively subtle histological abnormalities. While there has been some tau immunostaining reported (Ojo et al., 2013; Petraglia et al., 2014b), to date none of the models have recapitulated the *progressive* cortical and perivascular tau pathology that defines human CTE.

However, as noted above, most of the injured axons in rcTBI are not dilated. In ongoing work, we are attempting to determine whether hyperphosphorylated and/or aggregated tau accumulate in the non-dilated injured axons following rcTBI. Interestingly, a recent report described reduced behavioral impairments in tau knockout mice following a different rcTBI model (Cheng et al., 2014). Clearly, further investigations will be required to make a definitive determination of whether tau is a therapeutic target for rcTBI.

There are several possible non-mutually exclusive explanations for these negative results including insufficiently sensitive methods (the reason we are now trying array tomography-based approaches), latency from injury to development of tau pathology longer than the reasonable lifetime of mice, protective effects of anesthesia used during experimental injury, and a requirement for additional pathophysiological events or genetic factors.

It is apparent from the clinical experience of the 1st author and colleagues who care for retired professional athletes and military personnel that not all of these men who have sustained multiple concussive traumatic brain injuries go on to develop progressive

neurological and behavioral deterioration (unpublished observations). Many of them are cognitively normal and have good emotional balance. Others have deficits in cognitive function and emotional regulation that are static rather than progressive. Thus, a critical question is why some individuals appear resilient while others are more vulnerable to progressive deterioration following multiple concussive TBIs. We are interested in determining in both a clinical and experimental setting whether additional factors such as alcohol abuse, anabolic steroids, narcotics, sleep deprivation, sleep apnea, systemic injuries and inflammatory states contribute to axonal injury, tau pathology, and progressive neurological deterioration. Our conceptual model is that after injury, there may be two competing processes: clearance of pathological tau and trans-synaptic propagation of pathological tau. If trans-synaptic propagation of pathological tau species overwhelms clearance, progressive tau pathology and possibly neurological deterioration may result. Instead, if clearance prevails, the model would predict that deficits would recover over time or remain static, since some deficits are likely due to other, non-tau related pathology such as severed axons or neuronal cell loss. Thus, the biological factors contributing to clearance vs. propagation of tau pathology represent important targets for future investigation.

Intriguingly, experimental TBI has been recently shown to impair paravascular clearance of solutes from the brain, including exogenously injected tau (Iliff et al., 2014). This impaired clearance was exacerbated by genetic deletion of aquaporin-4, one of the major water channels in brain astrocytes. A logical hypothesis arising from this observation in mice would be that genetic polymorphisms in Acquaporin-4 (Dardiotis et al., 2014; Kleffner et al., 2008; Opdal et al., 2010; Sorani et al., 2008), other water channels, or regulatory pathways could affect the susceptibility or resilience to tau accumulation following concussive TBI in humans.

Intriguingly, the importance of small, oligomeric soluble assemblies of tau in the setting of TBI is beginning to be addressed (Hawkins et al., 2013). In many neurodegenerative diseases, toxicity appears more directly attributable to oligomeric assemblies of pathogenic proteins than to the immunohistochemically apparent deposits that define the pathological signatures of the conditions (Benilova et al., 2012; Esparza et al., 2013). Further work on the topic of oligomeric forms of tau after rcTBI (Lasagna-Reeves et al., 2011a; Lasagna-Reeves et al., 2011b; Lasagna-Reeves et al., 2012) may be critical for future mechanistic investigations and therapeutic development.

Amyloid-beta Pathology

After severe TBI, amyloid-beta accumulation has been reported in approximately 30% of cases (Roberts et al., 1991; Roberts et al., 1994). Amyloid-beta accumulation has also been reported in a variable fraction of CTE cases (McKee et al., 2012; Roberts et al., 1990). Whether the amyloid-beta pathology represents concomitant Alzheimer's disease or direct results from the concussive traumatic brain injury remains an open question. Repetitive concussive TBI in mice genetically engineered to overexpress amyloid precursor protein carrying mutations implicated in familial Alzheimer's disease has been reported to accelerate amyloid-beta pathology (Conte et al., 2004; Uryu et al., 2002). Amyloid-beta pathology due to rcTBI in mice carrying non-mutated human amyloid precursor protein has not been

reported to our knowledge. Furthermore, at least in the setting of more severe experimental TBI, the amyloid-beta pathology and the tau pathology appear to be mechanistically distinct (Tran et al., 2011a; Tran et al., 2011b). Specifically, we found that inhibition of amyloid-beta production did not affect TBI- related tau pathology in mice genetically engineered to produce both human amyloid precursor protein and tau (Tran et al., 2011a) and that the two types of pathology have distinct temporal and anatomical distributions (Tran et al., 2011b).

Microglial Activation

Multifocal microglial activation has been reported following human TBI (Smith, 2013) including concussive injury (Oppenheimer, 1968). Microgliosis appears to be long-lasting, though not indefinitely so, in injured white matter in humans (Smith et al., 2013). The specific role of microglia in CTE has not been directly addressed to our knowledge, though PET scan-based studies have indicated possible increases in the translocator protein binding ligand DPA-713 binding in former National Football League players, which may indirectly reflect microglial activation (Coughlin et al., 2014).

Microglial activation is a prominent and consistent feature in many animal rcTBI models (Table 2). For example, in our model, microglial activation persisted out to at least 7 weeks in white matter, whereas it peaked in the subacute period but resolved in gray matter regions (Shitaka et al., 2011). Our experimental observations led us to test the competing hypotheses that a) microglial activation contributes to traumatic axonal injury vs. b) microglial activation limits brain injury and contributes to regenerative responses. Our findings indicated that the answer appeared to be c) none of the above. Specifically, reduction in microglia using valgancyclovir administration to transgenic mice carrying herpes simplex thymidine kinase driven by the CD11b promoter had no effect on axonal injury following rcTBI (Bennett and Brody, 2014). There is a great deal of interest in the topic, as aspects of microglial reaction could represent targets for therapeutic intervention following TBI (Hailer, 2008; Perry, 2010) Importantly, subtypes of microglial activation after rcTBI have not been fully defined; there may distinct pro- and anti-inflammatory classes of microglia and different roles for inflammatory pathways at different stages of injury and neurodegeneration (Aguzzi et al., 2013). Interventions addressing specific microglial signaling pathways will be critical for future investigations.

Dendritic Spine Injury

In addition to axons, dendritic structures such as spines may be vulnerable to concussive injury because of their cytoskeletal architecture (Sala and Segal, 2014). Consistent with this idea, Golgi-stained mature spines in dentate gyrus granule neurons were reduced significantly 72 hours after controlled cortical impact TBI in mice (Gao et al., 2011). Similar findings were reported in neocortical layer II/III neurons and dentate granule neurons 24 hours after fluid percussion injury in rats (Campbell et al., 2012) and controlled cortical injury in mice (Winston et al., 2013). Interestingly, Golgi stained spines were increased in hippocampus 1 week after fluid percussion injury and in cortical layer IV/V injury 4 months after controlled cortical impact TBI (Campbell et al., 2012; Hoskison et al., 2009). It is not

clear whether the delayed increases represent a potentially epileptogenic or adaptive compensatory response.

Investigations of dendritic integrity after TBI have been relatively scarce, in part because methods to measure dendritic injury are less well developed than methods for axon injury. Golgi staining has been used to assess spine loss in other settings, but this method labels a non-random subset of pyramidal neurons and can be difficult to perform in a quantitatively reproducible manner. Diolistic labeling of neurons with dyes that fill the entire dendritic tree may represent a more truly unbiased and more consistent labeling approach (Gan et al., 2000). However, diolistic labeling of dendritic spines has not been used to assess the effects of TBI to our knowledge. Multiphoton microscopy in mice carrying fluorescent reporter genes in sparse neuronal subsets are beginning to be used to assess dendritic spine dynamics after TBI *in vivo* (Sword et al., 2013). Importantly, none of these assessments of dendritic spine integrity have been applied in animal models of repetitive concussive TBI to our knowledge.

Emotional Regulation Deficits

While much of the focus of investigations of the functional deficits due to severe TBI has been on motor and cognitive deficits, it is apparent that deficits in emotional regulation are prominent following rcTBI. These emotional regulation deficits have been brought into sharp focus by the mounting number of cases of suicide following rcTBI and the substantial disability attributable to post-traumatic stress disorder in military personnel with concussions sustained in the wars in Iraq and Afghanistan (Mac Donald et al., 2014a; Mac Donald et al., 2014b)(Mac Donald et al Brain 2015 *in press*). While the direction of causality in human patients cannot be determined with certainty, it is plausible that damage to frontal and basal forebrain emotional regulatory circuitry could underlie some of these deficits. Following blast-induced concussive TBI in a rat model, the injured animals displayed substantially greater persistent anxiety-like behaviors than controls, despite having been fully anesthetized during the blast event (Elder et al., 2012). However, these effects may not be unique to blast-injury. In our study of US military personnel injured in the wars in Iraq and Afghanistan by either blast-related mechanisms or non-blast-related mechanisms, we found indistinguishably high levels of overall disability, post-traumatic stress disorder, and depression independent of injury mechanism (Mac Donald et al., 2014b). PTSD and depression were significantly worse in both TBI groups than in deployed military controls with other injuries. The extent of depression was the strongest single correlate of overall disability.

However, severe PTSD and depression are not common in the short term after sports-related concussions. Thus, we reasoned that the combination of rcTBI and major environmental stress, such as commonly occurs during overseas deployment in a war zone, could interact synergistically to worsen emotional regulation. To test this in a controlled setting, we subjected mice to experimental rcTBI followed by foot shock stress in a conditioned fear paradigm. Contrary to our initial hypothesis, rcTBI did not affect extinction learning after foot shock. However, we found that the injury and foot shock together caused significant deficits in social interaction and prominent depression-like behavior, whereas neither injury

alone or foot shock alone did so (Klemenhagen et al., 2013). This supported the idea of synergistic interaction between rcTBI and additional environmental stress.

More recently, we have tested whether treatment with the antidepressant sertraline starting after the rcTBI and foot shock stress would significantly improve social interaction and alleviate depression-like behavior in 6-8 week old male C57Bl/6J mice. We found that rcTBI injury plus foot shock stress resulted in increased immobility in the tail suspension test compared to uninjured controls, as previously reported (Klemenhagen et al., 2013). Oral sertraline treatment reduced immobility time in the tail suspension test in a dose dependent fashion (Fig 3A). The tail suspension test is a classic behavioral test sensitive to the effects of clinically beneficial antidepressants. The maximum dose of 20 mg/kg/day fully normalized tail suspension performance relative to uninjured control mice. Sucrose preference, a measure of anhedonia and a depression-like behavioral endophenotype, did not differ between groups, and also was not significantly affected by rcTBI plus foot shock stress (Fig 3B). Furthermore, sertraline substantially increased social interaction time in rcTBI mice; in fact at the highest doses, social interaction was greater in the treated, injured, and shocked mice than in the uninjured controls (Fig. 3C). Olfactory function as assessed by the buried cookie test was not affected by either injury plus foot shock or any of the sertraline doses assessed (Fig 3D). Importantly, sertraline treatment was given in the drinking water, so it cannot be determined with certainty whether every mouse received the same amount of sertraline; blood levels were not assessed. Furthermore, treatment was given starting 7 days after injury for 3 weeks prior to the start of behavioral testing and administered through the testing period; it is not known whether these effects would generalize to delayed treatment starting months (or years) after injury. Thus, in conclusion, the combined rcTBI plus foot shock model provides a consistent and robust platform for preclinical testing of candidate therapeutics for mood and social deficits. Important future directions will include determining the extent to which these effects generalize across age, strain and gender. Ongoing efforts to test the time window during which treatments are effective and develop novel therapeutic strategies such as nootropic agents, epigenetic modulators, and synergistic combination therapeutics are warranted.

Conclusions

There is substantial interest in the acute and chronic effects of concussive traumatic brain injury, and a great number of questions remaining to be addressed. Some of the questions involving specific aspects of the injuries and therapeutic strategies can be answered with methods that while labor-intensive, are tractable within the near term (Box 1). However, other critical questions cannot be readily answered with existing methods, although new approaches currently in development may yield important discoveries in the next decade (Box 2). As technical advances in microscopy (e.g. super-resolution, implantable GRIN lenses, block-face scanning EM) come into widespread use, we will almost certainly develop a better understanding of the structural basis for concussive injury. Likewise, as direct manipulations of the function of specific cell types using genetic tools (optogenetic control of membrane potential and transcription, designer receptors exclusively activated by designer drugs) yields approaches relevant to TBI, we will be able to address experimental questions that are currently out of reach. Finally, since the ultimate goal of all research of

concussive TBI is to improve human health, discovery of directly translatable approaches to test specific hypotheses about pathophysiology in human patients (advanced imaging, genetics, physiological measures, microdialysis) and in human-derived cells will be of great relevance for therapeutic development and pharmacodynamic assessments of candidate therapeutics.

REFERENCES

Aguzzi A, Barres BA, Bennett ML. Microglia: scapegoat, saboteur, or something else? Science. 2013; 339:156–161. [PubMed: 23307732]

Andorfer C, Kress Y, Espinoza M, de Silva R, Tucker KL, Barde YA, Duff K, Davies P. Hyperphosphorylation and aggregation of tau in mice expressing normal human tau isoforms. Journal of neurochemistry. 2003; 86:582–590. [PubMed: 12859672]

Benilova I, Karran E, De Strooper B. The toxic Abeta oligomer and Alzheimer's disease: an emperor in need of clothes. Nature neuroscience. 2012; 15:349–357.

Bennett RE, Brody DL. Acute reduction of microglia does not alter axonal injury in a mouse model of repetitive concussive traumatic brain injury. Journal of neurotrauma. 2014; 31:1647–1663. [PubMed: 24797413]

Bennett RE, Mac Donald CL, Brody DL. Diffusion tensor imaging detects axonal injury in a mouse model of repetitive closed-skull traumatic brain injury. Neuroscience letters. 2012; 513:160–165. [PubMed: 22343314]

Blumbergs PC, Scott G, Manavis J, Wainwright H, Simpson DA, McLean AJ. Staining of amyloid precursor protein to study axonal damage in mild head injury. Lancet. 1994; 344:1055–1056. [PubMed: 7523810]

Blumbergs PC, Scott G, Manavis J, Wainwright H, Simpson DA, McLean AJ. Topography of axonal injury as defined by amyloid precursor protein and the sector scoring method in mild and severe closed head injury. Journal of neurotrauma. 1995; 12:565–572. [PubMed: 8683607]

Campbell JN, Register D, Churn SB. Traumatic brain injury causes an FK506-sensitive loss and an overgrowth of dendritic spines in rat forebrain. Journal of neurotrauma. 2012; 29:201–217. [PubMed: 21517673]

Cheng JS, Craft R, Yu GQ, Ho K, Wang X, Mohan G, Mangnitsky S, Ponnusamy R, Mucke L. Tau reduction diminishes spatial learning and memory deficits after mild repetitive traumatic brain injury in mice. PloS one. 2014; 9:e115765. [PubMed: 25551452]

Conte V, Uryu K, Fujimoto S, Yao Y, Rokach J, Longhi L, Trojanowski JQ, Lee VM, McIntosh TK, Pratico D. Vitamin E reduces amyloidosis and improves cognitive function in Tg2576 mice following repetitive concussive brain injury. Journal of neurochemistry. 2004; 90:758–764. [PubMed: 15255955]

Coughlin JM, Wang Y, Munro CA, Ma S, Yue C, Chen S, Airan R, Kim PK, Adams AV, Garcia C, Higgs C, Sair HI, Sawa A, Smith G, Lyketsos CG, Caffo B, Kassiou M, Guilarte TR, Pomper MG. Neuroinflammation and brain atrophy in former NFL players: An in vivo multimodal imaging pilot study. Neurobiology of disease. 2014; 74C:58–65. [PubMed: 25447235]

Creeley CE, Wozniak DF, Bayly PV, Olney JW, Lewis LM. Multiple episodes of mild traumatic brain injury result in impaired cognitive performance in mice. Academic emergency medicine : official journal of the Society for Academic Emergency Medicine. 2004; 11:809–819. [PubMed: 15289185]

Dardiotis E, Paterakis K, Tsivgoulis G, Tsintou M, Hadjigeorgiou GF, Dardioti M, Grigoriadis S, Simeonidou C, Komnos A, Kapsalaki E, Fountas K, Hadjigeorgiou GM. AQP4 Tag Single Nucleotide Polymorphisms in Patients with Traumatic Brain Injury. Journal of neurotrauma. 2014; 31:1920–1926. [PubMed: 24999750]

DeFord SM, Wilson MS, Rice AC, Clausen T, Rice LK, Barabnova A, Bullock R, Hamm RJ. Repeated mild brain injuries result in cognitive impairment in B6C3F1 mice. Journal of neurotrauma. 2002; 19:427–438. [PubMed: 11990349]

Elder GA, Dorr NP, De Gasperi R, Gama Sosa MA, Shaughness MC, Maudlin-Jeronimo E, Hall AA, McCarron RM, Ahlers ST. Blast exposure induces post-traumatic stress disorder-related traits in a rat model of mild traumatic brain injury. Journal of neurotrauma. 2012; 29:2564–2575. [PubMed: 22780833]

Esparza TJ, Zhao H, Cirrito JR, Cairns NJ, Bateman RJ, Holtzman DM, Brody DL. Amyloid-beta oligomerization in Alzheimer dementia versus high-pathology controls. Annals of neurology. 2013; 73:104–119. [PubMed: 23225543]

Friess SH, Ichord RN, Ralston J, Ryall K, Helfaer MA, Smith C, Margulies SS. Repeated traumatic brain injury affects composite cognitive function in piglets. Journal of neurotrauma. 2009; 26:1111–1121. [PubMed: 19275468]

Gan WB, Grutzendler J, Wong WT, Wong RO, Lichtman JW. Multicolor "DiOlistic" labeling of the nervous system using lipophilic dye combinations. Neuron. 2000; 27:219–225. [PubMed: 10985343]

Gao X, Deng P, Xu ZC, Chen J. Moderate traumatic brain injury causes acute dendritic and synaptic degeneration in the hippocampal dentate gyrus. PloS one. 2011; 6:e24566. [PubMed: 21931758]

Goldstein LE, Fisher AM, Tagge CA, Zhang XL, Velisek L, Sullivan JA, Upreti C, Kracht JM, Ericsson M, Wojnarowicz MW, Goletiani CJ, Maglakelidze GM, Casey N, Moncaster JA, Minaeva O, Moir RD, Nowinski CJ, Stern RA, Cantu RC, Geiling J, Blusztajn JK, Wolozin BL, Ikezu T, Stein TD, Budson AE, Kowall NW, Chargin D, Sharon A, Saman S, Hall GF, Moss WC, Cleveland RO, Tanzi RE, Stanton PK, McKee AC. Chronic traumatic encephalopathy in blast-exposed military veterans and a blast neurotrauma mouse model. Science translational medicine. 2012; 4:134ra160.

Hailer NP. Immunosuppression after traumatic or ischemic CNS damage: it is neuroprotective and illuminates the role of microglial cells. Progress in neurobiology. 2008; 84:211–233. [PubMed: 18262323]

Hawkins BE, Krishnamurthy S, Castillo-Carranza DL, Sengupta U, Prough DS, Jackson GR, DeWitt DS, Kayed R. Rapid accumulation of endogenous tau oligomers in a rat model of traumatic brain injury: possible link between traumatic brain injury and sporadic tauopathies. The Journal of biological chemistry. 2013; 288:17042–17050. [PubMed: 23632019]

Hoskison MM, Moore AN, Hu B, Orsi S, Kobori N, Dash PK. Persistent working memory dysfunction following traumatic brain injury: evidence for a time-dependent mechanism. Neuroscience. 2009; 159:483–491. [PubMed: 19167462]

Iliff JJ, Chen MJ, Plog BA, Zeppenfeld DM, Soltero M, Yang L, Singh I, Deane R, Nedergaard M. Impairment of glymphatic pathway function promotes tau pathology after traumatic brain injury. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2014; 34:16180–16193. [PubMed: 25471560]

Kanayama G, Takeda M, Niigawa H, Ikura Y, Tamii H, Taniguchi N, Kudo T, Miyamae Y, Morihara T, Nishimura T. The effects of repetitive mild brain injury on cytoskeletal protein and behavior. Methods Find Exp Clin Pharmacol. 1996; 18:105–115. [PubMed: 8740242]

Kleffner I, Bungeroth M, Schiffbauer H, Schabitz WR, Ringelstein EB, Kuhlenbaumer G. The role of aquaporin-4 polymorphisms in the development of brain edema after middle cerebral artery occlusion. Stroke; a journal of cerebral circulation. 2008; 39:1333–1335.

Klemenhagen KC, O'Brien SP, Brody DL. Repetitive concussive traumatic brain injury interacts with post-injury foot shock stress to worsen social and depression-like behavior in mice. PloS one. 2013; 8:e74510. [PubMed: 24058581]

Koffie RM, Hashimoto T, Tai HC, Kay KR, Serrano-Pozo A, Joyner D, Hou S, Kopeikina KJ, Frosch MP, Lee VM, Holtzman DM, Hyman BT, Spires-Jones TL. Apolipoprotein E4 effects in Alzheimer's disease are mediated by synaptotoxic oligomeric amyloid-beta. Brain : a journal of neurology. 2012; 135:2155–2168. [PubMed: 22637583]

Koffie RM, Meyer-Luehmann M, Hashimoto T, Adams KW, Mielke ML, Garcia-Alloza M, Micheva KD, Smith SJ, Kim ML, Lee VM, Hyman BT, Spires-Jones TL. Oligomeric amyloid beta associates with postsynaptic densities and correlates with excitatory synapse loss near senile plaques. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106:4012–4017. [PubMed: 19228947]

Lasagna-Reeves CA, Castillo-Carranza DL, Jackson GR, Kayed R. Tau oligomers as potential targets for immunotherapy for Alzheimer's disease and tauopathies. Current Alzheimer research. 2011a; 8:659–665. [PubMed: 21605039]

Lasagna-Reeves CA, Castillo-Carranza DL, Sengupta U, Clos AL, Jackson GR, Kayed R. Tau oligomers impair memory and induce synaptic and mitochondrial dysfunction in wild-type mice. Molecular neurodegeneration. 2011b; 6:39. [PubMed: 21645391]

Lasagna-Reeves CA, Castillo-Carranza DL, Sengupta U, Guerrero-Munoz MJ, Kiritoshi T, Neugebauer V, Jackson GR, Kayed R. Alzheimer brain-derived tau oligomers propagate pathology from endogenous tau. Scientific reports. 2012; 2:700. [PubMed: 23050084]

Laurer HL, Bareyre FM, Lee VM, Trojanowski JQ, Longhi L, Hoover R, Saatman KE, Raghupathi R, Hoshino S, Grady MS, McIntosh TK. Mild head injury increasing the brain's vulnerability to a second concussive impact. Journal of neurosurgery. 2001; 95:859–870. [PubMed: 11702878]

Longhi L, Saatman KE, Fujimoto S, Raghupathi R, Meaney DF, Davis J, McMillan BSA, Conte V, Laurer HL, Stein S, Stocchetti N, McIntosh TK. Temporal window of vulnerability to repetitive experimental concussive brain injury. Neurosurgery. 2005; 56:364–374. [PubMed: 15670384]

Mac Donald CL, Dikranian K, Bayly P, Holtzman D, Brody D. Diffusion tensor imaging reliably detects experimental traumatic axonal injury and indicates approximate time of injury. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2007a; 27:11869–11876. [PubMed: 17978027]

Mac Donald CL, Dikranian K, Song SK, Bayly PV, Holtzman DM, Brody DL. Detection of traumatic axonal injury with diffusion tensor imaging in a mouse model of traumatic brain injury. Experimental neurology. 2007b; 205:116–131. [PubMed: 17368446]

Mac Donald CL, Johnson AM, Nelson EC, Werner NJ, Fang R, Flaherty SF, Brody DL. Functional status after blast-plus-impact complex concussive traumatic brain injury in evacuated United States military personnel. Journal of neurotrauma. 2014a; 31:889–898. [PubMed: 24367929]

Mac Donald CL, Johnson AM, Wierzechowski L, Kassner E, Stewart T, Nelson EC, Werner NJ, Zonies D, Oh J, Fang R, Brody DL. Prospectively assessed clinical outcomes in concussive blast vs nonblast traumatic brain injury among evacuated US military personnel. JAMA neurology. 2014b; 71:994–1002. [PubMed: 24934200]

McKee AC, Cantu RC, Nowinski CJ, Hedley-Whyte ET, Gavett BE, Budson AE, Santini VE, Lee HS, Kubilus CA, Stern RA. Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. Journal of neuropathology and experimental neurology. 2009; 68:709–735. [PubMed: 19535999]

McKee AC, Stein TD, Nowinski CJ, Stern RA, Daneshvar DH, Alvarez VE, Lee HS, Hall G, Wojtowicz SM, Baugh CM, Riley DO, Kubilus CA, Cormier KA, Jacobs MA, Martin BR, Abraham CR, Ikezu T, Reichard RR, Wolozin BL, Budson AE, Goldstein LE, Kowall NW, Cantu RC. The spectrum of disease in chronic traumatic encephalopathy. Brain : a journal of neurology. 2012

Meehan WP 3rd, Zhang J, Mannix R, Whalen MJ. Increasing recovery time between injuries improves cognitive outcome after repetitive mild concussive brain injuries in mice. Neurosurgery. 2012; 71:885–891. [PubMed: 22743360]

Micheva KD, Smith SJ. Array tomography: a new tool for imaging the molecular architecture and ultrastructure of neural circuits. Neuron. 2007; 55:25–36. [PubMed: 17610815]

Mouzon BC, Bachmeier C, Ferro A, Ojo JO, Crynen G, Acker CM, Davies P, Mullan M, Stewart W, Crawford F. Chronic neuropathological and neurobehavioral changes in a repetitive mTBI model. Annals of neurology. 2013

Ojo JO, Mouzon B, Greenberg MB, Bachmeier C, Mullan M, Crawford F. Repetitive Mild Traumatic Brain Injury Augments Tau Pathology and Glial Activation in Aged hTau Mice. Journal of neuropathology and experimental neurology. 2013; 72:137–151. [PubMed: 23334597]

Opdal SH, Vege A, Stray-Pedersen A, Rognum TO. Aquaporin-4 gene variation and sudden infant death syndrome. Pediatric research. 2010; 68:48–51. [PubMed: 20351659]

Oppenheimer DR. Microscopic lesions in the brain following head injury. Journal of neurology, neurosurgery, and psychiatry. 1968; 31:299–306.

Perry VH. Contribution of systemic inflammation to chronic neurodegeneration. Acta neuropathologica. 2010; 120:277–286. [PubMed: 20644946]

Petraglia AL, Plog BA, Dayawansa S, Chen M, Dashnaw ML, Czerniecka K, Walker CT, Viterise T, Hyrien O, Iliff JJ, Deane R, Nedergaard M, Huang JH. The spectrum of neurobehavioral sequelae after repetitive mild traumatic brain injury: a novel mouse model of chronic traumatic encephalopathy. Journal of neurotrauma. 2014a; 31:1211–1224. [PubMed: 24766454]

Petraglia AL, Plog BA, Dayawansa S, Dashnaw ML, Czerniecka K, Walker CT, Chen M, Hyrien O, Iliff JJ, Deane R, Huang JH, Nedergaard M. The pathophysiology underlying repetitive mild traumatic brain injury in a novel mouse model of chronic traumatic encephalopathy. Surgical neurology international. 2014b; 5:184. [PubMed: 25593768]

Roberts GW, Allsop D, Bruton C. The occult aftermath of boxing. Journal of neurology, neurosurgery, and psychiatry. 1990; 53:373–378.

Roberts GW, Gentleman SM, Lynch A, Graham DI. beta A4 amyloid protein deposition in brain after head trauma. Lancet. 1991; 338:1422–1423. [PubMed: 1683421]

Roberts GW, Gentleman SM, Lynch A, Murray L, Landon M, Graham DI. Beta amyloid protein deposition in the brain after severe head injury: implications for the pathogenesis of Alzheimer's disease. Journal of neurology, neurosurgery, and psychiatry. 1994; 57:419–425.

Sala C, Segal M. Dendritic spines: the locus of structural and functional plasticity. Physiological reviews. 2014; 94:141–188. [PubMed: 24382885]

Shitaka Y, Tran HT, Bennett RE, Sanchez L, Levy MA, Dikranian K, Brody DL. Repetitive closed-skull traumatic brain injury in mice causes persistent multifocal axonal injury and microglial reactivity. Journal of neuropathology and experimental neurology. 2011; 70:551–567. [PubMed: 21666502]

Smith C. Review: the long-term consequences of microglial activation following acute traumatic brain injury. Neuropathology and applied neurobiology. 2013; 39:35–44. [PubMed: 23206160]

Smith C, Gentleman SM, Leclercq PD, Murray LS, Griffin WS, Graham DI, Nicoll JA. The neuroinflammatory response in humans after traumatic brain injury. Neuropathology and applied neurobiology. 2013; 39:654–666. [PubMed: 23231074]

Sorani MD, Zador Z, Hurowitz E, Yan D, Giacomini KM, Manley GT. Novel variants in human Aquaporin-4 reduce cellular water permeability. Human molecular genetics. 2008; 17:2379–2389. [PubMed: 18511455]

Sword J, Masuda T, Croom D, Kirov SA. Evolution of neuronal and astroglial disruption in the peri-contusional cortex of mice revealed by in vivo two-photon imaging. Brain : a journal of neurology. 2013; 136:1446–1461. [PubMed: 23466395]

Tran HT, Laferla FM, Holtzman DM, Brody DL. Controlled Cortical Impact Traumatic Brain Injury in 3xTg-AD Mice Causes Acute Intra-Axonal Amyloid-beta Accumulation and Independently Accelerates the Development of Tau Abnormalities. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011a; 31:9513–9525. [PubMed: 21715616]

Tran HT, Sanchez L, Brody DL. Inhibition of JNK by a Peptide Inhibitor Reduces Traumatic Brain Injury-Induced Tauopathy in Transgenic Mice. Journal of neuropathology and experimental neurology. 2012; 71:116–129. [PubMed: 22249463]

Tran HT, Sanchez L, Esparza TJ, Brody DL. Distinct Temporal and Anatomical Distributions of Amyloid-beta and Tau Abnormalities following Controlled Cortical Impact in Transgenic Mice. PloS one. 2011b; 6:e25475. [PubMed: 21980472]

Uryu K, Laurer H, McIntosh T, Pratico D, Martinez D, Leight S, Lee VM, Trojanowski JQ. Repetitive mild brain trauma accelerates Abeta deposition, lipid peroxidation, and cognitive impairment in a transgenic mouse model of Alzheimer amyloidosis. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2002; 22:446–454. [PubMed: 11784789]

Winston CN, Chellappa D, Wilkins T, Barton DJ, Washington PM, Loane DJ, Zapple DN, Burns MP. Controlled cortical impact results in an extensive loss of dendritic spines that is not mediated by injury-induced amyloid-beta accumulation. Journal of neurotrauma. 2013; 30:1966–1972. [PubMed: 23879560]

BOX 1: Open, experimentally tractable questions in the pathophysiology of rcTBI

1) Why does an initial concussive TBI that does not by itself cause persistent structural injury or functional impairment create a vulnerability to a second injury? Are cytoskeletal derangements, calcium homeostasis impairments, metabolic disruptions, blood flow abnormalities, microglial priming or other events responsible for the vulnerability to additional impacts?

2) Do subconcussive events cause vulnerability to concussive TBI? If so, by what mechanism and for how long?

3) Does rcTBI cause dendritic spine loss? If so, which regions are vulnerable and what are the temporal dynamics? Does spine loss correlate with behavioral impairments?

4) Can experimental therapeutics delivered at clinically realistic times after rcTBI alleviate long-term functional deficits in rigorous and directly translatable preclinical trials?

5) Does rcTBI cause persistent vascular reactivity impairments, and if so, what are the underlying mechanisms?

6) Does rcTBI cause substantial microthrombosis, and if so, what effect does this have on brain tissue integrity?

7) Do age and gender fundamentally affect the response to experimental rcTBI?

8) Does genetic vulnerability play a major role in the susceptibility to injury and response to concussive TBI? Large-scale multicenter studies involving thousands or tens of thousands of patients and careful clinical assessments will likely be required to obtain robust results.

BOX 2: Open questions regarding the pathophysiology of rcTBI that require additional methodological development

1) Which of the many pathophysiological events described or hypothesized to occur in animal models are most relevant to human rcTBI? How can these pathophysiological events be assessed in living human patients? Advanced neuroimaging, blood and CSF-based biomarkers, physiological monitoring methods, and pharmacological manipulations all may be brought to bear on this issue.

2) What additional events apart from rcTBI itself are required to cause the progressive tau pathology characteristic of CTE? A wide array of additional contributors have been proposed; systematic epidemiological studies will be required before meaningful controlled experimental protocols can be designed.

3) Do oligomeric assemblies of tau play a major pathophysiological role in the long-term progressive neurological deterioration that can follow rcTBI? Methods to accurately quantify endogenous oligomeric forms of tau and characterize their toxicity may shed light on the mismatch between toxicity and histopathology, by direct analogy to Alzheimer's Disease.

4) Do mood symptoms, anxiety, and depression following rcTBI arise from damage to limbic system and frontal lobe structures? Advanced imaging of periaqueductal gray, bed nucleus of the stria terminalis, amygdala subnuclei, orbitofrontal regions, hypothalamic subnuclei, and insula along with connecting fiber tracts will be challenging but theoretically feasible.

5) What are the roles of various subsets of microglia in the early, subacute, and chronic phases of rcTBI? New tools to mark and manipulate specific microglial activation states with good spatial and temporal control could unveil novel candidate therapeutic targets.

6) Do subconcussive injuries cause behavioral or histopathological abnormalities that are similar to or distinct from those caused by rcTBI? In our view, concussive injuries in animal models can be defined as injuries that cause a prolongation in recovery of mobility (longer than just anesthesia), and/or acute changes in neurological performance such as anterograde memory, balance, or motor behavior. A subconcussive injury, in our view, would be one that does not affect recovery time, memory, balance or motor behavior acutely. We are not aware of any animal models explicitly assessing long term effects of subconcussive injuries in animal models. This is an important area for future research.

Highlights

1) Concise review of selected topics in repetitive concussive traumatic brain injury

2) Animal models, approaches to assessing axonal injury and neurodegeneration.

3) Unpublished negative data on difficulties with experimental rcTBI tau pathology.

4) Unpublished data on sertraline treatment for depression-like behavior in rcTBI.

5) Open questions for future research.

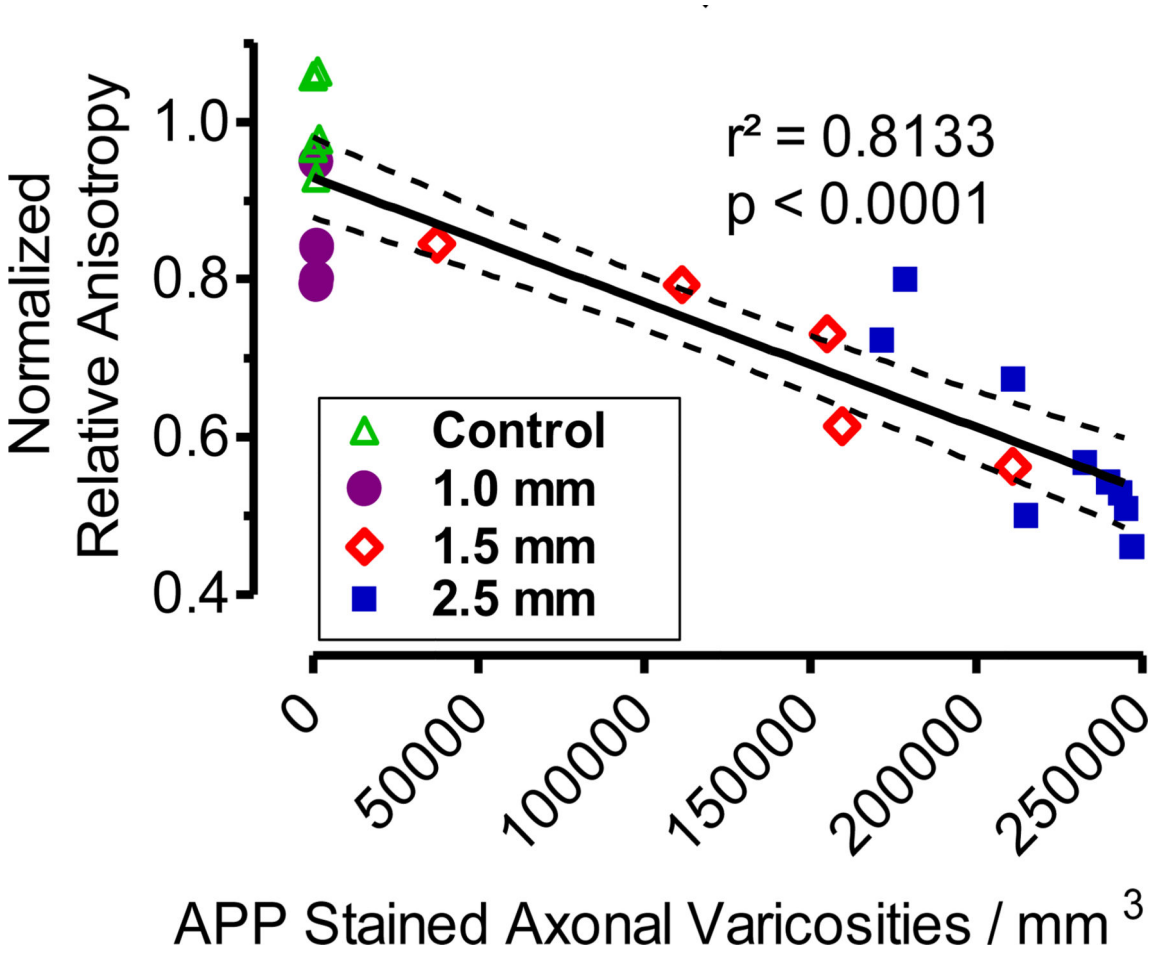

Figure 1. Diffusion tensor imaging in a mouse model of pericontusional traumatic axonal injury

Mice were injured with controlled cortical impact at 3 different severities (1.0 mm, 1.5 or 2.5 mm impact depth), scanned with DTI 24 hours later, and then sacrificed for quantitative histological assessment of axonal injury using stereological counting of APP stained axonal varicosities. Methods were otherwise as previously described (Mac Donald et al., 2007a; Mac Donald et al., 2007b). Notably, relative anisotropy was reduced in the least severely injured mice (1.0 mm) even though essentially no dilated, APP-immunoreactive axons were observed. Subsequent work has demonstrated the presence of non-dilated, APP-negative injured axons following less severe injuries. (Adapted from Brody et al, “Current and Future Diagnostic Tools for Traumatic Brain Injury: CT, Conventional MRI, and Diffusion Tensor Imaging” Handbook of Neurology, 2015 *in press*)

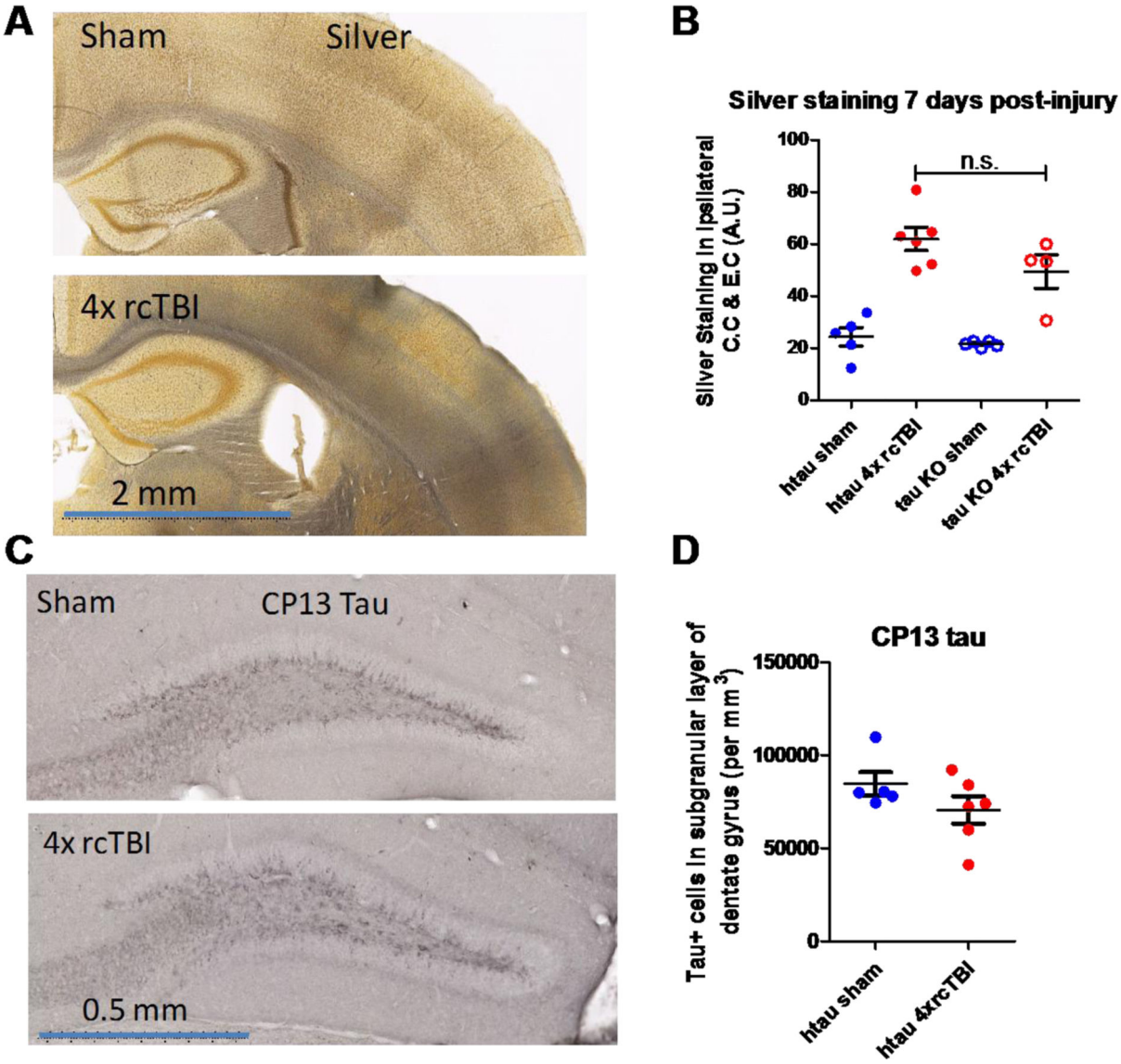

Figure 2. No effects of repetitive concussive TBI on tau immunostaining in hTau mice

6-8 week old hTau mice or tau knockout littermates were injured with 4 concussive impacts 24 hours apart, then sacrificed 7 days later. **A.** The injuries caused extensive silver staining in the cortex, corpus callosum (C.C.), external capsule (E.C.) and thalamus comparable to the results previously shown for 2 concussive impacts (Shitaka et al., 2011). **B.** Quantification of silver staining in the white matter by blinded observers by densitometry revealed substantially increased staining in injured mice compared with shams ($p<0.001$, 2-way ANOVA followed by Tukey post-hoc test. However, there was no difference in silver staining between the injured hTau mice and identically injured tau knockout littermates (A.U.: arbitrary units). **C.** Tau immunohistochemistry using CP13, a monoclonal antibody recognizing phosphorylated tau. No difference was apparent in the dentate gyrus between sham and injured hTau mice. **D.** Quantitative unbiased, blinded stereological analysis of CP13 positive cells in the subgranular layer of the dentate gyrus. No difference was found between groups ($p=0.19$, Student's t-test).

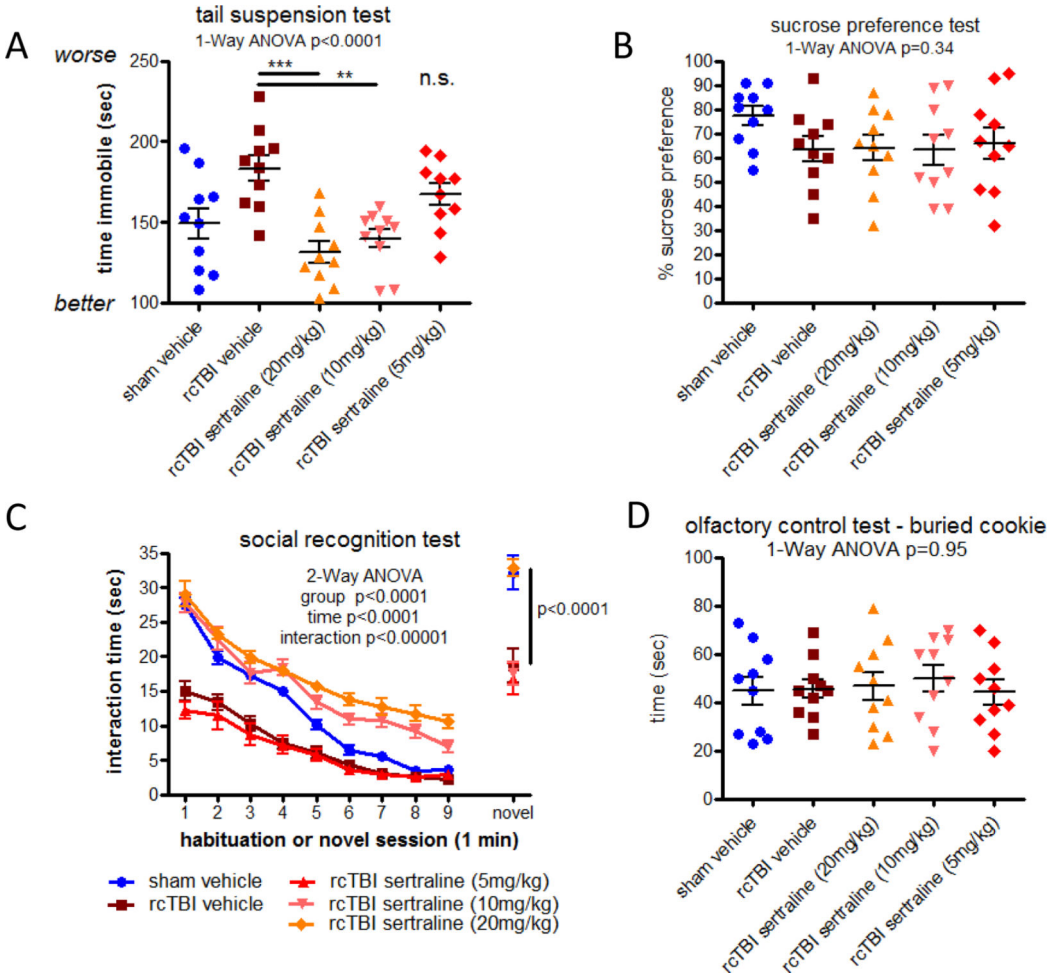

Figure 3. Sertraline treatment improved depression-like behavior and social interaction following repetitive concussive TBI plus foot shock stress

6-8 week old male C56Bl6 mice were subjected to 2 concussive TBIs 24 hours apart, then foot shock stress plus extinction training (Klemenhagen et al., 2013). Seven days later, mice were randomly assigned to sertraline vs. vehicle administered in the drinking water daily for 3 weeks prior to behavioral testing. **A.** Sertraline dose dependently reduced immobility time in the tail suspension test, as scored by a blinded observer. **B.** No effect of sertraline and little effect of injury plus shock stress in the sucrose preference test. **C.** Sertraline dose dependently increased social interaction in the social recognition test during both the initial 9 trials and the final trial with a second novel mouse, as scored by a blinded observer. **D.** No effect of injury plus stress or sertraline on olfactory function as measured using the buried cookie test.

Table 1

Approaches to assessing non-dilated injured axons following rcTBI

	Advantages	**Disadvantages**
Quantitative Electron Microscopy	Highest standard of sensitivity May provide insight into mechanisms (myelin damage, cytoskeletal disruption...)	Slow, labor intensive, expensive, prone to fixation artifacts, challenging to combine with molecular specific assessments Small regions assessed Manual counting of axons required
Silver staining of conventional tissue sections	Relatively fast and inexpensive Large regions of tissue assessed	Unknown mechanism of staining Nonspecific Not fully quantitative
Superresolution fluorescence microscopy	Potentially high sensitivity Potential to provide insight into mechanisms through molecular targeted probes.	Slow Very expensive Small regions assessed Unknown artifacts Will require extensive validation
Array tomography-based immunofluorescence microscopy	More sensitive than conventional fluorescent microscopy Faster than electron microscopy and superresolution microscopy Molecularly targeted probes Larger regions assessed than EM or superresolution microscopy High signal to noise allowing automated counting of injured axons	Unknown sensitivity to the smallest injured axons More labor intensive than conventional light microscopy Approximately 50% of antibodies work well in resin-embedded ultrathin sections.

Table 2

Animal models of repetitive concussive traumatic brain injury

Original Citation	Animal	Injury	Interval between injuries	# of Injuries	Histological Effects	Behavioral Effects
(Kanayama et al., 1996)	Adult rat	Lateral fluid percussion	24-96 hours	2-7	Abnormal MAP2 and neurofilament staining	Increased locomotor activity, less habituation
(Laurer et al., 2001)	Adult mice	Pneumatic closed-skull 6 mm rubber tip lateral impact	24 hours	2	Axonal injury (APP staining) in thalamus. Reduced MAP2 in dendrites	Worse neuroscore, rotorod and rotating pole performance
(DeFord et al., 2002)	Young adult mice	Weight drop with foam pad allowing some rotation	24 hours	4	*(No cell death or blood brain barrier effects)*	Impaired Morris water maze performance
(Creeley et al., 2004)	Young adult mice	Weight drop with firm support	24 hours	3	Multifocal abnormal de Olmos silver staining	Impaired Morris water maze performance
(Longhi et al., 2005)	Young adult mice	Pneumatic closed-skull 9 mm silicone tip lateral impact	3, 5, 7 days	2	Abnormal Fluoro-Jade in cortex, axonal injury (APP), reduced of MAP2 staining	Impaired Morris water maze & rotorod performance with 3-5 day intervals
(Friess et al., 2009)	3-5 day old piglets	Axial rotation, non impact	1 day, 1 week	2	Axonal injury (APP staining) at gray-white junction	Worse performance in T-maze and cognitive composite day 8
(Shitaka et al., 2011)	Young adult mice	Electromagnetic closed-skull 9 mm rubber tip lateral impact	24 hours	2	Axonal injury (silver staining, electron microscopy) and Persistent microgliosis	Impaired Morris water maze performance
(Meehan et al., 2012)	Adult mice	Weight drop allowing rotation	1 day 1 week, 1 month	1 3 5 or 10	*(No cell death, APP, Fluoro-Jade or TUNEL staining detected)*	Impaired Morris water maze performance with 1 day or 1 week intervals. 5 injuries at 1 day intervals still impaired 1 year later
(Mouzon et al., 2013)	Young adult mice	Electromagnetic closed-skull 5 mm metal tip midline impact	48 hours	5	Cerebellar hemorrhage, axonal injury (APP), microgliosis, and astrogliosis	Impaired rotorod and Barnes maze performance
(Petraglia et al., 2014a)	Adult mice without anesthesia	Lateral closed skull rubber impact onto helmet. Foam pad allowing rotational acceleration	6 per day × 7 days	42	Abnormal astrogliosis, microgliosis and tau immunohistochemistry reported separately (Petraglia et al., 2014b)	Abnormal neuroscore, Morris water maze, elevated plus maze, forced swim, tail hang, and non-REM sleep