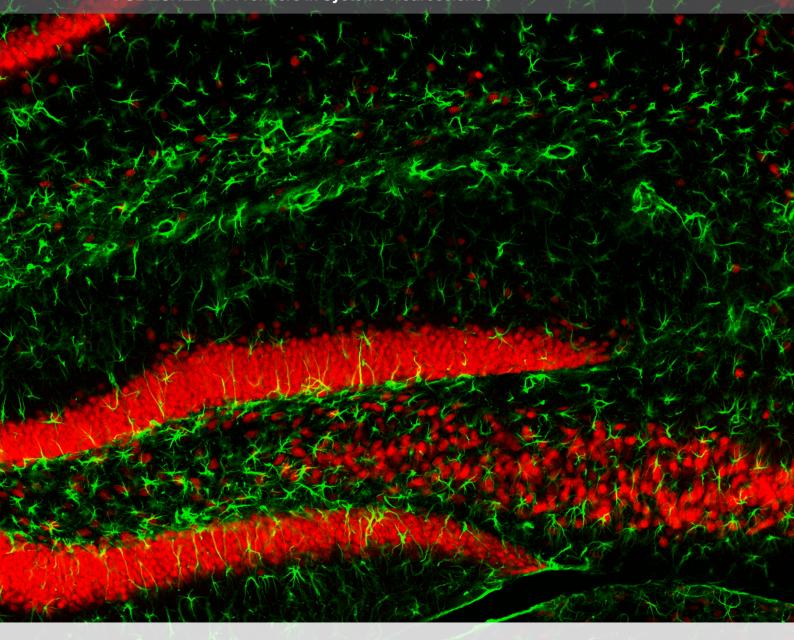
TRAUMATIC BRAIN INJURY AS A SYSTEMS NEUROSCIENCE PROBLEM

EDITED BY: H. Isaac Chen, John F. Burke and Akiva S. Cohen PUBLISHED IN: Frontiers in Systems Neuroscience







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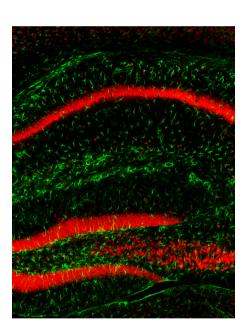
TRAUMATIC BRAIN INJURY AS A SYSTEMS NEUROSCIENCE PROBLEM

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Section of a naive mouse hippocampus depicting neurons (NeuN, red) and astrocytes (GFAP, green).

Cover picture provided by Guoxiang Xiong from Akiva Cohen's lab.

Traumatic brain injury (TBI) is traditionally viewed as an anatomic and neuropathological condition. Caring for TBI patients is a matter of defining the extent of an anatomical lesion, managing this lesion, and minimizing secondary brain injury. On the research side, the effects of TBI often are studied in the context of neuronal and axonal degeneration and the subsequent deposition of abnormal proteins such as tau. These approaches form the basis of our current understanding of TBI, but they pay less attention to the function of the affected organ, the brain. Much can be learned about TBI by studying this disorder on a systems neuroscience level and correlating changes in neural circuitry with neurological and cognitive function. There are several aspects of TBI that are a natural fit for this perspective, including post-traumatic epilepsy, consciousness, and cognitive sequelae. How individual neurons contribute to network activity and how network function responds to injury are key concepts in examining these areas. In recent years, the available tools for studying the role of neuronal assemblies in TBI have become increasingly sophisticated, ranging from optoge-

netic and electrophysiological techniques to advanced imaging modalities such as functional magnetic resonance imaging and magnetoencephalography. Further progress in understanding the disruption and subsequent reshaping of networks is likely to have substantial benefits in the treatment of patients with TBI-associated deficits.

In this Frontiers Topic, we intend to highlight the systems neuroscience approach to studying TBI. In addition to analyzing the clinical sequelae of TBI in this context, this series of articles explores the pathophysiological mechanisms underlying network dysfunction, including alterations in synaptic activity, changes in neural oscillation patterns, and disruptions in functional connectivity. We also include articles on treatment options for TBI patients that modulate network function. It is our hope that this Frontiers Topic will increase the clinical and scientific communities' awareness of this viable framework for deepening our knowledge of TBI and improving patient outcomes.

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Editorial: Traumatic Brain Injury As a Systems Neuroscience Problem

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Editorial on Research Topic

Traumatic Brain Injury As a Systems Neuroscience Problem

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Traumatic brain injury (TBI) has gained prominence in the public consciousness as a significant medical problem, especially in light of the recent conflicts in Iraq and Afghanistan and the ongoing discussions of head injuries in sports. Rightfully so, there has been significant energy invested in studying the perturbations of molecular cascades and cellular function in TBI (Mcintosh et al., 1998; Giza and Hovda, 2014; Boychuk et al., 2016) and the relevant neuropathological findings of this condition (Smith et al., 2013). Investigations in these areas have helped guide the clinical treatment of patients during the acute phase of injury and provided a link to long-term outcomes such as chronic traumatic encephalopathy and Alzheimer's disease. However, many clinical symptoms associated with TBI, including cognitive, neuropsychiatric, and consciousness disorders, and issues related to functional recovery from TBI are not easily understood within the frameworks of cellular biology and neuropathology alone. Systems neuroscience examines the activity of neural circuits and how they relate to behavior and function. TBI disrupts neural circuit function, and therefore examining TBI through the lens of systems neuroscience can generate new insights into the deficits experienced by patients and how these problems can be addressed. The objective of this Research Topic in Frontiers in System Neuroscience is to present some of the latest findings and views regarding the pathophysiology and treatment of TBI from a systems neuroscience perspective.

While myriad in presentation, many of the ailments that afflict TBI patients beyond the acute phase of the injury can be attributed to the failure of neural circuit systems. The deficits and disorders that mark the subacute and chronic periods of TBI are primary drivers of TBIassociated disability, which affect at least 5.3 million individuals in the United States (Thurman et al., 1999). This disability creates significant burdens for individual patients, their caregivers, and society at large, and it contributes significantly to the \$76.5 billion expended on TBI annually (CDC estimate; Injury Prevention and Control: Traumatic Brain Injury and Concussion, 2016). In patients with mild TBI's, upwards of 15% of patients experience persistent symptoms (Marshall et al., 2015), which can include cognitive and memory impairment, neuropsychiatric conditions (e.g., depression and post-traumatic stress disorder), and sleep disorders. With more severe injuries, these problems are accentuated, and other conditions, including movement disorders (Krauss, 2015), disorders of consciousness (Giacino et al., 2014) and post-traumatic epilepsy (Annegers et al., 1998), become more relevant. Outside the context of TBI, the abnormal neural circuitry underlying these various conditions have been the subject of significant study. However, pinpointing the network etiology of specific symptoms after TBI has been difficult because of the heterogeneous nature of the injuries and symptoms across patients. For example, the nature of the memory deficits created by TBI remains unclear, in part because both short-term and episodic memory, which are

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Chen HI, Burke JF and Cohen AS (2016) Editorial: Traumatic Brain Injury As a Systems Neuroscience Problem. Front. Syst. Neurosci. 10:100. doi: 10.3389/fnsys.2016.00100 supported by different neural circuits, are affected. This and other similar discrepancies highlight the need to investigate TBI as a systems neuroscience problem.

Given the above considerations, we believe that establishing the mechanisms by which traumatic disruption of brain networks induces deficits will require a multi-modal approach that cuts across disciplines. The first section of this Research Topic is comprised of three papers that offer different governing principles for understanding how TBI impacts neural circuit function. Bigler et al. describes how quantitative image analysis can be used to correlate changes in brain structure and connectivity to neuropsychological outcomes. Wolf and Koch posit that post-TBI deficits are due to disruptions in the timing of neuronal communication as a result of axonal injury. Carron et al. suggest that the symptomology of TBI can be viewed as aberrations of sensory system processing and that changes in cortical interneuron activity likely explain the hyperexcitability and alterations in neuronal encoding seen after TBI. These papers provide insight into how TBI perturbs brain network function and will, we hope, serve as guides for future investigations in this area.

Although the focus of this Research Topic is on the relationship between TBI and brain networks, network activity is ultimately built upon the function of individual neurons. As such, the second section of this Topic includes four papers that describe the effect of altered cellular metabolism and mitochondrial function on neural activity. Sun and Jacobs demonstrates how targeting cyclophilin-D and its effects on mitochondrial permeability transition pore opening could reverse TBI-induced abnormalities of intrinsic neuronal firing properties and reduce synaptic hyperexcitability. Continuing on the theme of mitochondrial dysfunction, Fischer et al. describe the correlation between TBI and increased mitochondrial fission, which may impair the survival of newborn neurons in the hippocampus. Wilson et al. explore how increased levels of phosphodiesterase isoforms may contribute to impaired hippocampal synaptic plasticity. Finally, Dash et al. show a decreased level of methionine and its metabolites in patients with severe TBI, which could lead to altered epigenetic regulation. These studies point to the many different cellular mechanisms that can contribute to neuronal, and thus neural circuit, dysfunction after TBI.

One of the most exciting aspects of studying TBI from a systems neuroscience perspective is the possibility of developing novel therapies specifically for circuit dysfunction. The third

and final section of this Research Topic contains four articles that examine therapeutic modalities based on how they affect brain network function. Pevzner et al. illustrate how the oscillatory activity of the brain is altered after TBI and how low-frequency stimulation of the medial septum could restore normal oscillatory rhythms in cognitive circuits. Girgis et al. survey the biochemical and circuitry changes in the injured hippocampus and review other potential stimulation targets. The article by Murugan et al. documents the effects of the flavonol compound kaempferol on reversing large-scale deficits in neural activity as measured by cerebral blood flow. Lastly, Butler et al. studied how inhibiting the mTOR pathway limited post-traumatic hippocampal neurogenesis and mossy fiber sprouting, a potential mechanism for suppressing post-traumatic epileptogenesis. These articles raise the possibility of a new generation of more effective interventions for TBI.

One of the primary reasons that TBI continues to have a widespread and devastating impact on patients is that there are few options for treating the long-term sequelae of this condition. Brain network activity is the closest biological correlate to clinical function, and thus it makes sense to study neural circuits and their dysfunction after TBI as a means of identifying new therapeutic targets. In this Research Topic, we have highlighted several different approaches for examining TBI-induced changes in the function of neural circuits and individual neurons. Future studies should continue this trend of studying network dysfunction after TBI and linking changes in neuronal metabolism and gene expression to neural circuit activity. Special attention will need to be paid to understanding how heterogeneous injuries can lead to common symptoms. We believe that this systems neuroscience approach will promote new interpretations of TBI, which will lead to novel therapeutic interventions, such as those presented in this Topic, and improved clinical outcomes for patients.

AUTHOR CONTRIBUTIONS

All 3 authors were co-editors on the Research Topic entitled, "Traumatic Brain Injury As a Systems Neuroscience Problem."

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Systems Biology, Neuroimaging, Neuropsychology, Neuroconnectivity and Traumatic Brain Injury

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The patient who sustains a traumatic brain injury (TBI) typically undergoes neuroimaging studies, usually in the form of computed tomography (CT) and magnetic resonance imaging (MRI). In most cases the neuroimaging findings are clinically assessed with descriptive statements that provide qualitative information about the presence/absence of visually identifiable abnormalities; though little if any of the potential information in a scan is analyzed in any quantitative manner, except in research settings. Fortunately, major advances have been made, especially during the last decade, in regards to image quantification techniques, especially those that involve automated image analysis methods. This review argues that a systems biology approach to understanding quantitative neuroimaging findings in TBI provides an appropriate framework for better utilizing the information derived from quantitative neuroimaging and its relation with neuropsychological outcome. Different image analysis methods are reviewed in an attempt to integrate quantitative neuroimaging methods with neuropsychological outcome measures and to illustrate how different neuroimaging techniques tap different aspects of TBI-related neuropathology. Likewise, how different neuropathologies may relate to neuropsychological outcome is explored by examining how damage influences brain connectivity and neural networks. Emphasis is placed on the dynamic changes that occur following TBI and how best to capture those pathologies via different neuroimaging methods. However, traditional clinical neuropsychological techniques are not well suited for interpretation based on contemporary and advanced neuroimaging methods and network analyses. Significant improvements need to be made in the cognitive and behavioral assessment of the brain injured individual to better interface with advances in neuroimaging-based network analyses. By viewing both neuroimaging and neuropsychological processes within a systems biology perspective could represent a significant advancement for the field.

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Bigler ED (2016) Systems Biology, Neuroimaging, Neuropsychology, Neuroconnectivity and Traumatic Brain Injury. Front. Syst. Neurosci. 10:55. doi: 10.3389/fnsys.2016.00055 Keywords: traumatic brain injury (TBI), neuroimaging, computed tomography (CT), magnetic resonance imaging (MRI), systems biology, connectivity, neuropsychology, quantitative image analysis

The International and Interagency Initiative toward Common Data Elements (CDE) for Research on Traumatic Brain Injury (TBI) and Psychological Health (see Menon et al., 2010) defines TBI as "... as an alteration in brain function, or other evidence of brain pathology, caused by an external force (p. 1637)" where severity is most commonly characterized by whether there was loss of consciousness (LOC) including its duration, post-traumatic amnesia (PTA) and/or Glasgow

Coma Scale (GCS) ratings. While these features of TBI are important descriptors of the injury they provide only limited information about underlying neuropathology, or how the injury may relate to outcome but often, are the only uniform descriptors of a brain injury used clinically or in research, especially in neuropsychological outcome studies. The problem with this approach is immediately grasped by viewing Figure 1. Patients with identical GCS scores, or whether LOC occurred or not, may have similar or widely diverse neuropathological findings on magnetic resonance imaging (MRI) at the same chronic stage post-injury. If a neuropsychological outcome study were to use only GCS, PTA, LOC or some similar injury severity rating, cases like in Figure 1 become lumped together with incredibly diverse underlying neuropathology. This diversity of pathology also means that any singular neuroimaging metric used to assess pathology will underestimate the totality of pathological effects or fail to even detect presence of a pathological change in the brain brought on by the trauma.

The basis for much of the confusion generated in the neuropsychological literature about TBI outcome is likely the result of combining cases with differing TBI-related pathology examined only with basic neuroimaging metrics. For example, in Figure 1 the axial images from a MR scan of two individuals who sustained severe TBI are shown on the right side of the figure. One demonstrates no observable gross pathology while profound abnormalities are distinctly visible in the other. In the child with extensive structural pathology there is parenchymal loss, shape distortion and multiple variations in MR signal intensity that deviate from the norm, each indicating differences in the types of neuropathological changes that have occurred. For the two cases with mild TBI (mTBI) shown on the left of the figure, one had a sizeable frontal lesion, the other no abnormality, just like one of the severe TBI cases (upper right). Also evident from viewing Figure 1 is that there is a tremendous amount of information in those images about the size, volume, shape, length, thickness, etc., of brain structures, as well as visible pathology when present, all of which can be quantified. Improved identification and quantification of brain images, including a multi-modality approach to comprehensively identify abnormalities should improve the predictive ability of neuropsychological outcome studies and likewise better inform treatment and follow-up for the TBI patient. However, what neuroimaging measures to use and within what framework TBI neuropathology is identified represent complex, unresolved issues and the basis for this review.

Masel and DeWitt (2010) argue that TBI should not be viewed as an event, but as a disease process (see also, Masel, 2015). This makes sense because even though TBI clearly has an exogenously defined onset, as stated in the definition above, the injury sets into motion a cascade of various pathological effects (Johnson et al., 2013, 2015; Smith et al., 2013; Armstrong et al., 2015, 2016; Mierzwa et al., 2015), some of which may be purely short-lived and transient, while others are chronic. Chronic effects from TBI are sufficiently common and disabling that TBI meets criteria as a disorder with a major worldwide disease burden (Olesen and Leonardi, 2003). Since there

is a time-dependent staging to injury effects, neuroimaging analyses need to be dynamic (Kim and Gean, 2011). If there are a multitude of pathological factors initiated by the injury, then characterizing them by various features extracted from neuroimaging variables should not be singular but as comprehensive and thorough as possible. As pointed out in Figure 1, it is a mistake to just characterize TBI by one of the markers of injury severity. It would be equally a mistake to characterize the neuroimaging identified neuropathology by a single measure (i.e., presence/absence of a focal lesion). But how should neuroimaging findings be analyzed, within what theoretical framework and how should these metrics be applied to outcome research and clinical use? What are some of the best ways to conceptualize traumatically induced neuropathology using current neuroimaging technology? These are the issues of this review.

Returning to Figure 1 the abnormalities that are highlighted reflect differences between each patient and likely relate to different aspects of TBI pathology. Given this striking heterogeneity, it immediately becomes apparent that there is no universally occurring "lesion" in TBI. It would also be unsatisfactory to approach this within a simple framework of the size or just where a definable abnormality may be located, which up to this time has been a common approach to neuropsychological outcome studies. Additionally, as will be explained more fully below, the information contained within a MR scan is unique to that individual, but most TBI studies approach neuroimaging analyses via group data comparisons. Whatever neuroimaging analysis tools emerge, they must be able to account for individual differences in brain structure and function but also appropriately identify all types of pathology potentially discernable from an image.

systems biology framework for understanding neuroimaging findings and their relevance to neuropsychology seems a most appropriate next step to improve understanding of the effects of TBI. Adapted from Vodovotz and An (2015), Figure 2 depicts a common "systems" approach applied to any disease or disorder. Such an approach emphasizes tissue, organ and systemic levels of an integrated system influencing health and dysfunction. As depicted in Figure 2, neuroimaging can inform every level of the system, but to understand the significance of the neuroimaging finding at each level within the system, one needs to know how neuropathological changes are manifested in scan findings and whether such findings influence behavior, emotion and/or cognition. So added to the schematic offered by Vodovotz and An (2015) is the potential value of neuroimaging which appears to be well equipped to address these three levels of a systems approach. When neuroimaging is combined with neuropsychological techniques, it would seem to be a recipe for a more comprehensive explanation of TBI outcome. Of course, the "systems" approach presented in Figure 2 begins at the tissue level that includes the cellular, metabolic and molecular and it is at these levels where the TBI story begins. What can neuroimaging inform about cellular pathology when the conventional MRI standard of image acquisition is based on an inferred slab of tissue that is a cubic millimeter thick?

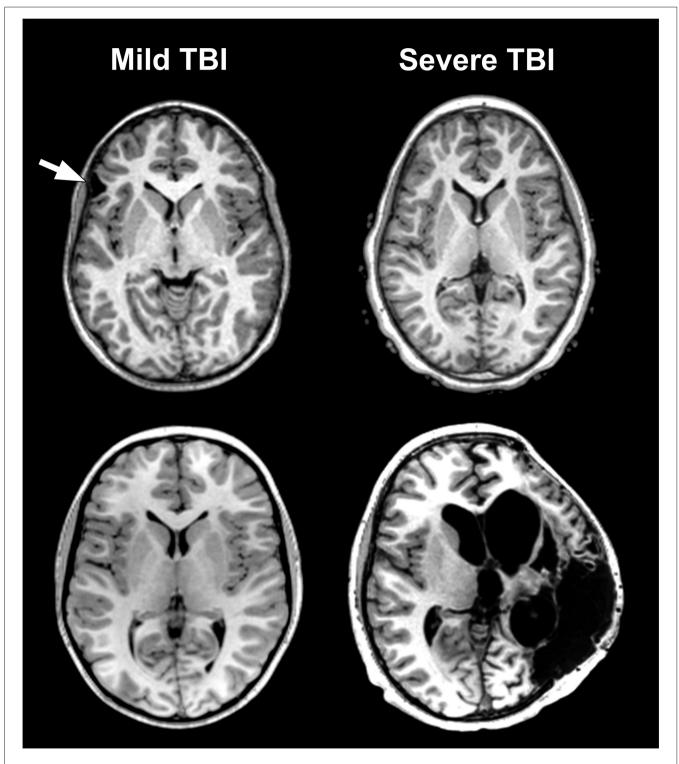


FIGURE 1 | The problem of traumatic brain injury (TBI) severity classification by using the Glasgow Coma Scale (GCS) is the wide disparity of structural pathology that may be present for a given classification level. Traditionally, mild TBI (mTBI) has been classified by GCS scores between 13–15. The arrow in the case presented in the upper left depicts a prominent focal area of frontal encephalomalacia as a residual from an old contusion in this individual who had a mTBI and an initial GCS = 14. Note that the asymmetry of the anterior horn of the lateral ventricular system on the side of the lesion that is likely a subtle reflection of greater parenchymal volume loss surrounding the side of the lesion. In contrast, the case in the lower left or the one in the upper right have no visible abnormality, despite GCS scores of 15 and 3, respectively. Finally, the obvious massive structural damage in the lower right is from a TBI patient with severe TBI and GCS of 3. The case in the upper left had a reported brief loss of consciousness (LOC) but the other individual with mTBI (lower left) did not. Both severe TBI cases also had positive LOC.

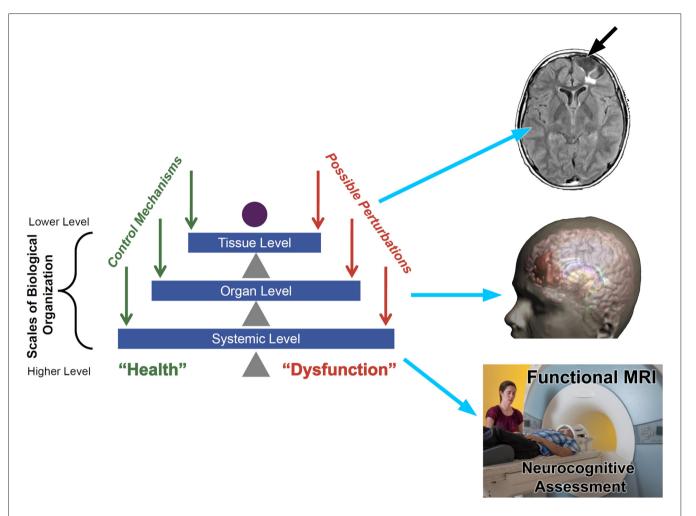


FIGURE 2 | Taken from Vodovotz and An (2015) the left side illustrates a pyramid view of a traditional systems biology approach from the tissue to systemic level where to the right, potential neuroimaging approaches to assess each level within the system is shown. Tissue Level: upper right depicts a fluid attenuated inversion recovery (FLAIR) sequence that shows tissue loss (encephalomalacia—white arrow) and underlying region of damaged white matter (WM; bright white region). Organ Level: whole brain is depicted in the middle as a 3-D reconstruction of the head and brain where the region in red depicts the overall extent of focal encephalomalacia that was shown at the tissue level. Systemic level: whole person being behaviorally and cognitively assessed using neurocognitive assessment including functional magnetic resonance imaging (fMRI) methods while in the scanner. The "systems" illustration on the left is taken from An et al. (2012) used with permission from e-Century Publishing.

THE INJURY AND THE BIOMECHANICS OF TRAUMA

TBI begins with the event that induces the injury and therefore mechanism of injury becomes a critical variable. What is quite astonishing is that even with the most precise experimental controls applied to animal models of TBI, where the identical weight-drop, fluid percussion, blast or other experimental condition is imposed the injury and resulting histology is never identically replicated (Statler et al., 2008). If the injury cannot be precisely replicated under strict experimental control, the diversity of circumstances and mechanical forces that lead to TBI in humans means that no two brain injuries are ever alike! Now add to this the fact that each brain develops within its own unique experience dependent world under unique genetic,

environmental, nutritional, emotional and socioeconomic forces, no two brains are ever alike. In fact, several studies (see Finn et al., 2015; Ueda et al., 2015) have demonstrated that imaging findings are so individualized by distinctive differences in brain morphology that each brain has its own "neural fingerprint."

The recent study by Bigler et al. (2016) is an example of the uniqueness of focal brain injuries and mechanism of injury within a large study of 251 pediatric cases where TBI was assessed, focused on identifying cases of mTBI (GCS \geq 13). All patients were assessed within an emergency department (ED) soon after the injury. In those meeting criteria for having sustained a TBI, there were over 30 different categories related to mechanisms of injury (falls, sports related, motor vehicle, etc.) and when visible MR pathology was identified, there were no

two children with pathology that was similar in size, location, distribution or identically overlapped. Also, when pathology was identified, it varied depending on the MR sequence used. All of this underscores the uniqueness of each TBI for each individual who sustains a brain injury. More to be written about later in the review.

Much of the pathology from trauma occurs as the result of tissue deformation that involves strain-related responses of the brain to impact dynamics influenced by the shape of the brain and its relation to the skull, meninges and vasculature (Bigler, 2007). The degree of deformation is influenced by accelerationdeceleration forces where the magnitude and directionality of change predict where the greatest shear-strain forces occur (Zhao et al., 2016). The biomechanical events associated with traumatic injury place tensile strain on axons which depending on the location and amount of those forces, neural tissue becomes deformed beyond biological tolerance resulting in axon damage and other ultrastructure pathology (Cloots et al., 2013; Wright et al., 2013; Sullivan et al., 2015). While true shear lesions occur (Peerless and Rewcastle, 1967), the term traumatic axonal injury or TAI may better characterize much of the microstructural damage because it includes a combination of pathological factors (Bigler and Maxwell, 2012).

To highlight the different sensitivities of MR technology in studying TBI, a case study will be used to illustrate what information can be extracted from a scan, some methods for image analysis and how different scan parameters lead to detecting different aspects of pathology. Another advantage for using a single case study opposed to group analyses is that there will be no need to provide additional demographic and injury details for a single subject, which would be required in a group analysis. Returning to what is outlined in Figure 2, the case study selected for an in-depth review should reflect the different levels of targeted information that a systems biology approach could identify to elucidate the effects of TBI. By using a case study approach, specific neuroimaging details about pathological identification that are unique to the individual can be extracted that otherwise would be lost in group data comparisons. Nonetheless, each of the points discussed in the identification of neuroimaging based pathology in this case study approach provides the basis for further empirical investigation at a group level. In-depth review of neuroimaging findings in TBI are covered in the original text by Gean (1994) titled Imaging of Head Trauma and recently updated (see Gean, 2015).

The child participant selected and highlighted as the case study in this review was from the Social Outcomes of Brain Injury in Kids (SOBIK) investigation, details of which have been previously published (Bigler et al., 2013; Dennis et al., 2013; Yeates et al., 2013). Hereafter, this will be referred to as the "TBI Case Study". All aspects of the study had institutional review board approval and followed all ethical guidelines for human neuroimaging and neuropsychological studies. MRI findings reported herein were obtained from a 1.5 Tesla General Electric Signa Excite scanner using the following image sequences and parameters: sagittal acquisition 1.2 mm thick T1 FSPGR IR (repetition time (TR): 3.86 ms, echo time (TE): 1.47 ms); 5.0 mm

thick dual echo proton density/T2 acquired in the axial plane (PD settings at 2800.00 ms for TR and 30.00 ms for TE); T2 settings at 2800.0 ms for TR and 90.0 ms for TE; Fluid attenuated inversion recovery (FLAIR) sequence was 3.0 mm thick (TR: 10002.00 ms, TE 111.89 ms) and the gradient recalled echo (GRE) sequence was also 3.0 mm thick with a TR: 567.0 ms with a TE of 15.0 ms. The SOBIK study was in part classroom based and therefore children within the study had to have sufficiently recovered from their TBI to be mainstreamed into some level of regular classroom placement. When scanned and tested at 12 years 4 months of age this male child obtained the following scores on the Wechsler Abbreviated Scale of Intelligence (WASI): full scale standard score of 109 (WASI Vocabulary T-score = 51, WASI Matrix Reasoning T-score = 59) with a Wechsler Intelligence Scale for Children—IV Edition Processing speed Index (PSI) score of 94. The child was originally injured when 8.5 years of age.

Clinically, computed tomography (CT) is typically the first scan done when someone is acutely injured and meeting criteria for neuroimaging. Figure 3 is the day-of-injury (DOI) CT scan in the TBI Case Study child who had sustained a severe TBI (GCS = 3) as the result of a motor vehicle accident. DOI imaging is always minutes to hours after the initial injury so clinical neuroimaging never captures the immediate effects of the trauma. In this child, based on available information, the initial CT was performed at least an hour after injury, where at the scene as well as ED, GCS was rated a three. As depicted in Figure 3, CT findings demonstrated scattered hemorrhagic shear lesions and contusions. In the multisite NIH sponsored "Transforming Research and Clinical Knowledge in TBI (TRACK-TBI)" study intraparenchymal shear injuries as viewed in Figure 3 were classified as "hemorrhagic axonal injury" (see Yuh et al., 2013). Also, within the TRACK-TBI study, TAI and diffuse axonal injury (DAI) were defined by NIH CDE criteria where 1 to 3 foci were designated as TAI and >4 foci as DAI (Duhaime et al., 2010). These lesions are thought to occur as a consequence of shear forces deforming parenchyma and blood vessels sufficient to mechanically disrupt and/or tear tissues and the vasculature, along with surface contusions against the boney cranial fossa (Bigler, 2007). Importantly, note in the two axial views, in radiological perspective with the patient's right side is on the viewer's left, that the soft tissue swelling (see red arrow), where initial impact occurred, is on the right. This swelling is in the contralateral posterior aspect of the other hemisphere, opposite to where the greatest amount of traumatic shear/contusion injury occurred in the inferior left frontal region. This represents a classic coup (right parietal, initial impact) with greatest injury in a contrecoup distribution involving the left frontal lobe, as depicted in the upper right image of Figure 3. Note also, that there is a small area of hemorrhage underneath the site of the coup impact area on the right which could represent a region of contact surface contusion, but the majority of the hemorrhagic frontal lesions are within the parenchyma, with some extending to the left temporal lobe as well.

Viewing these types of focal injuries, the next important consideration to understand is the distribution of biomechanical

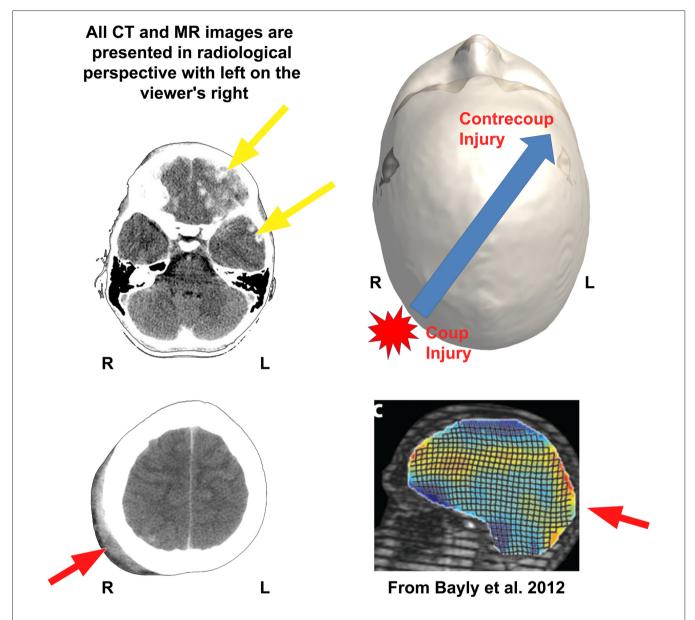


FIGURE 3 | The shear-strain effects of impact head injury are shown in the case study presented in this review. The day-of-injury (DOI) computed tomography (CT) of this child when originally assessed in the emergency department (ED) shows the initial impact—coup injury with soft-tissue swelling outside the skull (red arrow, bottom left or red star in upper right)—influences the direction of greatest biomechanical deformation of brain parenchyma but it occurs opposite the point of impact as the contrecoup injury in the left frontal region (top yellow arrow). The red arrow in the bottom left CT shows soft tissue swelling of the scalp over the posterior right skull signifying the general area of head impact. (Note that the CT images on the left are in radiological perspective). Some of the energy of that impact becomes translated forward and opposite (contrecoup) resulting in multiple areas of surface contusion and hemorrhagic shear injury in the left frontal (top yellow arrow) and temporal lobe regions (bottom yellow arrow in the upper left CT). Although there is subtle hemorrhage (white arrow, lower left image) associated with the coup injury, the greatest amount of damage occurs secondary to the contrecoup injury (yellow arrow), where there is extensive hemorrhagic contusion and shearing involving the inferior left frontal and temporal areas. These regions of damage over time result in extensive encephalomalacia and degenerative changes as depicted in Figures 4, 6-8. The upper right image is a dorsal view of a 3-D recreation of this child's head showing the point of impact and direction of the brain displacement resulting in the contrecoup injury (to be consistent with the radiological perspective of CT presentation on the left, please note that in the 3-D reconstruction of the head in this Figure, left is on the viewer's right). Reproduced with permission from Bayly et al. (2012) in the lower right corner is a biomechanical demonstration of brain deformation that occurs with a head drop of 2 cm. Note that the

distortion of the brain that results in this type of damage. Understanding the biomechanics of injury provides clues as to which regions within the brain will receive the greatest shear/strain; hence, where likely pathology may be observed

as a result of the greatest deformation of brain parenchyma. Much of the shear/strain pathology occurs at the cellular level, but understanding where and how the macroscopic lesions occur informs where other cellular pathology may reside.

Bayly et al. (2012) modeled an occipital impact using a "tagged" MRI protocol and examined deformation strain within the brain. While in the MR scanner, this involved a slight head drop into a sling resulting in \sim 2-3 g, where, g is the acceleration of gravity. This is also depicted in Figure 3. With such minor g-forces, no injury occurs and the brain tolerates minimal amounts of movement like this on a regular basis such as with jumping and landing. Nonetheless, the tagged lines which should appear as a rigid grid of intersecting parallel and perpendicular lines, if not deformed, show a wave action throughout the brain with a distinct coup to contrecoup motion. For the actual acceleration/deceleration injury that occurred in the "TBI Case Study" within milliseconds there was rapid and massive movement of the brain within the cranium to produce this lesion pattern which would have put considerable strain across deep white matter (WM) regions including the corpus callosum and especially long coursing fasciculi and subcortical areas, along with the secondary frontal impact. As depicted in Figure 3, because of where the scalp soft-tissue swelling was located, a point of impact was likely on the right posterior lateral aspect of the head with energy translated forward, after the initial impact. Because of the position of the anterior and middle cranial fossa, with this kind of impact there will likely be deformation against boney ridges (Bigler, 2007). As will be shown with MRI studies, depicted in subsequent illustrations, this kind of coup-contrecoup pattern of injury results in multiple isolated as well as dispersed lesions more than just the hemorrhagic shear injuries within the frontal lobe readily viewed in Figure 3. Indeed, part of the reason for this child having lost consciousness with persistent low GCS for several days is the likely disruption of upper brainstem WM tracts that involve the reticular activating as well as diffuse thalamic

projection system (Jang et al., 2015), along with the generalized cerebral edema, for which this child underwent shunt placement for better management of intracranial pressure (see Figure 4). Significant strain effects will not necessarily produce classic shear lesions, but may nonetheless induce cytoskeletal changes or alter synaptic integrity that may render inefficiencies in neural conduction. This combined with inflammatory changes result in complex cellular and metabolic pathologies occurring throughout the injured brain. As compelling as the CT images are that a major aspect of trauma-related pathology has been captured, as will be shown the coarse abnormalities in Figure 3 are just the beginning. Remember, the premise in this review is that lesion evolution in TBI is dynamic and especially in the first weeks to months post-injury, ever changing. Therefore, whatever neuroimaging feature is captured at a given time point is but a snapshot at that point in time postinjury.

There is another element associated with the biomechanics of brain injury also revealed by the tagged lines in the model shown in **Figure 3** which shows that the movement is never uniform and varies across different brain regions and sites depending on the moment (within milliseconds) from the point of impact to when parenchymal distortion occurs. As has been shown by finite modeling (Kraft et al., 2012), axons are therefore not uniformly affected within a strain field. So depending on the impact energy translated to the brain, the unique and individualized conformity of the brain within the cranial vault, the rotation and distribution of energy forces and brain displacement, some axons may be injured, others not affected. Just these factors alone create heterogeneity so that no two brain injuries are ever identical.

Figure 4 also depicts follow-up CT imaging within the first 2 months of injury along with a T1 weighted MR

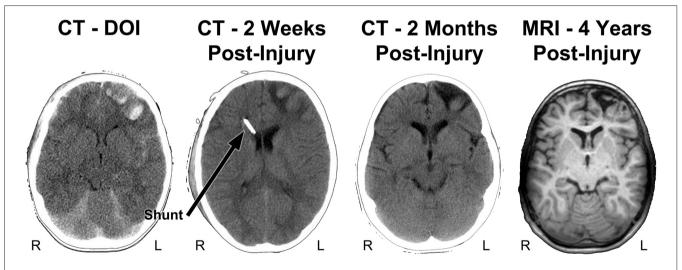


FIGURE 4 | Parenchymal changes over time from the DOI scan to 4 years post-injury. The DOI through the scan at 2 months are all from CT and the far right image is a T1-weighted MRI, at 4 years post-injury. In contrast to the CT images shown in Figure 3, the slice level of these images are all at the level of the anterior horn of the lateral ventricle. Note that where the most prominent acute hemorrhages and frontal contusions occur on the DOI scan that this also becomes the center-point for tissue dissolution (encephalomalacia) over time. Shortly after the DOI scan was obtained the child received an intraventricular shunt to aid in intracranial pressure reduction and monitoring. By 2 months post-injury the focal encephalomalacia is very prominent (white arrow) and there is also prominence of the Sylvian fissure (black asterisk) along with ventricular enlargement, indicative of some generalized cerebral volume loss. R, right; L, left.

image at a generally similar level obtained 4 years post-injury. As reflected in the initial images not only are the prominent frontal hemorrhages visible but the brain is swollen, there is subarachnoid blood and loss of sulcal definition throughout the brain and an initial decrease in ventricular size because of generalized cerebral edema. Taken together these initial findings are consistent with diffuse, generalized swelling of the brain. The shunt catheter is viewed in the right anterior horn of the lateral ventricle, used to assist in managing intracranial pressure at the 2-week post-injury scan. The inflammatory aspect of brain trauma represents another pathological feature of the brain injury especially influential at the cellular level in terms of metabolism and blood flow, often considered as a secondary cause of damage following the initial brain injury (Bramlett and Dietrich, 2015). While this review will remain focused on just the structural side of neuroimaging, there are numerous neuroimaging methods including MR spectroscopy, MR-based blood flow measures along with positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging that can be used in studying TBI (Amyot et al., 2015; Irimia and Van Horn, 2015; Sundman et al., 2015; Wilde et al., 2015; Currie et al., 2016). These later techniques provide more direct information about actual brain activity and metabolic functioning, which is not reflected in just a structural MR scan (Irimia and Van Horn, 2015; Wilde et al., 2015; Koerte et al., 2016). It is beyond the scope of this review to go into any detail of these techniques in reference to neuropsychological outcome studies, but the reader is directed to an entire issue of Neuropsychology Review edited by Sullivan and Bigler (2015) that summarizes the utility of these measures in neurocognitive and neurobehavioral studies.

Beyond the initial impact dynamics and focal pathologies that occur on the DOI, as a consequence of the generalized cerebral edema secondary injury and brain insult occurs (Yu and Kobeissy, 2015). Accordingly, some non-specific cerebral damage evolves over time that is a consequence of the primary and secondary effects of the trauma. As shown in Figure 4 these changes over time can be assessed with structural imaging, often reflected as changes in brain volume. Viewing the DOI CT findings in **Figure 4**, the anterior horns of the lateral ventricular system have a rather slit-like appearance, probably a reflection of compression from generalized cerebral edema when initially scanned. However, at all follow-up time periods the ventricular system is larger, which can be quantified by measuring what is referred to as the ventricle-to-brain ratio or VBR. VBR can be used to measure how much the ventricular system changes with edema or can be used as a global index of cerebral atrophy (Bigler et al., 2004). When the VBR was done at 4 years post-injury, this child's VBR was elevated in excess of two standard deviations compared to a normative sample, signifying generalized volume loss.

WHAT LESION TO MEASURE?

As shown in **Figure 4**, within 2 weeks significant parenchymal changes appear in the form of tissue degradation from the DOI scan, reflecting the dynamic features of what was the

original traumatic injury. As already stated, quantitatively there is significant loss of parenchymal volume that can be measured as well as viewed. Since CT imaging is based on tissue density, the darker appearance in the 2-week and 2-month follow-up CT scans, where prominent hemorrhagic lesions in the left frontal lobe were originally identifiable on the DOI CT scan (bright white within brain parenchyma in Figures 3, 4), reflects loss of tissue density that evolved over time. This signifies that tissue dissolution along with absorption of much of the extravasated blood has occurred, all-the-while intracranial pressure is going down and the child is coming out of coma and improving cognitively. These observations underscore the dynamic changes and wide variability that occurs when trying to pin down and measure a particular injury-related pathology. Since major aspects of the changes visualized at 2 months become the chronic structural abnormalities viewed in the MR scan at 4 years post-injury, extremely complex histopathological, phagocytic, neuroinflammatory, vascular and structural changes are dynamically occurring in a relatively short period of time.

So when does one establish what is the lesion in TBI and when and how should it be measured? As shown in Figure 4 at 2 months post-injury a distinct region of frontal encephalomalacia has emerged (arrow in upper right image of the CT at 2 months post-injury), with what appears to be a very distinct boundary, by CT standards. Image analysis tools are readily available that could outline the boundaries of encephalomalacia and by knowing the thickness of the CT image and the distance between each scan slice, a volume estimate of the damage could be calculated. Since on CT regions of encephalomalacia reflect CSF prominence one can be certain that pathological changes in brain parenchyma would be captured by performing such an analysis. But is a clearly defined CT region of identifiable encephalomalacia in TBI the sole lesion and the best method for characterizing the damage?

The answer is no, because CT's dependence on tissue density alone provides just a unidimensional image of brain pathology. Turning to the schematic in Figure 5, too often when TBI is discussed it has been discussed and modeled only as an axonal injury within the context of DAI. However, the basic schematic shown in **Figure 5** (adapted in part from Marklund and Hillered, 2011; Gandy et al., 2014; Ling et al., 2015) shows not only the neuron, but glia including the oligodendrocyte derived myelin along with capillaries that are as small and delicate as the neural structures they feed and with which they functionally interact. There is also the extracellular matrix whose role is only becoming known, but equally important and probably involved in the pathological effects of TBI as well (Benarroch, 2015). Parenchymal damage from trauma affects all of these cellular components and fortunately, MRI has a differential capability in detecting different tissue types and related pathology. The CT in this "TBI Case Study" objectivity shows a large area of frontal encephalomalacia but does not show any associated pathology that can readily be separated into more specific components of damage related to WM, gray matter and hemorrhage as well as metabolic or blood flow changes that can be achieved with MRI. As shown in Figure 5 (see also Figures 6, 7), different MR sequences have different capabilities of detecting these different

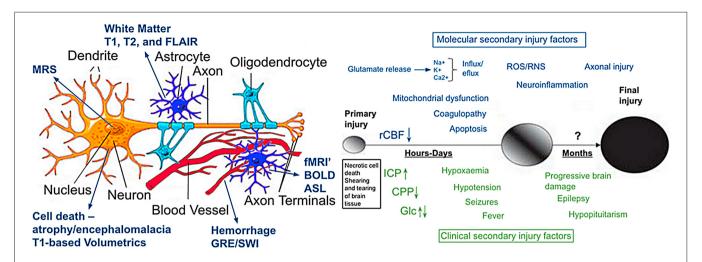


FIGURE 5 | The illustration on the left is modified from Marklund and Hillered (2011) and Ling et al. (2015) and depicts a typical CNS neuron with supporting cells and blood supply (used with permission). Although an over simplification, also outlined in the illustration is how different imaging techniques tap different components of cellular or vascular integrity. The image to the right is from Gandy et al. (2014) which they used to portray the molecular pathogenesis of TBI and CTE. It illustrates current concepts of mechanisms underlying primary and secondary injury mechanisms in TBI and how they change overtime. At early times after injury, glutamate release and ionic disturbances (Na⁺, Ca⁺ and K⁺) disrupt energy metabolism and cause other metabolic disturbances that lead to decreases in cerebral blood flow. Mitochondrial dysfunction causes increases in reactive oxygen (ROS) and nitrogen species (RNS) that can cause further cellular injury. Tissue damage evokes neuro-inflammatory changes that emerge later. Injury may be exacerbated by secondary clinical factors including hypoxemia, hypotension, fever and seizures. These secondary molecular and clinical factors lead to progressive tissue damage. Abbreviations: Ca²⁺, calcium ions; CPP, cerebral perfusion pressure; Glc, Glucose; ICP, intracranial pressure; K⁺, potassium; Na⁺, sodium; rCBF, regional cerebral blood flow. Used with permission from the British Journal of Pharmacology and Wiley Publishing.

tissue types and pathologies that relate to the source of pathology at the cellular level from TBI. At the macroscopic level, this is shown in **Figure 6** which depicts different aspects of trauma related pathology based on different MR sequences, which likely provides unique information about the injury beyond what can be gathered from just CT. Furthermore, a systems biology perspective would suggest that patterns of predominately differing pathology likely have different influences on outcome so they would need to be examined both separately as well as within the overall "system." Accordingly knowing whether predominate patterns of damage were expressed via changes in WM, gray matter or hemorrhagic pathology or combinations would likely differentially influence outcome.

Taking this latter point further, Figure 7 more specifically depicts how MRI can assist in identifying different microstructural components of the damage from TBI. Using a schematic from Armstrong et al. (2015, 2016) that illustrates how damage from TBI and shear injury may appear at the cellular level, what has been inserted into their schematic is the "TBI Case Study" MR scans that depict different pathologies. In the classic sense of stretch/deformation injury that produces DAI, as previously stated this has been operationalized as centered on the GRE or susceptibility weighted imaging (SWI) MR scan (see Yuh et al., 2013), based on the number of so-called microhemorrhages (hemosiderin foci) identified. As shown in Figures 6, 7B these hypointense (dark) signal abnormalities, also referred to as "microbleeds" show up as punctate small lesions thought to indicate where capillary rupture has occurred from shear injury or as in **Figure 6**, larger areas where prior contusion has occurred. In theory, if sufficient to shear blood vessels then sufficient to injure axons, with presumed DAI pathology to be present in association with punctate microhemorrhages. In the child featured in the "TBI Case Study" there is also clearly cortical damage, with extensive hyperintense white matter (WMH) signal abnormality observed on the FLAIR MR sequence (see Figures 6, 7D). As a consequence of the cortical damage, associated with deformation, contusion, hemorrhage and edema this WM damage as shown in the schematic may be associated with more widespread pathology and not just selective axonal damage (Figure 7D). Using FLAIR imaging, there is also the punctate WMH signal abnormality that shows up within deep WM as presented in Figure 7C in this child. WMHs have been associated with TBI, especially when they are observed at the border of the gray-white matter junction (Riedy et al., 2016), but they do occur in healthy, asymptomatic individuals with no history of head trauma, and are therefore nonspecific findings (Bigler, 2013). However, in the "TBI Case Study" highlighted in this review, the WMH signal abnormalities likely relate to the trauma sustained from the head injury but their clinical significance has not been fully determined. While GRE/SWI identified microbleeds (hemosiderin deposition) in association with WMHs relate to DAI/TAI as Riedy et al. (2016) have shown, WMHs and SWI-defined trauma-related microbleeds may not overlap (Liu et al., 2016). Therefore, at the systems level detection of abnormalities by these differing MR sequences, they may be tapping different cellular pathologies. Since in Figure 7C, there is no identifiable hemosiderin deposition in the region adjacent to the WMH does this punctate

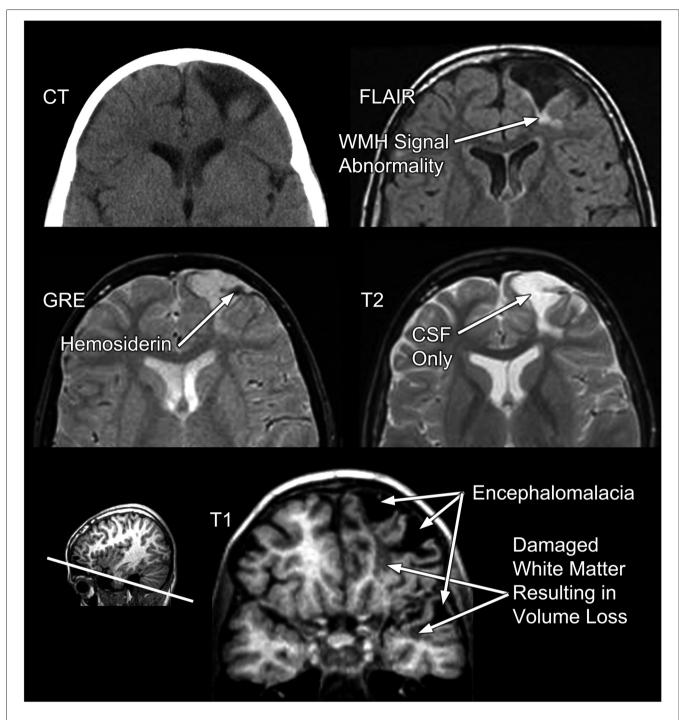


FIGURE 6 | In the same TBI patient as presented in Figures 3, 4 different MRI sequences yield different facets of the brain injury and residual pathology. FLAIR, fluid attenuated inversion recovery; MRI sequence GRE, gradient recalled echo MRI sequence. "L" refers to the patient's left.

WMH reflect a focal shear lesion or some sort of other focal lesion within the WM? In the Armstrong et al. (2015, 2016) conceptualization a focal WM disruption may reflect underlying axonal pathology that is different than TAI. Where there is more confluent hyperintense signal on FLAIR imaging, this suggests a more extensive degradation of WM integrity which is likely

a combination of multiple factors not just shearing (Bigler, 2015). When viewed where the major cortical encephalomalacia has occurred, as depicted in **Figure 7D**, beneath the large frontal defect, these WM changes likely evolve from multiple pathological factors. Clearly there was cortical destruction, but also from the DOI CT, there were shearing events that

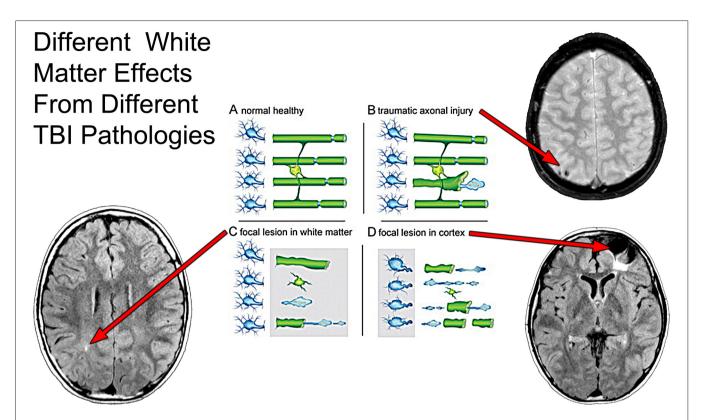


FIGURE 7 | As taken from Armstrong et al. (2015, 2016) where the effect of axon degeneration on the myelin-oligodendrocyte unit in different pathological scenarios following TBI. Schematic of a myelin-oligodendrocyte unit in the normal adult brain (A) or after different pathologies associated with TBI (B-D). In the healthy condition (A), neurons (blue) in the cerebral cortex (left side of panel) extend axons that project through the WM (right side of panel). Myelin and oligodendrocytes are shown in green. Each oligodendrocyte forms myelin sheaths around multiple axons. Nodes of Ranvier (blue) are specialized regions of axon between adjacent internodal lengths of myelin. Traumatic axonal injury (B) causes axon degeneration, as illustrated by a single damaged axon among a set of adjacent intact axons. Axon damage from traumatic axonal injury often initiates at nodes of Ranvier. Damaged axons swell, fragment, and form end bulbs with accumulations of organelles and cytoskeletal elements. Double-layered myelin sheaths often extend out from degenerating axons. The length of these myelin figures exceeds that expected from collapse of the myelin sheath around a degenerating axon. TBI can cause focal lesion areas in WM (C, gray box). Focal lesions, for example from microhemorrhages or neuroinflammation, damage a high proportion of axons, oligodendrocytes, and myelin. The cerebral cortex can undergo similar focal tissue destruction (D, gray box), particularly in cortical regions underlying the site of an impact to the head. Axons become disconnected from damaged cortical neurons. Degeneration of a high proportion of axons in a WM tract leads to subsequent myelin degradation and oligodendrocyte death. These three scenarios (B-D) focus on damage to the neuron and axon to show the relationship to the myelin-oligodendrocyte unit. Scenarios (C,D) illustrate loss of myelin as a result of overt tissue damage that includes loss of axons. None of the examples illustrates actual demyelination, i.e., loss of myelin around an intact axon, which can also occur after TBI. Superimposed on this schematic from Armstrong et al. (2015) are different MRI sequences likely sensitive to the different WM pathologies as outlined by Armstrong et al. where a focal WM hyperintensity may reflect focal WM damage (lower left image), whereas a hypointense lesion pattern may indicate TAI (upper right panel) but when there is cortical destruction underlying WM degrades as well, shown by the hyperintense signal just underneath the region of focal encephalomalacia. Used with permission from Elsevier Publishing.

occurred within the WM along with mechanical deformation of the cerebral cortex and contusion. So the WM degradation in **Figure 7D** may be a combination of cortical damage and neuronal loss as well as specific DAI and more general TAI effects within the frontal WM. Indeed, Smitherman et al. (2016) have shown that in terms of coarse prediction of pediatric outcome (Glasgow Outcome Scale-Pediatrics, GOS-P) from TBI total FLAIR-identified WM pathology was the best predictor of global outcome, but not necessarily specific neuropsychological findings.

So are the scan images present in **Figure 7**, sufficient to capture the "lesion", or is there more pathology? No, the answer is that more analysis and quantification is needed.

Returning to the T1-weighted image from an oblique coronal slice at the bottom of **Figure 6**, even at this coarse level there is distinct anatomical definition between the gray cortical ribbon and underlying WM. Furthermore, where the cortical damage has occurred there is visible reduction in the thickness of the cortical mantel that remains (compare the thickness of the right hemisphere cortical ribbon to the more damaged left). Using the T2-weighted images at the same level also helps define boundaries between CSF and what may be parenchyma (refer to **Figure 6**). Using information from these two sequences permits quantification of overall parenchymal volume loss in this region along with CSF volume increases. This was depicted three-dimensionally in **Figure 2** (the left frontal encephalomalacic

lesion is depicted in red in the "Organ" level illustration). Also the T1-weighted image can be segmented into white and gray matter and then using an open-source program like FreeSurfer¹, major ROIs can be identified, parcellated and classified such that volume can be calculated for each ROI (Bigler, 2015). Since the volume of the brain and all of its component parts are age and development dependent by assessing age-specific, healthy, typically-developed individuals a comparison sample can be generated where each ROI is compared to a normative sample. Size relates to function in a positive fashion although from a neuropsychological perspective size/volume correlates typically account for less than a third of the known variance between size/volume and function (Bigler, 2015).

From this kind of volumetric approach using automated image analysis techniques, several 100 brain ROIs can be derived, but the individual compilation of such measures becomes cumbersome when attempting to visualize where traumatic changes have occurred. Another approach is to use 3-D techniques that warp the individual brain to a normative template where a Jacobian-modulated extraction of how the

patient brain has to be altered to fit the template graphically will display where differences reside in the individual patient compared to the control sample (Avants et al., 2011a,b; Tustison et al., 2014; Khan et al., 2015). Using such an approach if there is substantial loss of parenchymal volume in the patient, the warping to fit the normative standard will depict volume reductions. This is commonly shown in shades of blue that reflect how significant the regional loss is, with the most intense blue representing the greatest volume loss or difference from the normative template. Oppositely, increases are signified by "warm" colors with the greatest increases shown as dark orange to red. Up to this point the "lesion" in this research participant has be highlighted by focal characteristics of signal differences visibly based on the native MR sequence, with the frontal encephalomalacia dominating the coarse visual changes. However, what these Jacobian maps depicted in Figure 8 show not only the expected cortical volume loss from the obvious focal lesions but actually extend to volume differences to regions where there were no visible lesions. For example, in the coronal map on the left side of Figure 8, note that in the right frontal lobe it has less volume as well, even though the T1 anatomical image does not reflect focal damage. When the ventral surface is shown (middle view) the most intense blues are in the left

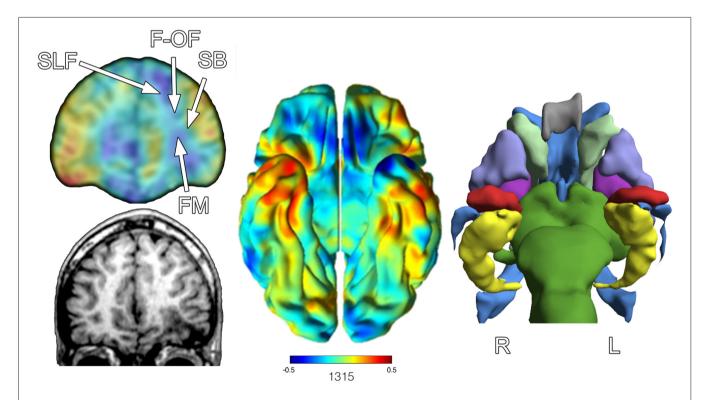


FIGURE 8 | The image on the left shows at a coronal slice level the Jacobian warping to fit the TBI patient brain to the normative control template. An actual coronal T1-weighted slice at an approximate level from the anatomical MRI is presented in the lower left. The arrow points to the region of encephalomalacia. The middle image is the full Jacobian analysis depicting the ventral surface of the brain where the degree of "blueness" demonstrates where volume loss has occurred, where oppositely warm colors reflect increase. The image to the right is from FreeSurfer output to generate a 3-D image of various ROIs: red = amygdala, yellow = hippocampus, purple = putamen, gray = corpus callosum, light green = caudate, blue = ventricle, violet = globus pallidus, darker green = brainstem.

Note that the left hippocampus, amygdala and putamen are smaller in appearance, empirically confirmed based on FreeSurfer volumetric findings in reference to an age- and demographically-matched control sample. R, right, L, left. White arrows in the upper left image depict general location of four major fasciculi that interface with the frontal lobe. SLF, Superior Longitudinal Fasciculus; F-OF, Fronto-Occipital Fasciculus; SB, Striatal Bundle; FM, Forceps Minor.

¹http://freesurfer.net

inferior frontal and temporal polar areas, nicely corresponding to where the obvious encephalomalacia has occurred but much of the inferior, orbitofrontal region of the right is also reduced. The visible effects of volume loss within subcortical areas as depicted from the FreeSurfer output as shown in **Figure 8** on the right side of the figure, demonstrates visibly smaller left hippocampus, amygdala and putamen, when compared to right hemisphere counterparts as well as to a normative sample. So not only are there "lesions" in the traditional sense of being visibly identifiable as shown in **Figures 4**, **6**, **7**, there are widespread volumetric changes that have pathologically altered brain structure.

One explanation for volume loss and changes in shape that occur distal to the focal pathology is the loss in connectivity (Hayes et al., 2016). Also shown in **Figure 8** is the relative position of major fasiculi that interface the frontal lobe with the rest of the brain. Because of the focal pathology on the left, WM tracts that would normally interconnect with the inferior frontal lobe on the right via the forceps minor (FM) drop out affecting the volume and shape of structures that were once interconnected.

As already mentioned, the GRE sequence is particularly sensitive to parenchymal iron deposition and identifying regions of hemosiderin and likely prior hemorrhage (Chappell

et al., 1992). Of particular interest from a neuroinflammatory perspective is that presence of hemosiderin may relate to localized neuroinflammation (Schrag et al., 2010; Logsdon et al., 2015). Therefore, where GRE or SWI-the SWI sequence is superior to the standard GRE—findings detect prior hemorrhages from TBI may serve a dual role: (1) demonstrates the location of prominent shearing; and (2) identify locations potentially more susceptible to inflammatory response because they indicate where focal injury has occurred. Figure 9 also includes the locations of microbleed and focal areas of hemosiderin deposition, which as visualized in this illustration may be independent of where either WM pathology or focal atrophy have occurred. This would argue that GRE/SWI abnormalities should be analyzed separately and any study that examines just one of these MR sequences will not be addressing all of the different ways in which the brain may be damaged and that damage quantified.

Does the 3-D approach presented in **Figures 2**, **8**, **9** now capture the totality of the "lesions" associated with TBI in this case study? No.

Degeneration that occurs distal to the site of axonal injury is referred to as Wallerian degeneration, but the role that such degeneration plays in TBI is not fully understood (Maxwell, 2015). Neuronal health and integrity is dependent on cellular

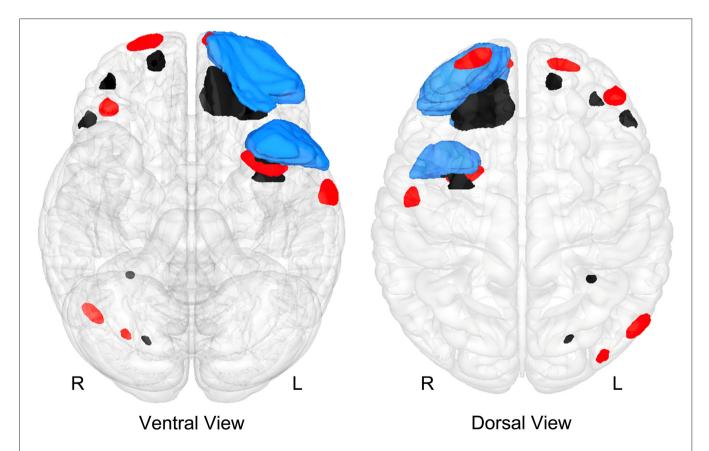


FIGURE 9 | A "glass-brain" image of the Case Study Child with TBI depicting where focal abnormalities could be isolated as identified by T1 and T2 imaging for encephalomalacia (blue), region of focal hemosiderin deposition (red) and WM hyperintensities (black). Noteworthy is that while the majority of these focal lesions involve frontal and temporal lobe regions, there is little overlap of the lesions.

interaction and when a single neuron within a network of neurons is injured, loss of trophic input to subsequent neurons in the chain will either disrupt the network, requiring network adjustment and work-around to maintain function, or the potential for downstream neuronal cell death due to lack of input (Nave, 2010; Conforti et al., 2014). What this means from a "systems" perspective is that the neuroimaging identified lesion does not occur in isolation, with potential influences very distal to where the lesion may be. As a consequence of variations of Wallerian effects, volume loss, structural changes in shape and contour may occur distal to wherever a more focal pathology may be identified, as explained above and shown in **Figure 8**; therefore, measuring just where the focal pathology is located may be misleading and incomplete in describing the totality of the effects of a focal lesion.

For example, the Jacobian-warped image of this TBI child's brain in Figure 8 (upper left image) shows volume reduction in multiple regions of the right frontal lobe that did not sustain the kind of focal damage that occurred on the left. Also of interest, note that there are also regions of volume increase as reflected in areas of red-orange (middle ventral view of Figure 8). Specifically, the temporal polar area of the right is larger than the age-appropriate pediatric template from control children without brain injury. There are no identifiable lesions in the right temporal area, but the left was injured. Since the MRI was obtained 4 years post-injury, does this represent regions of developmental compensation in the less affected right temporal lobe? Does hypertrophy in areas not or less injured reflect mechanisms of recovery? Adaptation? Not known, but an interesting speculation. What can be said quantitatively though, is rather startling. Based on the FreeSurfer lesion quantification, total lesion volume loss in this child (defined as the total of CSF in regions of focal pathology, total WM hyperintense signal and abnormal GRE findings) is close to a 100 cc, a volume loss at about 9% of total brain volume. If one now takes a conservative estimate of \sim 80,000 neurons with 4.5 million synapses per 1 mm of neural tissue (see Insel and Landis, 2013), the observable damage within these different illustrations would reflect billions of damaged or lost cells and trillions or more of disrupted or lost synaptic connections.

Despite the various objective abnormalities that these neuroimaging studies demonstrate, by GOS-P standards the child featured in these analyses has had a good outcome. As previously mentioned testing at age 12 revealed an above average FSIQ of 109. This child was also adequately functioning within a regular public school classroom placement. Given the ROI volume loss and numerous focal lesions, a conundrum emerges as to what significance to give to the abnormalities or should this be discussed in terms of potential compensatory changes and resiliency that have occurred over the 4 years since being injured? From a "systems biology" perspective, living systems do so through adaptation, so what neuroimaging shows should not just be where pathology may reside, but also how the system adapts.

From the above discussion and what has been illustrated in Figures 1-8, neuroimaging provides excellent methods for

representing TBI-related pathology at the tissue and organ level, essential from a systems biology perspective as presented in **Figure 2**. The question of what is the lesion, how measured and expressed clinically and in research has still not been answered because part of that definition will be how pathology at the tissue and organ (brain) level affects neurobehavioral functioning at a systemic level. As such, the next "systems" level discussion turns to one of neural networks and neuroimaging, in an attempt to address the bottom rung of the systems pyramid as depicted in **Figure 2**.

SYSTEMIC LEVEL IN HEALTH AND DYSFUNCTION: BRAIN IMAGING OF NETWORKS

The lesions and structural abnormalities as shown so far can be measured in MR signal intensity, volume, surface area, contour, thickness of a cortical region and their location all accurately quantified and depicted in 3-D. However, if an identifiable but abnormal signal from a particular MR sequence constitutes a "lesion" not detectable by another MR sequence means that how a "lesion" is defined and measured depends on the sequence and what is being measured. Thus, integration of all neuroimaging information needs to include all MR sequences, not just limiting consideration of information from one. As shown in **Figure 9** the locus and distribution of focal abnormalities is entirely dependent on the MR sequence where much of these different abnormalities do not overlap in the "TBI Case Study" presented in this review.

Furthermore, the neuroimaging presentation thus far has focused on just the structural side of neuroimaging and lesion identification, but not on the potential consequences of how lesions disrupt networks, from small local networks, to large integrated networks regulating behavior, cognition and emotion. The traditional size, volume and location of trauma-related abnormalities are just part of the story. Probably even more important at the systemic level, is how a lesion affects the neural network (Catani and Thiebaut de Schotten, 2008). Fortunately, there are numerous neuroimaging techniques that provide methods for defining networks and assessing their integrity (Sorg et al., 2015).

Figure 10 uses the MR technique referred to as diffusion tensor imaging (DTI) to illustrate aggregate WM tracts in the brain of the left hemisphere in a healthy individual. Returning to the structural imaging in the TBI Case Study with severe injury, as impressive as the focal pathology may be, it is not the focal damage, per se, that is most disruptive to functional outcome but how focal damage and related pathologies affect pathways and neural networks. In Figure 10, the upper left sagittal T1-weighted MRI depicts the frontal and temporal polar damage with the red line depicting the angle of the cut for the coronal image shown in the bottom left of Figure 10. Viewing the extent of the focal damage in the context of DTI-derived pathways, all of the input and output connections would be affected. Two major pathways shown in Figure 10—the arcuate (part of the superior longitudinal

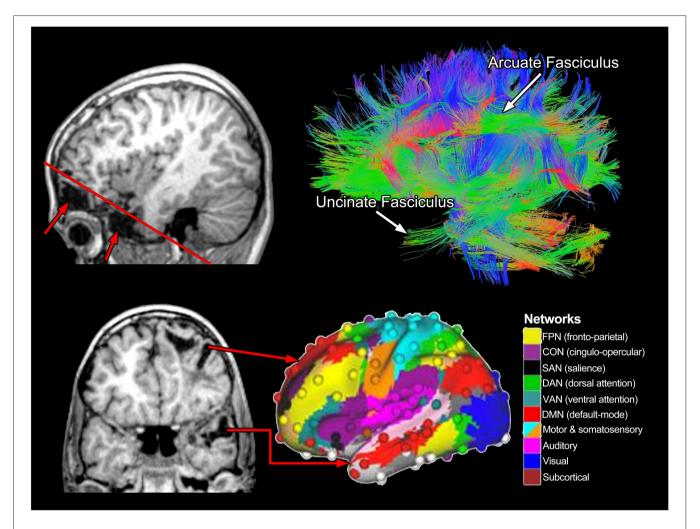


FIGURE 10 | The upper right image represents a lateral view of whole-brain tractography from diffusion tensor imaging (DTI) where two major fasciculi are identified. The color schema reflects fiber tract orientation where green reveals anterior-posterior coursing tracts, blue vertically oriented and warm colors (orange-red), reflect laterally oriented tracts. The upper left sagittal view shows the inferior frontal (top arrow) and temporal (bottom arrow) lobe encephalomalacia with the oblique red line showing the angle of the coronal cut as shown in the bottom left T1-weighted MRI. As adapted from Cole et al. (2013) and used with permission from Nature Publishing Group is a representation of various brain networks, color coded by networks identified in the right panel. The red arrows in the lower left panel come from distinct areas of damage that would likely affect various networks associated with frontal and temporal lobe function.

fasciculus [SLF]) and uncinate fasciculi (temporal lobe to orbitofrontal connectivity)—both would be disrupted along with the frontal and temporal distribution of the cingulum bundle (buried and not distinctly visible in Figure 10 (see figure caption), inferior occipitofrontal fasciculus (again buried and not specifically viewable in Figure 10 but courses along the base of the frontal and occipital lobes). These pathways actually participate in multiple networks and should not be viewed, as in earlier days of neuroscience, as being dedicated to a specific function, but to multiple cognitive and behavioral functions.

Beyond the scope of this review, but briefly summarized herein there is a tremendous amount of neuroimaging research on brain networks that involves functional MRI (fMRI) often combined with other electrophysiological measures and techniques like DTI. **Figure 10** also presents the cortical network

schematic developed by Cole et al. (2013), representative of 10 different networks derived from a combination of these techniques. From this perspective, whatever lesion or pathology may exist from a TBI, the neurobehavioral consequences will be in terms of which networks are damaged, by how much and how adaptive the networks may be after being injured (see Hayes et al., 2016).

One important feature of the Cole et al. (2013) investigation was the central role of the frontoparietal network (FPN) as an integrative and across-network connectivity center. Figure 11, adapted from Cole et al. (2013) portrays a simple network connectivity map that depicts the central role of the FPN within the overall integrated whole-brain network. In the TBI Case Study by using abnormal FreeSurfer (>2.0 SD's below an age-matched norm) volumetric ROI findings combined with where the most significant cortical

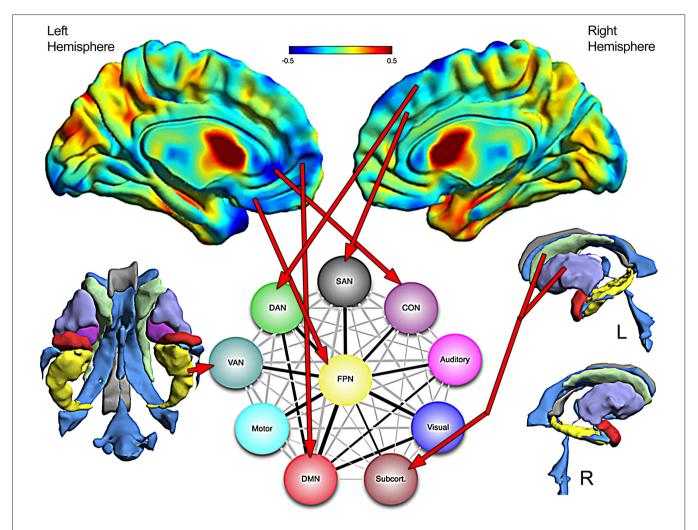


FIGURE 11 | The top images are the Jacobian warped brain maps that reflect volume loss or increase in relation to a normative template (see Figure 8 for color description). Assuming that where significant volume reduction has occurred would reflect damage to networks dependent on those areas, a schematic network map from Cole et al. (2013) is used to project where damage is and what networks would be affected. The FreeSurfer derived 3-D images of the ventral view of subcortical areas is shown on the left (without the brainstem—see Figure 8) and lateral views on the right, with red arrows pointing to networks that are likely affected by the damage. Network diagram is used with permission from Nature Publishing Group.

volume losses were identified via Jacobian warping as indicators of residual damage which network affected can be plotted (red arrows in Figure 11). From a systems biology perspective, identifying pathology is just part of the challenge, but how pathology influences the organism at the systemic level (as highlighted in Figure 2) is the most important challenge because therein is the link between damage, neural networks and behavior. In the case of TBI this would be to utilize neuroimaging information about pathology—not just were visible lesions are but where volume loss has occurred as well-and use that information in the examination of cognition, emotion and behavior. How to integrate this with neurobehavioral outcome, to be discussed in the next section, will require a major conceptual shift in how neuropsychology has approached assessment.

THE LIMITS OF TRADITIONAL NEUROPSYCHOLOGICAL ASSESSMENT

To objectively study human behavior it has to be measured in some fashion, which includes metrics to assess cognitive and emotional functioning. This was a major aspect of Marr's (1971) original outline for a systems approach related to cognition—the necessity of accurately measuring the target behavior. To achieve the initial goals of characterizing the neurocognitive and neurobehavioral correlates of the brain when damaged, neuropsychology emerged mid-20th century in an era where non-invasive neuroimaging did not exist. Out of necessity, the founders of this field had to be creative in terms of natural observation and capitalized on coarse measures of brain damage, like aphasia, hemiplegia or hemisensory deficits from TBI and other disorders (i.e., stroke, neoplasm) to standardize

certain neuropsychological measures (see Bigler, 2009). Using a normative approach with test scores established in age-typical healthy controls, neuropsychological assessment techniques were developed where the influence of brain damage/dysfunction was viewed in terms of discrepant performance from the norm for a particular measure like memory or perceptual processing (Lezak et al., 2012). In the formative years of clinical neuropsychology the interpretation of such findings was viewed from a perspective of lateralization, localization and modular control (Ross and Long, 1990; Ross et al., 1990), but not networks (Catani et al., 2012). Interestingly, in terms of blunt dissection techniques all of the major fasciculi that now can be so eloquently shown in vivo with DTI techniques, as presented in Figure 10 were known by early 20th Century. Major speculations about their role in behavior were central to connectionist theories of brain function, championed by Geschwind's seminal contributions beginning in the 1950's and extending into the beginnings of neuroimaging, including TBI (Rubens et al., 1977). However, even in Geschwind's era, the emphasis was more on where a particular lesion could be disrupting a pathway or pathways rather than the multiple ways in which pathology and lesions may be expressed in TBI (Filley, 2010, 2011).

Despite 21st Century technology and the technological feats of modern neuroimaging, the basic "tools" of neuropsychological assessment still use a "paper-and-pencil" foundation introduced by Alfred Binet in the early 1900's (Binet and Simon, 1905). In most cases if a task is timed, it is measured in seconds to minutes with the use of a hand-held stop watch, whereas neural processing occurs in millisecond timeframes. For many disorders this traditional neuropsychological approach works well, just like the current reflex hammer and stethoscope that came into general use about the same time and have required no digital update. However, in TBI, as shown in this review, abnormalities are dynamic, ever changing and affecting the brain in unique ways where at each time point postinjury there is a mixture of pathology in conjunction with adaption and recovery. Is the neuropsychological task measuring a deficit, adaptation or an unaffected function? However, an even bigger problem for conventional neuropsychological assessment is that except for certain aspects of motor, sensory and some language tests, standardized traditional assessment methods have not been designed to specifically assess network integrity as outlined in Figure 10. Most traditional assessment tools do not tap singular networks, but a multiplicity of them working in concert. Furthermore, neuropsychological test scores are typically reported as aggregate scores reflective of an operationally defined construct in reference to a normative standard not specifically tied to a particular brain variable. For example, in the TBI Case Study presented herein what is the relevance of the FSIQ score of 109 in reflecting how and where the brain has been damaged along with what neural systems are affected without knowing information gathered from neuroimaging? As already presented the neuroimaging in this child shows extensive damage, so what information does an IQ metric convey about network damage and the underlying pathology sustained by this child? The answer is that by itself and without neuroimaging findings factored in, not much.

To further illustrate this point, a speed of processing metric will be used. Processing speed deficits represent a common finding in those with TBI, particularly related to the diffuseness of injury and amount of WM damage (Lezak et al., 2012). In the TBI Case Study the child's PSI on the WISC-IV was 94, which is a standard deviation (15 points) below the FSIQ score. While the PSI score remains in the low average range, being one standard deviation below overall IQ would be consistent with reduced processing speed as a consequence of the severe TBI. From the pathology shown in Figure 11, such a result is not surprising given the frontal damage, the fact that there is reduced volume in the anterior corpus callosum and cingulate as shown by the Jacobian warping, along with quantitative analyses that show more than a standard deviation reduction in overall WM volume. But which of these regions and precisely which major brain fasciculi are affected cannot be ascertained with a traditional neuropsychological approach like this. All that can be concluded is that processing speed is reduced, but without further integration of both structural and functional neuroimaging whether this is universally reduced processing speed or regional cannot be determined. What is needed is a systems approach where functional techniques like fMRI and/or electrophysiological procedures are incorporated into a multimodal structural MR that more specifically assesses pathway integrity related to function and directly measure specific network efficiency and processing speed. For example, the current review concentrates mostly on anatomical features of structural imaging where anatomical findings do not necessarily reveal function; but fMRI and electrophysiological techniques reveal patterns of activation and neural engagement measured with millisecond precision directly extracted from brain activation patterns. From a systems perspective if these different levels of structural and functional neuroimaging could be integrated it should lead to an improved understanding as to how the damaged brain is functioning.

If the neuropsychological assessment tool is to be integrated into a multimodal assessment technique, this also means abandoning 100-year old traditional methods. For example, the PSI task used with this child measures a myriad of cognitive functions, not how they are interconnected or processing time between them. Returning to Figures 10 and 11, processing speed indices need to be developed more specifically that tap within and between networks as well as those that assess overall network integration and integrity and do it in real time brain processing speed, which is in milliseconds. Cognitive neuroscience paradigms for assessing processing speed attempt to be more domain and network specific and therefore tend to use reaction time and/or some event-related electrophysiological or fMRI blood oxygen level dependent (BOLD) activation tasks (Dockree and Robertson, 2011; Nilsson et al., 2014). Using the above mentioned techniques combined with MR-based functional connectivity (fc) mapping the potential for clinical application

is being realized (McAndrews, 2014). This work includes having the patient not even engaged in a cognitive task, but using resting state (rs) fc mapping techniques to derive brain networks (Barkhof et al., 2014; Tracy and Doucet, 2015). These rs-fcMRI studies have not found their way into broad clinical application at this point, but that is just a matter of time before there is a more complete integration across cognitive neuroscience, clinical neuropsychology and functional neuroimaging to the point cognition and behavior are entirely assessed within the domain of brain imaging. The next step, within a systems biology framework would be to concomitantly develop such measures specific to the TBI patient and track how neural systems and networks either come back online, adapt or remain impaired. The next section provides some thoughts on how this might be accomplished.

SYSTEMS BIOLOGY AND NEUROPSYCHOLOGY

This review began with the very basic model offered by Vodovotz and An (2015) as to what should constitute a translational systems biology approach to studying a disorder. As presented in this review contemporary and advanced neuroimaging methods certainly provide critical information at the tissue, organ and systemic level. But there remain major gaps, with a most prominent one being how to measure cognition and neurobehavioral outcome and link such findings to neuroimaging variables.

Lisman (2015) reviews 21st Century challenges for understanding the brain as it relates to cognition using the systems perspective introduced by Marr (1971) which emphasized a neural network approach. As previously stated, central to Marr's (1971) systems approach is defining how behavior and cognition can be measured. Expanding on the foundation laid by Marr (1971), Lisman outlines the following systems framework to better understand cognition: "First, the functional properties of the process must be defined and behaviorally characterized. Next, the computational algorithm that performs that process must be identified. Finally, how neurons and their network connections lead to the execution of that algorithm must be determined (p. 864)". Juxtaposed to these statements are those of Cipolotti and Warrington (1995) on the neuropsychological assumption that "brain damage can selectively disrupt some components of a cognitive system (p. 655)" and thereby influence test performance which in turn acts as a marker of impaired brain function.

As discussed in the previous section, traditional neuropsychological techniques mostly tap global functions. For a neuropsychological test to be useful in specifically assessing a TBI patient, it must reliably assess the cognitive/behavioral dimension it was purportedly designed to assess and equally important, exhibit decrements in performance levels when those neural systems are damaged. Most traditional test methods in neuropsychology have been developed based on presumed domains of cognitive function, like memory

or executive ability, respectively, often thought to reflect "frontal Lobe" and "temporal lobe" integrity. However, using executive functioning as an example of what is wrong with current use of neuropsychological measures in TBI, as shown in **Figure 10**, there are regions within the frontal, parietal and temporal lobe and their connectivity that make up the executive network. Taking the TBI Case Study presented in this review, what is not known is how do the different lesions and abnormalities as shown in **Figures 8–10** influence test performance. Is test performance more influenced by the WM pathology, the focal encephalomalacia, the loci of hemorrhagic lesions or the regional and whole brain degenerative changes that occurred or the integration of all of these pathologies?

Neuroimaging has a distinct role to play in how to best characterize and quantify "brain damage" and relate such changes in neuropsychological test performance but the field has only recently begun to address these issues (Irimia and Van Horn, 2015). Tradition is hard to break but old tests developed during a non-digital era are likely not the answer. New assessment techniques aided by advances associated with computer-based assessments, especially those using virtual techniques (Parsons, 2015) will likely be capable of better defining "the functional properties of the process" as pointed out by Lisman. Integrated with functional neuroimaging and electrophysiological measures, neuroimaging methods could also be instrumental in developing "computational algorithms" to more fully explain the workings of these neural systems that guide cognition and behavior. Interestingly, algorithms applied to modeling processing speed within networks already have been introduced (van der Helm, 2012). Similarly neuroimaging algorithms that improve detection of WM pathology related to slowed processing predictive of cognitive impairment are being developed (Jokinen et al., 2015).

However, to fully achieve the "computational algorithms" that addresses how "neurons and their network connections" lead to cognition and behavior will require animal models. Fortunately, such translational approaches are being developed with similar structural and fMRI and related neuroimaging techniques used with both animals and humans to study the effects of TBI (Kim et al., 2014; Gozzi and Schwarz, 2015; Meabon et al., 2016).

Another criticism with traditional neuropsychological assessment has been that it is overly focused on assessing cognition, especially within a strictly controlled environment, such as a laboratory setting. Unfortunately, as Parsons (2015) points out lab-based neuropsychological assessment does not necessarily mimic real-world circumstances where lots of competing sensory stimuli, intra-individual variables and environmental conditions may be present. The potential to overcome some of these limitations may be accomplished with virtual assessments within a neuroimaging environment, especially if those assessments were expanded to include emotional stimuli and their influence on standard measures of memory, executive, language and visual-spatial functions. Traditional neuropsychological assessment often ignores or

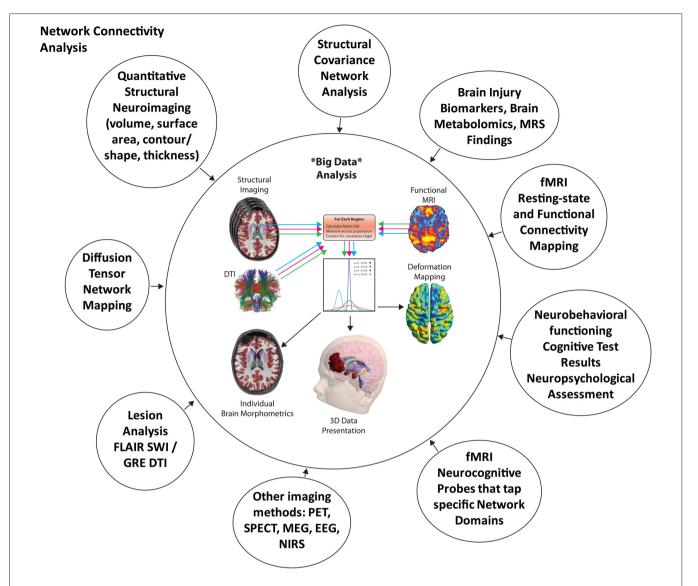


FIGURE 12 | Using neuroimaging findings to better understand the effects of TBI within a systems biology approach, the various circles highlight different neuroimaging and assessment methods that tap different levels of systems relations and/or pathology that could contribute to an integrative analysis of outcome from brain injury. At this point, this is merely a schematic representation showing how normative databases could be generated from different neuroimaging methods that yield unique information. For example, the different types of scans and scan sequences can be mined for structural, DTI and functional imaging metrics so that individual comparisons can be made within each data point in reference to a normative sample (center boxes reflecting statistical comparisons to normative samples), with the output displayed on a 3-D image of the brain. For this illustration just the structural defect in the left frontal region is highlighted in the 3-D image at the bottom. The colored lines represent different metrics extracted from the scan such that a statistical comparison can be made between a normative imaging dataset where individual patient neuroimaging data can be contrasted. Ultimately using neurocognitive probes based on some activation technique from functional neuroimaging integrated with correlative techniques that examine neuropsychological test findings and network integrity results, such findings could be presented in terms of their clinical relevance by a color-coded method that highlights where the most robust relations are.

incompletely assesses the emotional state of the patient, or when it is evaluated, is done purely by the patient completing a questionnaire. Likewise, processing speed is currently measured as a separate metric, not part of each domain being assessed and certainly not in the context of how fluctuations in emotion may affect cognition. All of this is probably limiting our understanding of how brain, mind and cognition function normally as well as in those with TBI (see Cromwell and Panksepp, 2011).

DATA PRESENTATION AND CONCLUSIONS

As reviewed to this point, there are elegant neuroimaging methods differentially sensitive to trauma-related pathologies associated with TBI and likewise there are excellent methods to quantify these abnormalities but how should they be shown and integrated with neuropsychological findings? In the very beginning of neuroimaging, scan findings used in

neuropsychological outcome studies of TBI involved simple and typically singular metrics like presence/absence of an abnormality on CT or a global measure of brain atrophy (Cullum and Bigler, 1986; Levin et al., 1987). With 21st Century advances, how to handle "Big Data" is now at the forefront with the neuroimaging field likely pulling from genomic, proteomic and other large scale research endeavors to generate the best methods for image display (Toga et al., 2015; Das et al., 2016). As shown in Figure 12 a wealth of image analysis tools exist that can be applied to the quantification of structural and functional effects of TBI, but there is no agreed upon method to integrate these data into a meaningful presentation that capitalizes on all of the available information. From the automated to semi-automated methods now available for image analysis, a single case like that used in this article could involve thousands of data points, just from the perspective of the structural neuroimaging findings whereas if functional neuroimaging were added tens of thousands of data points could be part of the algorithm. The end product for the patient, however, should be something that distills all of this information into something that is relevant to outcome and straightforward to interpret.

Neuropsychology as currently applied can only infer that a particular low score actually reflects an impairment but neuropsychological test findings are not capable of specifying which neural structure(s) or systems are specifically affected without neuroimaging. This needs to be the first step of integration. From a systems biology approach this begins at the tissue level and below (see Figure 2). As of this writing DTI and MRS techniques are the only ones considered to more directly tap the "micro" environment of the brain. Accordingly, for the TBI patient, the first level of analysis may be something that assesses WM patency and general metabolic integrity of the brain. If TBI is selectively disruptive to WM then may be the analysis begins with these MRI methods that examine basic microstructure and metabolic integrity. Both of these techniques also provide some metrics that potentially address neuroinflammation and neuroinflammatory changes over time. Next, volume, thickness, shape and contour of a structure have general implications for parenchymal integrity at the organ level. SWI and FLAIR sequence findings have specific relevance within the domain of traumatic lesions, where lesion localization, size and type likely also relate to findings at the tissue and organ level (Kuceyeski et al., 2015). It may be that the proximity to a focal lesion is an important variable that alters regional tissue shape, size and contour. There are metrics within each approach that can be compared to age, sex and demographic specific normative samples, which could then be used to define where neuroanatomical and neuropathological differences exist in the individual who has sustained a TBI. Combining DTI and other morphometric analyses with rs-fcMRI mapping could approach network analyses and connectivity mapping, showing strength and weaknesses of frank abnormalities within a network in the TBI patient. The end point of such analyses would address the tissue and organ level of brain structure and ultimately shown in some 3-D format an easily visualized image of where pathology resides and its relation to outcome, as has been presented for the case study (see Figures 8, 9, 11, 12).

Next, to understand function at the systemic level using neurocognitive and neurobehavioral test results along with functional activation imaging findings, these tissue and organ level data points would be statistically assessed in relation to the neurocognitive and neurobehavioral test results using machine learning and probabilistic frameworks for classification and differentiation. Features of such an approach have been undertaken for degenerative diseases (Klöppel et al., 2012), neuropsychiatric disorders (Wu et al., 2016), stroke (Kuceyeski et al., 2015) and even TBI (Mitra et al., 2016). At this time all of these studies are mostly demonstrations of "proof of concept" as there is no uniform or agreed upon standard as how these complex analyses should be done and data displayed.

While the above outline is overly broad, it does attempt to take a systems biology approach starting at a tissue level of analysis moving to whole brain and neuropsychological integration. Such an ambitious endeavor will require large sample sizes to begin the process of extracting the most meaningful neuroimaging variables that relate to neurocognitive and neurobehavioral outcome in TBI. Such efforts have actually begun (Mirzaalian et al., 2016; Wilde et al., in press). Despite these obstacles, using such a perspective, should yield novel insights in how best to extract the most meaningful and predicative information from a scan. In that sense, this review returns to Figure 2 where the most important presentation of data may come via which level in the system is being addressed and how that level has been affected, adapted or returned to baseline. The current challenge to the TBI investigator and clinician is how to best bring these rich data gathering methods together to better understand TBI and help improve diagnosis, treatment and recovery.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Disruption of Network Synchrony and Cognitive Dysfunction After Traumatic Brain Injury

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Traumatic brain injury (TBI) is a heterogeneous disorder with many factors contributing to a spectrum of severity, leading to cognitive dysfunction that may last for many years after injury. Injury to axons in the white matter, which are preferentially vulnerable to biomechanical forces, is prevalent in many TBIs. Unlike focal injury to a discrete brain region, axonal injury is fundamentally an injury to the substrate by which networks of the brain communicate with one another. The brain is envisioned as a series of dynamic, interconnected networks that communicate via long axonal conduits termed the "connectome". Ensembles of neurons communicate via these pathways and encode information within and between brain regions in ways that are timing dependent. Our central hypothesis is that traumatic injury to axons may disrupt the exquisite timing of neuronal communication within and between brain networks, and that this may underlie aspects of post-TBI cognitive dysfunction. With a better understanding of how highly interconnected networks of neurons communicate with one another in important cognitive regions such as the limbic system, and how disruption of this communication occurs during injury, we can identify new therapeutic targets to restore lost function. This requires the tools of systems neuroscience, including electrophysiological analysis of ensemble neuronal activity and circuitry changes in awake animals after TBI, as well as computational modeling of the effects of TBI on these networks. As more is revealed about how inter-regional neuronal interactions are disrupted, treatments directly targeting these dysfunctional pathways using neuromodulation can be developed.

Keywords: TBI, concussion, axonal injury, synchrony, ensemble coding, oscillations, limbic system, hippocampus

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INTRODUCTION

Traumatic brain injury (TBI) can disrupt cognitive processing and memory function for many years post injury (Capruso and Levin, 1992; Ryan and Warden, 2003; Monti et al., 2013). TBI is a complex, heterogeneous disorder with many factors contributing to a spectrum of severity from concussion (mild TBI) to severe brain injury. Injuries across this spectrum may include damage to the white matter, where many axons of the brain coalesce into tracts that run throughout the brain (Johnson et al., 2013b). Diffuse axonal injury, detected histopathologically post mortem, is the hallmark neuropathology resulting from inertial brain injury (Adams et al., 1982; Gennarelli et al., 1982). Axonal injury can also result from ischemia, raised intracranial pressure, and other complications of diffuse brain injury. Injury to axons can lead to disruption

in the channels, ionic homeostasis and myelination necessary for action potential propagation.

Unlike an injury to only a discrete brain region, axonal injury is fundamentally an injury to the substrate by which all networks in the brain communicate. There has recently been shift away from the brain as a "computer" analogy-a collection of discrete processors that shuttle packets of information between them in various stages of analysis. Rather, the brain is now depicted as a series of dynamic, interconnected networks with emergent properties that communicate via long axonal pathways grouped into white matter tracts (Alivisatos et al., 2012). This architecture has been termed the "connectome" due to its complexity and the connected network structure that it represents (Sporns et al., 2005; Van den Heuvel and Sporns, 2011). Neurons communicate via these pathways and encode information within and between brain regions in a number of ways, many of which are timing dependent. Our central hypothesis is that traumatic injury to axonal connections may disrupt the exquisite timing of neuronal communication within and between brain networks, and that this may underlie aspects of post-TBI cognitive dysfunction.

We will briefly review the evidence for axonal injury and memory and executive function impairment following TBI. We then describe some of the basic mechanisms of network level communication, how they depend on precise timing, and how failure of these mechanisms may lead to cognitive problems. As an example, we then focus our discussion on hippocampal network communication on multiple scales and present specific hypotheses regarding how this may be disrupted after TBI.

DIFFUSE AXONAL INJURY IS THE HALLMARK OF INERTIAL TRAUMATIC BRAIN INJURY

A series of studies have demonstrated that inertial brain injury leads to mechanical injury to axons resulting in axonal pathology (Gennarelli et al., 1982; Graham et al., 1988; Adams et al., 1989; Margulies et al., 1990; Smith et al., 1997). Diffuse axonal injury is the hallmark multi-focal neuropathology of inertial injury, and is detected post mortem using antibodies against transported proteins that accumulate due to cytoskeletal damage or secondary disconnection (for review see Johnson et al., 2013b). More recently, other populations of injured axons have been defined, suggesting that those with transport disruptions may only be a portion of the total injured population (Johnson et al., 2016). Degenerative processes associated with axonal injury may be long-lasting, as it has recently been demonstrated in a chronic TBI human cohort that active axonal degeneration is occurring up to 18 years post injury (Johnson et al., 2013a).

While diffuse axonal injury is a common neuropathological consequence of moderate to severe injury, increasing data suggest that damage to axons may also be an important aspect of mild TBI, or concussion. Axons are preferentially vulnerable to biomechanical forces during inertial rotation, which are a primary or contributing factor to TBIs at every

severity, including concussion (Holbourn, 1943, 1945; Strich, 1961; Adams et al., 1982; Gennarelli et al., 1982; Browne et al., 2011). Due to the anisotropy of white matter tracts and the viscoelastic properties of axons, they are selectively vulnerable to rapid mechanical forces such as shear and tensile strain during inertial injury (Smith and Meaney, 2000; Johnson et al., 2013b). The role of axonal injury and its prevalence in mild TBI is an active area of investigation, but different lines of evidence are converging to suggest its involvement. There are only a few post-mortem studies of mild TBI with small cohorts, but those that have been performed detected axonal injury in many cases (Blumbergs et al., 1989, 1994). Recent advances in imaging such as diffusion tensor imaging (DTI) have suggested that white matter abnormalities correlate with cognitive deficiencies in mild TBI (see below), although the correlation between DTI and axonal pathology remains to be determined (Miles et al., 2008; Wu et al., 2010; Jorge et al., 2012; Siman et al., 2013; Rabinowitz and Levin, 2014). One supporting factor is the existence of blood biomarkers, some of which are breakdown products from axonal injury, and that correlate with outcome and cognitive measures post concussion (Siman et al., 2013, 2015; Shahim et al., 2014).

MEMORY AND EXECUTIVE FUNCTION ARE PERSISTENT COGNITIVE DEFICITS AFTER TBI

Hippocampal dependent memory impairment after TBI is perhaps one of the most commonly modeled aspects of clinical TBI. Persistent memory impairment after TBI, including after mild TBI, represents significant morbidity: 25% of soldiers suffering only a diffuse TBI returning from assignment have significant memory impairments (Hoge et al., 2008), and many civilians have persistent impairment decades after their injury (Draper and Ponsford, 2008; Monti et al., 2013). Interestingly, in one of the only reports that examined mild TBI pathology in humans after death from unrelated injuries, it was noted that the fornix, the major output tract of the hippocampus, had axonal pathology in all of the five cases examined (Blumbergs et al., 1994). The fact that memory impairment and axonal pathology in memoryrelevant structures both occur in milder TBI cases without more severe focal injuries to the brain suggests that memory impairment after TBI may be due in part to communication disruption within and between brain regions as a result of axonal injury.

Executive function is also persistently impaired following TBI (McDowell et al., 1997; Draper and Ponsford, 2008). Patients with TBI have demonstrated deficits in working memory paradigms, including the n-back task (Sanchez-Carrion et al., 2008), a form of memory task requiring prefrontal executive function. Furthermore, severity of executive function deficits correlates with the degree of frontal white matter abnormalities in human DTI (Kinnunen et al., 2011). Interactions between the prefrontal cortex (PFC) and the hippocampus are known to be central to the integration of executive function and

memory. DTI in humans suggests that limbic white matter pathways, including the cingulate bundle which connects the PFC and the hippocampus, are damaged following diffuse TBI (Niogi et al., 2008a,b; Spitz et al., 2013) and that this damage correlates with memory and attention impairment in humans (Niogi et al., 2008b). Finally, cognitive processing speed, which supports all executive and memory functions, has been demonstrated as a deficit post-injury (Draper and Ponsford, 2008), with hippocampal fornix axonal dysfunction suggested as one potential substrate (Fisher et al., 2000; Mathias et al., 2004; De Monte et al., 2006; Yallampalli et al., 2013).

NETWORK DYSFUNCTION FOLLOWING AXONAL INJURY

How might diffuse axonal injury following TBI contribute to the well-established deficits in memory and executive function? Answering this broad question is one major goal of TBI research and requires the formulation and testing of an extensive series of hypotheses that span multiple scales of organization, from subcellular changes in the cytoskeleton, to intrinsic properties of individual neurons, to the formation of local "neuronal ensembles" (see below), and to the function of regional and global neural networks (i.e., the connectome).

Axonal Injury Disrupts Timing of Neuronal Signal Conduction

At the cellular and subcellular scale alterations in intrinsic neuronal properties and conduction delays following TBI have been examined using ex vivo brain slice preparations from rodent TBI models, or *in vitro* neuron culture preparations that have been mechanically injured. Axonal injury can lead to secondary changes in the channels, ionic homeostasis and myelination necessary for timely and robust action potential propagation. In vitro models have demonstrated that there is substantial ionic disruption at lower levels of axonal strain (Yuen et al., 2009), while at higher strains Na+ channel disruption leads to calcium influx and changes in the channel subunit distribution (Wolf et al., 2001; Iwata et al., 2004). These changes in the axons after injury could lead to a total disruption of signal transmission initially, and then compromised or delayed propagation over long periods of time due to compensatory channelopathies (Yuen et al., 2009).

Depolarization due to ionic imbalance could also underlie synaptic communication deficits, as the loss of driving force may affect the calcium influx necessary for synaptic release (Reeves et al., 2005; Goforth et al., 2011). Previous TBI models have demonstrated changes in axonal conduction in both myelinated and unmyelinated axons in the collosum (Reeves et al., 2000, 2005; Colley et al., 2010), as well as in presynaptic fiber volley amplitudes in various models, all of which could disrupt precise signal timing and integration of inputs (Norris and Scheff, 2009; Reeves et al., 2000; but see Cole et al., 2010). While understanding cellular mechanisms of

altered action potential conduction velocities and neuronal firing properties links traumatic axonal injury to timing disruption in neuronal communication and may lead to secondary injury prevention strategies, it does not address how these abnormalities disrupt network level function leading to cognitive deficits.

Global Functional Brain Networks are Altered Following TBI

At the global scale, non-invasive functional imaging such as functional magnetic resonance imaging (fMRI) is used to determine global or regional brain network differences between human TBI patients and healthy controls, which are then correlated to cognitive dysfunction. Structural imaging techniques such as DTI can delineate broadly the white matter patterns of injury and attempt to link them to both cognitive dysfunction (see above) as well as network abnormalities determined by fMRI (MacDonald et al., 2008; Palacios et al., 2012; Tang et al., 2012) and for review see Xiao et al. (2015). Recent advances in functional imaging analysis techniques have capitalized on the inherent fluctuations in regional brain activity during quiet rest to identify brain regions that fluctuate together (are "functionally connected") and which become relatively deactivated when the brain engages in non-self-referential goal-directed tasks. This major network of activity has been called the default mode network (DMN), thought to represent an intrinsic core network in the absence of significant sensory input (Raichle, 2015). The DMN generally includes bilateral frontal, prefrontal and parietal regions as well as the cingulate, and has been identified in humans, non-human primates and rodents (Xiao et al., 2015). DMN activation, deactivation and connectivity have all been found to be altered in TBI patients compared to healthy controls and correlated with cognitive impairment (Bonnelle et al., 2011, 2012; Sharp et al., 2011; Palacios et al., 2012; Xiao et al., 2015). While these methods have been utilized to generate generalized hypotheses about connectivity and treatment after TBI (Ham and Sharp, 2012), they do not easily suggest any specific therapeutic targets as they lack a well-defined relationship to the presumed underlying abnormalities in neuronal communication.

Understanding Network Timing Dysfunction May Lead to New Therapeutic Strategies

The predominant treatments that have been developed over the last 20 years for TBI have centered around sparing neurons from degeneration post injury, although recently attention has been paid to the axonal connections between neurons as well (Smith et al., 2013). However, the success of translation has been poor for most agents, and few were designed to modulate axonal dysfunction or its downstream effects. There are also currently no treatments for mild TBI that are considered effective, particularly for post-concussive symptoms. We propose a systems approach that lies between the micro scale of sodium channel disruption

and the macro scale of global functional brain connectivity to address the following questions:

- How does pathological alteration in the sub-second timing of neuronal communication lead to impaired formation of "neuronal ensembles" (see below) as well as the local and global breakdown of interactions between ensembles?
- How does this network dysfunction lead to memory and executive function impairment seen after TBI?

We propose that hypotheses directed at understanding the timing relationships between networks of neurons at the millisecond level, and how disruption of this timing after TBI leads to cognitive impairment, may offer an important and largely unexplored pathway towards novel therapeutics aimed at restoring lost function via new electrophysiological targets. For example, animal models of TBI demonstrate a loss of theta frequency power in the hippocampus. Stimulation of medial septal projections to the hippocampus within the theta frequency range, but not outside of it, has been shown to recover behavior in these models (Lee et al., 2013, 2015). We propose to develop directed therapies that are predicated on a detailed understanding of the electrophysiological impairments following injury.

CONSTRUCTION AND INTEGRATION OF NEURONAL ENSEMBLES REQUIRES PRECISION TIMING

Neuronal Ensembles

Perception of sensory stimuli, short and long-term memory, and even habits are thought to be encoded and represented by distributed neural assemblies termed "ensembles" across the brain (Pennartz et al., 1994; Buzsáki and Chrobak, 1995; Sutherland and McNaughton, 2000; Buzsáki, 2004). Multiunit recordings have recently identified the neuronal activity underlying many of these representations during awake behavior (Laubach et al., 2000; Hoffman and McNaughton, 2002; Barnes et al., 2005). Memory has long been described by Hebbian processes of changing connection strength between neurons, and more recently the "engram" or distributed neuronal memory trace associated with some aspects of memory has been causally demonstrated using optogenetic and other techniques (Hebb, 1949; Lashley, 1950; Liu et al., 2012; for review see Josselyn et al., 2015; Tonegawa et al., 2015).

Many interesting questions remain, however, including how shorter-term memory in the hippocampus is integrated with other memories and moved out to the cortex, and how this distributed memory is then represented in the cortex. How are spatial (across the brain or brain regions) and temporal (over time) representations of the above examples (perception, etc.) coordinated over such long distances? How is it that ensembles in the cortex receive discrete contextual information from groups of cells in the hippocampus such as "place" cells? One mechanism for coordinating different regions of the brain and transmitting information between them is an oscillation, a foundational concept in neuroscience and

neurology whose mechanistic role in these processes has been debated since its discovery. Another concept for distributed representations of information in the cortex are "syn-fire chains", a model that demonstrated that a synchronously firing series of neurons could propagate (or transmit) information through noisy layers (Abeles et al., 1993; Aertsen et al., 1996; Diesmann et al., 1999). These concepts are not mutually exclusive, but both require exquisite timing and ensembles of coordinated cell firing. These ensembles must be coordinated across great physical lengths, potentially including across hemispheres, suggesting that traumatic injury to the conduits of this precise information exchange could lead to significant impairment.

Oscillations and Entrainment are Fundamental Mechanisms of Neuronal Communication at Multiple Scales

Synaptic communication between neurons within local ensembles, or across long distances between ensembles, requires the conduction of current along axons and the release and binding of neurotransmitters at synaptic junctions leading to current flow. If sufficient coordinated input current flows into an ensemble of neurons, that neuronal ensemble may alter its output (firing pattern) to be more synchronous with the dominant currents it is receiving. A single neuron has many sources of current, both excitatory (depolarizing) and inhibitory (hyperpolarizing) from many local and distant brain regions that are integrated over time. One can envision that if one or more afferent white matter tracts have been injured, the inflow of synaptic currents to a given neuronal ensemble may no longer be sufficiently coordinated to maintain its precisely timed output, leading to functional impairment.

The combined current influx into a localized brain region can be measured as the local field potential (LFP). Cortical LFP can be measured with scalp EEG, subdural electrode grids, or with individual electrodes placed in the brain. The LFP of deeper structures (in the hippocampus, for example) requires depth electrodes that penetrate the structure. Patterns of oscillations in the LFP within different frequency bands have been observed in all mammalian species studied and are linked to behavioral states (Buzsáki et al., 2012). For example, in primates, alpha (8-12 Hz) oscillations are observed to dominate the occipital lobe during quiet rest with the eyes closed (Jurko et al., 1974), a rhythm proposed to arise from thalamocortical projections in the absence of sensory input (Buzsáki and Watson, 2012). Theta oscillations (4-10 Hz in rats) in the hippocampus have been observed to dominate during navigation, memory tasks, and rapid eye movement (REM) sleep (Buzsáki and Moser, 2013) and appear to depend on projections from the medial septum (Buzsáki, 2002; see below).

LFP oscillations represent dominant, coordinated inflow of current into the region being measured, not necessarily the pattern of firing of the local neurons (Buzsáki et al., 2012). Only if those inputs are strong enough and sufficiently synchronized will they influence when the local neurons fire. For example, when a particular oscillating input dominates current inflow,

local neurons may preferentially fire more often during a particular phase of that oscillation, called entrainment. There may be multiple local and distant sources of oscillatory input at different frequencies, all of which are influencing the timing of local neuronal firing. In this way the resulting pattern of firing is an integration of the influences of many disparate brain regions, as well as the local connections in the ensemble, in combination with the cell's intrinsic properties. Furthermore, this varies over time: different behavioral states or actions may be characterized by the dominance of one oscillation over others at any given moment. As described below, this is the case in the hippocampus. The patterned influence of current inflow from many regions on the subsequent firing pattern of a neuronal ensemble is a major proposed mechanism by which information is coordinated and transmitted between brain regions, and one that relies critically on precise, sub-second timing.

Spike Timing Dependent Plasticity

Another axonal timing dependent phenomena, spike-timing dependent plasticity (STDP), is a well described phenomenon whereby neurons that fire reinforce the synapses on their own dendrites using back-propagating actions potentials (Tritsch and Sabatini, 2012). In this manner, those inputs onto the dendrites that collectively led to the firing of a neuron are strengthened. The timing of the dendritic inputs and the timing of the resulting firing of the neuron have been proposed as one basis for memory formation in the hippocampus. When specific axons are temporarily or permanently dysfunctional, this may drastically affect the ability of the neurons to integrate incoming information over time and appropriately reinforce the relevant synaptic inputs. At another level, if this were to occur to numerous cells at the same time, the loss of the ensemble activity that represents a "memory" may be temporarily or permanently disrupted, as would the ability to encode new memories.

TIMING DEFICITS IN HIPPOCAMPAL NETWORKS HYPOTHESIZED AS MECHANISM UNDERLYING MEMORY AND EXECUTIVE DYSFUNCTION AFTER TBI: FROM LOCAL TO GLOBAL

The hippocampus is one of the most studied brain regions in both TBI and memory due to its central role in short-term and spatial memory and its demonstrated vulnerability during TBI. Hippocampal networks are TBI-relevant examples of the processes described above, allowing us to develop specific hypotheses about hippocampal network dysfunction following TBI at both local and global scales that can be tested using a systems neuroscience approach. Beyond the hippocampus proper, wider hippocampal networks that include reciprocal connections to entorhinal cortex, striatum, septal region, amygdala and PFC are known to be important for memory and executive functioning. Some of these connections may be preferentially injured following TBI, as discussed above (see "Memory and Executive Function

are Persistent Cognitive Deficits after TBI" section). Further, much is known about the role of oscillatory activity and precision timing within hippocampal networks as they relate to memory formation, consolidation and executive function.

Two Complementary Network States Characterize the Awake Hippocampus and May Become Dysfunctional After TBI

In the awake animal, two dominant electrophysiological states have been described in the hippocampus. In the rodent, theta frequency band oscillations (approximately 4-10 Hz) dominate the hippocampal LFP during spatial exploratory activity, memory tasks, and REM sleep (Buzsáki, 2002). Immobility, eating, grooming, and non-REM slow wave sleep are dominated by slower, large amplitude irregular LFP activity punctuated by high amplitude spikes called sharp waves (Buzsáki, 2015). It is thought that during exploratory, theta-dominant activity, sequences of "events" are linked to form a memory. These temporally linked events could be a series of locations visited by a rat in a box that are integrated to form an internal map of the rat's environment, or as proposed by Buzsáki and Moser (2013) they could be more complex cognitive episodes combined to form an episodic memory, such as the steps in tying one's shoe (Buzsáki, 2005). In contrast, there is evidence that during sharp wave dominant states of immobility or non-REM sleep, specific sequences of neuronal firing representing these episodic memories, are "re-played" in a temporally compressed manner and may be undergoing some form of consolidation and redistribution to other connected areas of the brain (Lee and Wilson, 2002; Foster and Wilson, 2006; Diba and Buzsáki, 2007; Girardeau et al., 2009; and for review see Eichenbaum, 2000; Carr et al., 2011). The coincidence of memory dysfunction and sleep disturbance after TBI is intriguingly suggestive of a failure of one or both of these hippocampal states, or the balance between them.

The Exploratory State: Place Cells, Theta Oscillations and Memory Formation

Perhaps the most well-studied neuronal function to date is that of the hippocampal place cell. First described in rats by O'Keefe and Dostrovsky (1971), place cells are pyramidal cells in the hippocampus that modulate their firing rate based on the position of the animal within its spatial environment (O'Keefe and Dostrovsky, 1971). A given ensemble of place cells will increase their firing rate if the animal traverses one or more locations within its environment, termed the cells' place field. In this way a map of an animal's environment and its current position therein is maintained by a collection of neuronal ensembles. As an animal moves along a particular path in its environment, a sequence of place cell ensembles will fire in an order that represents the path taken. A memory of the animal's traveled path is therefore formed, represented as a temporal sequence of place cell ensemble firings. This basic mechanism can be extended and applied to other

episodic memories, such as tying a shoe (Buzsáki and Moser, 2013).

Although the mechanisms are poorly understood, it is clear that the formation of a place cell is dependent on coordinated input into the hippocampus from "grid cells" found in the entorhinal cortex, via direct and indirect projections to the CA1 hippocampal subfield along the perforant and temporoammonic pathways. Lesions to the direct projections from entorhinal cortex to CA1 result in impaired place cell formation (Tonegawa and McHugh, 2008), and inducing trauma to the perforant pathway leads to impaired spatial memory in rodents (Skelton, 1998; Perederiy and Westbrook, 2013). Furthermore, DTI evidence of perforant pathway injury has been correlated with memory impairment in patients with severe TBI (Christidi et al., 2011). We therefore hypothesize that axonal injury to perforant pathway projections would lead to impaired place cell formation and spatial memory deficits after TBI (McNaughton et al., 2006a).

A critical property of hippocampal place cells is that they are synchronized, or entrained, to the hippocampal theta oscillation during exploration. The phase of the theta cycle at which a place cell fires also changes in a predictable way as the animal moves through the place field of that cell, termed "phase precession" (O'Keefe and Recce, 1993). It is thought that theta oscillations in the hippocampus provide a structure into which neuronal firing sequences such as place cell ensembles are organized, compress the time scale of ensemble sequences such that synaptic plasticity can take place, and provide a structure for synchronization with other brain regions (Buzsáki and Moser, 2013). It is therefore critical that the timing of synaptic currents into place cell ensembles is maintained with precision. Studies in animals and humans have shown that learning trials in a memory task occurring during periods of time when theta power is increased in the hippocampus lead to improved memory performance (Seager et al., 2002; Sederberg et al., 2003; Merkow et al., 2014). Additionally, there is early evidence that hippocampal theta power is reduced in the lateral fluid percussion model of TBI and that this may correspond to reduced memory performance (see below; Fedor et al., 2010; Lee et al., 2013, 2015; Paterno et al., 2016).

The origin of the hippocampal theta oscillation is complex, but it is clear that it is dependent on extra-hippocampal synaptic inputs. A key structure with reciprocal GABAergic connections to the hippocampus via the fornix is the medial septum/diagonal band of Broca (MS-DBB; Freund and Antal, 1988; Tóth et al., 1993). Rhythmically firing cells in the MS-DBB are entrained to hippocampal theta oscillations and persist in their rhythmicity even when theta oscillations no longer dominate the hippocampus (Petsche et al., 1962). Both physical and drug-induced lesions of the MS-DBB, as well as lesions of the septohippocampal projections of the fimbria-fornix, abolish theta oscillations in the hippocampus (Petsche et al., 1962; Stumpf et al., 1962; Buzsáki et al., 1983; Buzsáki, 2002; McNaughton et al., 2006b). This and other evidence led to the original conceptualization of the MS-DBB as the rhythmic "pacemaker" of hippocampal theta oscillations (Buzsáki et al., 1983), although it is now believed that theta arises from reciprocal inhibitory connections between the MS-DBB and hippocampus along with cholinergic septohippocampal tone, entorhinal cortical inputs, recurrent collateral projections from CA3, and intrinsic properties of intra-hippocampal interneuron inhibitory circuits (Dragoi et al., 1999; Buzsáki, 2002; Vandecasteele et al., 2014) Interestingly, it has recently been demonstrated that bidirectional long-range GABAergic projections exist between the entorhinal cortex and the hippocampus and contribute to theta generation, suggesting another vulnerable pathway for theta disruption via axonal injury (Melzer et al., 2012).

Not only are intact connections between the hippocampus and MS-DBB necessary for hippocampal theta oscillation generation, but their loss and subsequent functional restoration with electrical stimulation are linked to memory performance deficits and subsequent recovery, respectively (McNaughton et al., 2006b). There is also early evidence for the relevance of this circuitry dynamic in TBI models. Lee et al. (2013, 2015) conducted a series of experiments in rats after lateral fluid percussion injury demonstrating both a reduction in performance on the Barnes Maze (a rodent spatial memory task) and a reduction in hippocampal theta power, as well as the temporary restoration of theta power and maze performance with theta frequency stimulation of the MS-DBB. Furthermore, while theta power in the hippocampus was reduced after TBI it remained unchanged in the MS-DBB, as did theta coherence between MS-DBB and the hippocampus, suggesting a specific disruption of the septohippocampal circuitry (Lee et al., 2015). This reduction in theta power after fluid percussion injury has also been described in a mouse model that demonstrated sleep disruption after TBI (Lim et al., 2013).

We therefore hypothesize that reductions in theta hippocampal power observed post-TBI may be related to axonal injury of numerous pathways projecting into the hippocampus, in addition to hippocamposeptal reciprocal projections. Furthermore, reductions in theta power may lead to impaired entrainment and therefore impaired temporal organization of place cell ensembles, or a reduction in phase precession, which ultimately leads to impaired memory formation and impaired spatial navigation.

Place Cells and Cross Frequency Coupling

The synchronization between oscillations of different frequencies within and between brain regions is termed cross-frequency coupling (CFC) and has recently received a great deal of attention (for review see Hyafil et al., 2015). The representation of multiple items during working memory, the parsing out of various stimuli, and long-distance communication have all been linked to CFC (Hyafil et al., 2015). One form of CFC, phase-phase coupling, may be particularly sensitive to timing disruptions and may therefore be affected by TBI and axonal injury. Theta and gamma (~40 Hz) oscillations in particular have been demonstrated to couple in this manner, and have been proposed to form a "code" whereby multiple items can be represented in working memory

in an ordered fashion (Lisman and Buzsáki, 2008; Lisman and Jensen, 2013).

In this model, not only do place cell ensembles (or cell ensembles representing any memory item) fire in temporal sequence entrained to the theta oscillation (see above), but each individual ensemble is locked to a cycle of the gamma oscillation. Several gamma cycles are, in turn, phase-locked to a single theta cycle. In this way gamma oscillations serve to organize neuronal ensembles representing individual memory items, while the phase relationship to theta oscillations serves to organize these memory items into a defined temporal sequence, forming the basis of episodic memory and potentially serving as the structure that allows distribution across brain regions (Lisman and Jensen, 2013). Theta-gamma CFC has been implicated in both working and long-term memory operations in rodents and humans, and in both hippocampus and cortex (Canolty et al., 2006; Tort et al., 2009; Maris et al., 2011; Friese et al., 2013). Theta-gamma CFC has recently been demonstrated to increase in healthy human volunteers during an n-back task that required ordering of information in working memory (Rajji et al., 2016). We hypothesize that timing delays due to axonal injury and dysfunction may lead to the disruption of the appropriate encoding of this ordered information via CFC, and that this mechanism may underlie aspects of TBI-related deficits in working memory.

The source of gamma oscillations is thought to be intrinsic properties of GABAergic interneurons and therefore a "locally generated" phenomenon (Hyafil et al., 2015). Interestingly, there is evidence to suggest that hippocampal interneurons may be preferentially injured following TBI (Lowenstein et al., 1992; Almeida-Suhett et al., 2015). We therefore hypothesize that hippocampal gamma oscillation power may also be reduced following TBI, leading to impaired theta-gamma coupling and a reduction in the temporal structure necessary for the formation of episodic memory.

The Sharp Wave-Ripple State: the "Deafferented Hippocampus"

The theta dominant state of the hippocampus depends on integrating input from many regions and is active during spatial exploration and REM sleep. In contrast, the sharp wave-ripple (SPW-R) state of the hippocampus is thought to be internally generated, dominant during quiet rest, eating, grooming, and deep, non-REM sleep (Buzsáki, 2015). Indeed, recordings from fetal hippocampi that have been implanted into adult rats (and therefore deprived of natural inputs from other brain regions) reveal a dominance of sharp waves and the synchronization of pyramidal neuronal firing to them, persisting even during spatial exploratory activity that normally suppresses sharp waves in favor of theta dominance (Buzsáki et al., 1987a,b). Electrophysiologically, the SPW-R state occurs when hippocampal afferent inputs are reduced (Buzsáki, 2015). It is dominated by irregular activity punctuated by large amplitude sharp waves 40-100 ms in duration, thought to originate from the CA3 region. Brief episodes of fast oscillations (150-240 Hz) occur in the CA1 region at the peaks of the sharp waves, termed ripples (Buzsáki, 1986). These ripples are thought to be generated by fast local interneuron synaptic inhibition onto CA1 pyramidal cells in response to Schaffer collateral synaptic input from CA3. This inhibition leads to strong entrainment of the pyramidal cells to the ripple (Buzsáki, 2015).

The functional significance of the hippocampal SPW-R state is not well-understood, but there is increasing evidence that it may play a critical role in memory consolidation and re-distribution to extra-hippocampal brain regions (Girardeau et al., 2009; Buzsáki, 2015). Remarkably, it has been shown in rats that the specific temporal sequence of place cell firing that occurs when a rat moves through its environment, initially synchronized to theta oscillations (see above), is replayed in exact reverse sequence immediately after the spatial experience (Foster and Wilson, 2006; Diba and Buzsáki, 2007; Girardeau et al., 2009) and during slow wave sleep (Lee and Wilson, 2002), as well as in forward sequence immediately prior to the repetition of that spatial experience (Diba and Buzsáki, 2007). However, these place cell sequence replays occur during ripple oscillations, temporally compressed approximately twenty-fold, in bursts of approximately 100 ms (Lee and Wilson, 2002). This precisely-timed process is thought to play a role in the consolidation and possibly distribution of episodic memory to a wider neocortical network (Logothetis et al., 2012), particularly as ripples have been detected in other brain regions, and as the artificial elimination of sharp wave ripples during posttraining sleep leads to impairment of subsequent memory performance in rats (Wierzynski et al., 2009; Girardeau et al., 2009).

The consequences of TBI on the SPW-R state are potentially numerous. First, we hypothesize that axonal injury to intrahippocampal projections such as the Schaffer collateral system from CA3 to CA1, or the disruption of interneuronal circuitry (particularly in light of evidence for preferential loss of hippocampal interneurons; Lowenstein et al., 1992; Almeida-Suhett et al., 2015), may lead to a reduction in ripple generation and/or an impaired ability for place cells to entrain to them. This may lead to memory consolidation impairment. Second, we hypothesize that injury to efferent axonal projections from the hippocampus (via the fornix, for example) may disrupt the transmission of ripple-associated relay events to the neocortex, leading to impairment of memory redistribution. Third, we hypothesize that widespread loss of synaptic input into the hippocampus (e.g., entorhinal projections) due to axonal injury could inappropriately bias the hippocampus towards its deafferented, synchronous state, the SPW-R state, which is further supported by a recent set of experiments demonstrating pathological high frequency activity after perforant path disruption (Ortiz and Gutiérrez, 2015). This could potentially cause widespread memory formation and consolidation difficulties, sleep cycle disturbance, and epileptiform activity, all symptoms classically observed after TBI. A recent study combining single cell recordings in the hippocampus with resting state fMRI in non-human primates demonstrated a consistent increase in DMN activity after hippocampal ripple events (Kaplan et al.,

2016). This finding coupled with previous work demonstrating inappropriately persistent DMN activity during cognitive tasks in patients with TBI (Bonnelle et al., 2012) suggests a global network related to an internal hippocampal SPW-R state that is unable to disengage appropriately with cognitive demand.

Finally, as discussed, the hippocampal SPW-R state is internally generated and thought to be the most synchronous state of the hippocampus (Buzsáki, 2015), which may be of particular relevance to post-traumatic epilepsy. Ripples share some characteristics with pathological high frequency oscillations (pHFOs) seen during interictal periods of epilepsy that are predictive of seizure onset (a hyper-synchronous state; Bragin et al., 2010). Others have proposed that pHFOs may be generated by the disruption of firing reliability associated with the SPW-R, leading to offset ripple oscillations (Foffani et al., 2007; Ibarz et al., 2010). This suggests that epileptogenesis could result in part from a hippocampal network in an abnormally persistent SPW-R state, or one with disrupted timing patterns leading to pathological oscillations.

Global Hippocampal Network Dysfunction After TBI

The hippocampus is part of the larger limbic system, including the PFC, the amygdala and the striatum. There are numerous examples in the limbic system of how oscillations in one region can influence neuronal ensembles in another, suggesting that this system is highly interconnected by axonal pathways carrying timing dependent information. We hypothesize that cognitive processes subserved by these interconnected pathways could become impaired after TBI.

Hippocampal Prefrontal Cortical Synchronization and Working Memory

Projections from the hippocampus to the PFC and the striatum carry output that is entrained to hippocampal theta oscillations. This theta-entrained output leads to the entrainment of target (i.e., PFC and striatal) neurons to hippocampal theta (Berke et al., 2004; Jones and Wilson, 2005a). In rodent models theta oscillations in the PFC in addition to the hippocampus are important for spatial navigation and spatial working memory (Jones and Wilson, 2005b; Benchenane et al., 2010; Spellman et al., 2015). Jones and Wilson demonstrated that when rats must make a choice about the correct location of a food reward using working memory, two things happen between the PFC and the hippocampus: first, the normally distinct theta oscillations of each structure synchronize, and second, the firing of PFC neurons becomes entrained to the theta oscillations in the hippocampus (Jones and Wilson, 2005b). Importantly, this synchronization and entrainment was only observed when the rat made the correct choice and progressively became stronger up to the point of decision. Along with rodent studies supporting the role of theta synchronization between hippocampus and PFC during working memory, recent advances in human magnetoencephalography (MEG) using source localization have demonstrated that temporal lobe theta amplitude successfully predicts integration of memories, and that this involves increased hippocampal-PFC theta coupling (Backus et al., 2016). In light of human structural evidence that PFC-hippocampal white matter tracts may be preferentially impaired following TBI (see "Memory and Executive Function are Persistent Cognitive Deficits after TBI" Section), these studies lead us to hypothesize that axonal injury following TBI may lead to impairments in working memory due to an inability of the hippocampus and PFC to couple at the appropriate time via theta oscillations, either due to latency or conduction issues along the pathways between these structures, or due to a primary reduction in hippocampal theta power, or both. We therefore predict reduced coupling of hippocampal and PFC theta oscillations, and reduced context appropriate entrainment of PFC neurons to hippocampal theta oscillations post-TBI.

Hippocampal, Prefrontal Cortical and Amygdalar Synchronization and Affective Memory: TBI and Post-Traumatic Stress Disorder Comorbidity?

Recent work from a number of laboratories has examined the interactions between the limbic structures known to be involved in another type of learning called "fear conditioning". This classical conditioning paradigm is performed by pairing a cue to an aversive stimulus, after which presentation of the cue without the stimulus then elicits a fear response (Maren, 2001). This paradigm has been described as a model of post-traumatic stress disorder (PTSD), as the effects of this pairing are long lasting, and can only be extinguished by numerous presentations of the cue without the stimulus. The strongest link between limbic areas during acquisition and extinction of fear memory appears to be between the PFC and amygdala, as increases in both theta and gamma oscillation synchrony have been reported within and between these structures during various stages of a fear conditioning task (Likhtik et al., 2014; Stujenske et al., 2014). Consolidation of fear memory involves theta oscillatory synchronization between the hippocampus and amygdala, which appears in the days after training (Seidenbecher et al., 2003). In contrast, oscillatory synchrony occurs between all three structures during REM sleep after training, which has been suggested as a predominant time when memory is consolidated, suggesting a specific role for oscillation synchronization in the consolidation of fear memory (Popa et al., 2010). While the mechanisms underlying this long distance synchronization remain to be elucidated, it is clear that behavioral state and learning changes are correlated with changes in oscillations shared by or propagated between these limbic regions during fear and anxiety associated behavior.

PTSD is a frequent comorbidity with TBI in the military population, with almost 35% of mild TBI exposed Veterans reporting qualifying symptoms associated with their service in theater (Galarneau et al., 2008; Stein and McAllister, 2009). However, a great deal of controversy remains over whether mild TBI contributes to the susceptibility for PTSD, or whether TBI mechanistically underlies some aspects of presenting PTSD symptoms (Hoge et al., 2008; Stein and McAllister, 2009). The presenting symptomatology of PTSD

(i.e., emotion dysregulation, sleep and cognitive deficits) may have an underlying basis in the biomechanical disruption by TBI of the pathways coordinating limbic regions and their oscillatory network interactions described above. TBI may also disrupt the substrates for the consolidation processes necessary for the predominant treatment for PTSD, extinction therapy.

A SYSTEMS NEUROSCIENCE APPROACH TO TBI

All cognition and memory is formed from neural ensemble coding, which has a spatial and temporal component distributed throughout the brain and that is linked via axonal connections. TBI has the potential to disrupt the spatiotemporal interaction between ensembles due to changes in axonal latency, via axonal loss due to dysfunction or degeneration, leading to changes in synaptic and intrinsic neuronal properties. As we have described, timing disruptions in hippocampal networks from the local to the global level may both explain some of the persistent symptoms following TBI, including memory and executive function impairment, and suggest therapeutic strategies for intervention and recovery of lost function.

We have argued that axonal injury to pathways within the hippocampus such as Schaffer collaterals, as well as pathways that connect the hippocampus to other larger networks such as the perforant pathway, the fornix, and the cingulate bundle (connecting the hippocampus to the PFC) leads to timing delays in neuronal communication particularly relevant to TBI symptoms. Several overarching hypotheses emerge:

- TBI induced axonal injury results in loss of, or derangement in synchronization within intra-hippocampal connections. This may affect proper hippocampal oscillatory function, including the temporal organization of neuronal ensembles involved in memory construction (e.g., place cells).
- TBI induced axonal injury results in loss of, or pathological delay in synaptic input into the hippocampus. This may affect organizing oscillations from extra-hippocampal sources (e.g., theta), proper formation of neuronal ensembles encoding spatial or episodic memories, and the coordination of both.
- TBI induced axonal injury to long-range connections between the hippocampus and other distant structures may impair coordinated oscillatory activity between these regions and adversely affect executive functions such as working memory, or affective processes such as fear conditioning.

Neuromodulation Strategies Based on Neuronal Timing Disruptions

Neuromodulation strategies arising from these hypotheses would not only reveal anatomical targets for stimulation or inhibition, but also suggest timing frequency parameters for such interventions. For example, much of the history of deep brain stimulation for movement and psychiatric disorders, a classic neuromodulation therapy, has focused on anatomical targets and has largely employed an empirical method for determining stimulation parameters (Lozano and Lipsman, 2013). We propose a fundamentally different strategy, one that

employs an understanding of the timing deficits within neuronal circuits and then seeks to restore them, or compensate for them. For example, one might envision providing electrode stimulation of the septo-hippocampal pathway at a theta frequency, either by medial septal stimulation, or direct fornix stimulation, to restore organizing theta oscillations in the hippocampus to support memory function, a strategy that has already been employed in rodent models (Lee et al., 2013, 2015). This could be further developed into a closed loop system that might limit theta stimulation to periods of low SPW-R activity, or in response to increased entorhinal cortical activity.

An approach based on dysfunctional neuronal timing may also lead to molecular therapeutic strategies. It has been proposed that myelination and related latency, or signal conduction speed, may in fact be a plastic phenomena, suggesting that there may be some control exerted by the neuron over how fast a signal can be propagated to its targets (Pajevic et al., 2014). If demonstrated conclusively, this could be a mechanism for repair after TBI that is already being utilized by the brain after injury, and one that could be exploited for therapeutic applications should there be widespread latency delays. Latency delays have been demonstrated in human TBI utilizing evoked potentials, and have been correlated with outcomes in some studies, but whether these latency changes in evoked responses are diagnostic remains controversial, and they have been used predominantly in moderate to severe TBI (Rappaport et al., 1991; Keren et al., 1991; Lew et al., 2004; Morgalla and Tatagiba, 2014). These results have not been reproduced in animal models to date, presumably due to the axonal distances required to demonstrate a detectable change in latency. These should be undertaken in large animal models in order to measure latency effects and test therapeutic ideas for restoring axonal function.

Animal Models of Axonal Injury, Human Confirmation of Network Dynamics

Animal models of pure diffuse axonal injury remain rare due to the difficulty in generating the biomechanical forces necessary to induce inertial injuries. However, existing inertial injury models in gyrencephalic species have demonstrated similar axonal pathology to the patterns and distribution of human diffuse axonal injury (Smith et al., 1997). At lower levels of rotation, presumed to model mild TBI, axonal pathology was still widely detected (Browne et al., 2011). However, although not models of inertial injury, axonal loss and injury also occur in many rodent TBI models, suggesting that they can be utilized to test selected hypotheses presented here in affected pathways (Johnson et al., 2015). Similarly, a significant limitation of our understanding of network oscillations and entrainment is that it is also based on rodent studies, although many of the mechanisms we describe have been observed in humans and non-human primates, as noted in previous sections. Thus, it is critical that mechanisms of network synchrony are verified in higher-order species, including humans, and that methods are developed for electrophysiological investigations in gyrencephalic animal models of traumatic axonal injury more representative of the

human condition. Developing these strategies and the detailed understanding that will support them requires the tools of systems neuroscience, including electrophysiological analysis of both single neuron behavior and circuitry changes after TBI in awake animals, as well as computational modeling to develop more specific experimental predictions. As more is revealed about how regional neuronal interactions are disrupted via axonal injury, treatments directly targeting these dysfunctional pathways using neuromodulation can be developed.

CONCLUSION

All cognition and memory is formed from neural ensemble coding, which has a spatial and temporal component distributed throughout the brain and that is linked via axonal connections. TBI has the potential to disrupt the spatiotemporal interaction between ensembles due to changes in axonal latency, or via axonal loss due to dysfunction or degeneration leading to changes in synaptic and intrinsic neuronal properties. In order to better understand these changes in the brain post injury, we must begin to use the tools of systems neuroscience to "listen to the neuronal ensemble" as well as to begin to modulate

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these connections so that we may better understand how to develop both neuromodulatory and molecular therapies for this condition.

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JAW and PFK conceived of and wrote the article.

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Traumatic Brain Injury and Neuronal Functionality Changes in Sensory Cortex

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Traumatic brain injury (TBI), caused by direct blows to the head or inertial forces during relative head-brain movement, can result in long-lasting cognitive and motor deficits which can be particularly consequential when they occur in young people with a long life ahead. Much is known of the molecular and anatomical changes produced in TBI but much less is known of the consequences of these changes to neuronal functionality, especially in the cortex. Given that much of our interior and exterior lives are dependent on responsiveness to information from and about the world around us, we have hypothesized that a significant contributor to the cognitive and motor deficits seen after TBI could be changes in sensory processing. To explore this hypothesis, and to develop a model test system of the changes in neuronal functionality caused by TBI, we have examined neuronal encoding of simple and complex sensory input in the rat's exploratory and discriminative tactile system, the large face macrovibrissae, which feeds to the so-called "barrel cortex" of somatosensory cortex. In this review we describe the short-term and long-term changes in the barrel cortex encoding of whisker motion modeling naturalistic whisker movement undertaken by rats engaged in a variety of tasks. We demonstrate that the most common form of TBI results in persistent neuronal hyperexcitation specifically in the upper cortical layers, likely due to changes in inhibition. We describe the types of cortical inhibitory neurons and their roles and how selective effects on some of these could produce the particular forms of neuronal encoding changes described in TBI, and then generalize to compare the effects on inhibition seen in other forms of brain injury. From these findings we make specific predictions as to how non-invasive extra-cranial electrophysiology can be used to provide the high-precision information needed to monitor and understand the temporal evolution of changes in neuronal functionality in humans suffering TBI. Such detailed understanding of the specific changes in an individual patient's cortex can allow for treatment to be tailored to the neuronal changes in that particular patient's brain in TBI, a precision that is currently unavailable with any

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THE CLINICAL PROBLEM OF TRAUMATIC BRAIN INJURY (TBI)

Traumatic brain injury (TBI) is caused by direct blows to the head or inertial forces during relative head-brain movement. TBI can result from physical trauma to the head, in sports accidents, physical abuse, motor vehicle accidents, military conflict and terrorist activity. The first three account for most TBI in civilians and the latter two for a large increase in TBI in defense personnel and civilians (Narayan et al., 2002; Werner and Engelhard, 2007; Myburgh et al., 2008; Park et al., 2008; Risdall and Menon, 2011). TBI is a major global health issue with an incidence of at least 200/100,000 population and mortality rate for severe TBI of 20-30% in developed countries and up to 90% elsewhere (VNI, 2007/2008; Helps et al., 2008; Faul et al., 2010; Risdall and Menon, 2011). An estimated 52,000 people with TBI die annually in the USA, with TBI being a contributing factor in up to 30.5% of all injury-related deaths (Faul et al., 2010). It is a continuing problem for victims, families and the community since even mild TBI may result in life-long disability, with enormous social and medical burdens; total life-time expenses in moderate-to-severe TBI were estimated to be \$8.6 billion in 2008 in Australia alone (Collie et al., 2010) and \$60 billion inclusive of direct and indirect medical costs in the USA in 2000 (Corso et al., 2006). Even mild TBI is associated with high rates of cognitive impairment, often affecting young people with a long life ahead.

Current treatment options in TBI are scarce and of limited effectiveness. To date there has been no successful Phase III clinical trial of a therapy and there are no FDA-approved therapies for mitigating the effects of TBI. In particular, with respect to drug therapies, in December 2014 the PROTECT III Phase III Randomized Controlled Trials (RCT) of progesterone treatment for TBI report noted "more than 30 clinical trials have investigated various compounds for the treatment of acute TBI, yet no treatment has succeeded at the confirmatory trial stage" (Wright et al., 2014). These past translational failures may have arisen from not having sufficient fine grain detail of the different pathophysiologies or differential prognosis in different injury models—likely because of not using techniques that provide fine grain resolution of brain activity changes in different forms of TBI. We argue here that electrophysiological monitoring of neuronal activity, especially cortical neuronal activity, provides a mechanism to do so individually in TBI patients. To establish this point we will review the understanding that has been gained from use of systems neuroscience techniques to study the effects of TBI on cortical neuronal functionality.

This review is couched in the context that much is known of molecular and anatomical changes post-TBI but, until recently, almost nothing was known of the changes in cortical neuronal function and information processing that cause long-term cognitive, motor and sensory deficits and their evolution over time. Given that effective connectivity and optimal network structure is essential for proper information processing in the brain, and functional brain abnormalities are associated with pathology in connectivity and network structures, people with

mild to moderate diffuse TBI can recover simple motor skills such as grip strength and finger tapping (Haaland et al., 1994) in the first year after trauma. Even after severe forms of TBI motor impairment show a pattern of improvement (Walker and Pickett, 2007) possibly due to development of compensatory behaviors and uninjured intact motor areas providing recovery via intracortical connectivity with other cortical regions and/or through their direct corticospinal projection pathways (Nudo, 2013), but have prolonged cognitive deficits (Strich, 1956; Bawden et al., 1985; Povlishock, 1993; Haaland et al., 1994; Gagnon et al., 1998; Graham et al., 2000; Werner and Engelhard, 2007; Draper and Ponsford, 2008; Little et al., 2010; Fozouni et al., 2013; Bayley et al., 2014). Cognition encompasses abilities such as attention, memory, language, reasoning and problem solving, and is essential for almost every aspect of our lives. It is impacted after even mild TBI, with impairment in memory, speed of information processing and executive functioning being strongly associated with the degree of functional outcome following injury, affecting many areas including employment, education, and social participation (Strich, 1956; Bawden et al., 1985; Haaland et al., 1994; Gagnon et al., 1998; Graham et al., 2000; Draper and Ponsford, 2008; Little et al., 2010). Thus the cognitive deficits caused even by mild TBI have wide ranging effects on every facet of a patient's life.

Commonly-used methods such as behavioral assessment and structural neuroimaging do not allow for precise monitoring of the evolution of TBI-induced changes in neuronal functionality. Only electrophysiology can provide the high-precision information needed to monitor and understand the temporal evolution of neuronal functionality changes. We have used this technique in rats to detail changes in cortical neural function at different time points after either closed skull TBI or open skull TBI (Alwis et al., 2012, 2013; Johnstone et al., 2013, 2014, 2015; Yan et al., 2013). Our studies in animal models of closed-skull and open-skull TBI have now provided compelling evidence that TBI alters sensory cortical neuronal functionality (Alwis et al., 2012, 2013; Johnstone et al., 2013, 2014, 2015; Yan et al., 2013). We will review these and other relevant studies in a systems neuroscience framework after a brief review of neuronal changes in TBI.

A BRIEF OVERVIEW OF TBI EFFECTS ON BRAIN NEURONS

During head trauma, inertial forces from rapid acceleration-deceleration and rotation of the brain lead to diffuse TBI, where there is diffuse axonal injury (DAI); tearing injuries to axons, causing axonal swelling and disconnection (Povlishock, 1993; Gaetz, 2004; Werner and Engelhard, 2007). Many molecular cascades are activated to cause secondary brain damage via multi-factorial processes including oxidative stress, excitotoxicity, hypoxia-ischemia, inflammation and edema, leading to continuous changes in axon pathology over minutes-to-weeks (Povlishock, 1993; Gaetz, 2004; Werner and Engelhard, 2007). The molecular and anatomical changes in TBI follow a complex time course, and neuron pathology evolves from

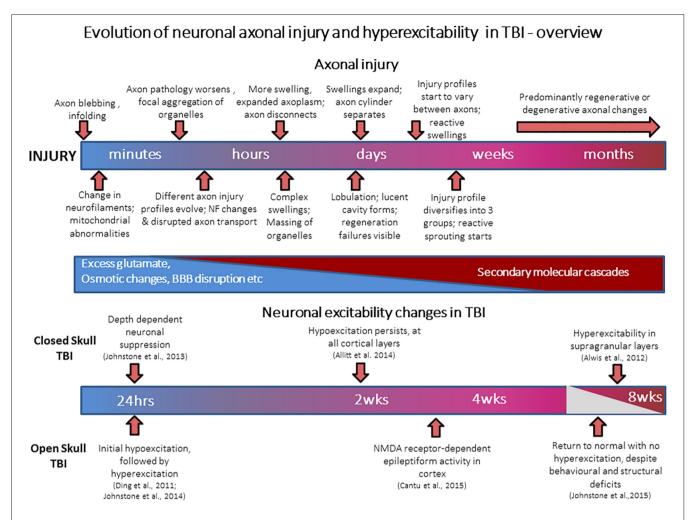


FIGURE 1 | Evolution of neuronal axonal injury and hyperexcitability after traumatic brain injury (TBI). The schematic figure summarizes events occurring during the progression of axonal injury from acute post-injury periods (minutes to hours), through to late post-injury periods (weeks to months). Also presented is a summary of known electrophysiological changes in neuronal excitability in cortex after TBI, and the time-points at which they occur.

minutes to weeks post-trauma, as shown in the simplified summary of Figure 1. Very severe head injury causes lesions (focal TBI) visible on standard imaging but diffuse TBI is believed to be underdiagnosed as it is not visible with CT or MRI [now there is some success with diffusion tensor imaging (DTI), for white matter injury (Fozouni et al., 2013)]. Focal injury includes contusions, which are usually superficial bruises of the brain affecting the cortex and in more severe cases the underlying white matter itself. About 70-75% of human TBI cases show diffuse TBI and \sim 20-25% suffer focal and diffuse injury. The fluid percussion (FP) and controlled cortical impact (CCI) models are representative open skull injury models while the weight drop impact acceleration (WDIA) model is representative of closed skull injury model, the effects of which are similar to those which occur in motor vehicle and sporting accidents. Diffuse and focal TBI have some overlapping behavioral deficits (Gaetz, 2004; VNI, 2007/2008; Helps et al., 2008; Faul et al., 2010; Risdall and Menon, 2011), but also differ in outcomes since focal injury also leads to brain lesions, neuro-degeneration, and behavior changes like epilepsy generally not associated with diffuse TBI.

During the phase of rapid acceleration-deceleration and brain rotation, shear-tensile forces can cause direct severing of axons, an effect referred to as primary axotomy (Corbo and Tripathi, 2004). Such direct severing of axons, causing them to retract and form a retraction ball, is only found in the most severe cases of TBI (Povlishock and Katz, 2005). More commonly, in mild and moderate forms of TBI there is secondary axonal swelling and disconnection, a process referred to as secondary axotomy (Pettus et al., 1994; Büki and Povlishock, 2006). Secondary axotomy appears due to several mechanisms (Farkas and Povlishock, 2007). One mechanism involves axonal swelling and subsequent axotomy due to the disruption by the primary insult of underlying axoplasmic cytoskeletal components (Maxwell et al., 1997; Farkas and Povlishock, 2007). As delivery of substances continues through normal transport kinetics in a disrupted cytoskeleton, intracellular protein and cellular organelles accumulate along sections of the axon, leading to

progressive swelling and collapse several hours after initial injury (Kelley et al., 2006; Farkas and Povlishock, 2007; Greer et al., 2013). This accumulation makes it possible to microscopically identify DAI by axonal retraction bulbs. Other mechanisms of axonal disconnection include altered membrane permeability, activation of cysteine proteases, cytoskeleton breakdown and mitochondrial swelling. In this instance, DAI is not detected by hallmark neuropathological swollen bulbs, as there is a conversion of anterograde to retrograde transport which blocks the occurrence of swelling (Povlishock and Katz, 2005), but β-amyloid precursor protein, an indicator of impaired axonal transport, accumulates at the site of axotomy (Gentleman et al., 1993). After axonal severing, downstream axonal segments will undergo Wallerian degeneration, as early as 1-3 h after trauma, but even up to several months post-impact (Kelley et al., 2006).

The exact progression of these changes will vary between patients with injury type and severity, and patient-related factors. Knowledge of how these changes translate to the changes in neuronal information processing that cause long-term cognitive, motor and sensory deficits is critical for prognosis, to ensure therapy is appropriate for brain processes occurring *at that time* in each patient.

SENSORY CORTEX AS A SYSTEMS NEUROSCIENCE TEST BED

Our internal world and our responses to the external world are often driven by the input we receive of that external world. Thus, our underlying thesis is that many of the prolonged cognitive, sensory, movement and memory deficits after TBI are exacerbated from deficits in sensory processing. This hypothesis is framed in the context of the accumulating evidence in humans and animals that the prolonged cognitive, sensory, movement and memory deficits after TBI may flow-on from deficits in sensory processing (Strich, 1956; Bawden et al., 1985; Haaland et al., 1994; Gagnon et al., 1998; Graham et al., 2000; Draper and Ponsford, 2008, 2009; Ponsford et al., 2008; Little et al., 2010; Bayley et al., 2014), even in mild/moderate TBI (Strich, 1956; Bawden et al., 1985; Haaland et al., 1994; Gagnon et al., 1998; Graham et al., 2000; Draper and Ponsford, 2008; Little et al., 2010). It is also the case that sensory cortex is a good model system for studying post-TBI deficits in cortical processing because of its many features of topographic organization, its well-characterized neuronal responses to a wide range of sensory features, and the fact that sensory stimuli can be precisely quantified and reproduced between test sessions and animals.

To study the underlying neuronal functionality changes that underlie these deficits, we have conducted studies using an animal model, the rat barrel [somatosensory] cortex, a major sensory processor receiving tactile input from the face whiskers and guiding behaviors like perception, social interactions, navigation and guidance, and sensory and motor learning. The rodent whisker-recipient cortex (the postero-medial barrel sub-field (PMBSF) of somatosensory cortex the so-called "barrel" cortex) has been studied intensively over the past

few decades owing to its distinctive structural and functional organization. It contains a highly organized somatotopic map of the rodent's facial whiskers in the mystacial pad (Schubert et al., 2001). The mystacial pad system comprises short (microvibrissae) and long (macrovibrissae) whiskers arranged in an organized fashion along the snout in a grid-like pattern of arcs and rows (Woolsey and Van der Loos, 1970). Afferent information from each whisker projects via brainstem and thalamic nuclei to the PMBSF (details below).

Like other areas of the neocortex, neurons in PMBSF are arranged in layers that connect to different cortical and subcortical regions (Mountcastle, 1997). The neocortex comprises layers I-VI, with the cell-sparse layer I being the most superficial and layer VI the deepest and lying just above the white matter. In PMBSF, cells in the main thalamic input layer IV are clustered in a distinctive barrel-like manner (hence "barrel" cortex) with cells arrayed around a cell-sparse hollow. Each barrel receives input from a specific whisker, the Principal Whisker (PW; details below). The cells directly above and below each barrel represent a functional column extending from supra (layers I-III) to infragranular (V-VI) layers (Mountcastle, 1997). Barrels are separated by cell-sparse septa which are responsible for extra-columnar processing of information, and transfer of signal between adjacent barrels (Welker and Woolsey, 1974; Alloway, 2008). Afferent information from each whisker projects via brainstem and thalamic nuclei to an individual "barrel" in layer IV (Alloway, 2008) to define the PW of that barrel. Cells in a single functional column aligned with a specific barrel respond best to the same PW (Schubert et al., 2001) although they also respond to adjacent whiskers.

Input from the thalamus is relayed to granular layer IV, which mainly projects to layer II/III neurons, as well as other layer IV neurons. Supragranular neurons are responsible for integrating information both within a single functional column, as well as across adjacent cortical column, hence receiving inputs from other supragranular neurons, as well as pyramidal neurons from granular layer IV (Petersen, 2007, 2009). Output pathways for these supragranular neurons include projections to supragranular neurons in adjacent barrels, as well as projections to infragranular layers (LV; Welker et al., 1988; Hoeflinger et al., 1995; Petersen, 2007, 2009; Crochet and Petersen, 2009). Infragranular layers (Layers V and VI) are involved in output to other cortical areas such as secondary somatosensory cortex (S2) and the motor cortex (Fabri and Burton, 1991; Hoeflinger et al., 1995).

Three parallel afferent pathways carry tactile information from the whisker follicle to the cortex, with the pathways involving different brainstem and thalamic nuclei. The leminiscal pathway travels via the principal nucleus of the trigeminal and the dorsomedial section of the ventral posterior medial (VPM) nucleus of thalamus, to terminate densely in layer IV barrels of the PMBSF cortex. This pathway also has secondary projections that terminate in layers III, Vb, and VI in the same vertical column as the layer IV barrel. The paralemniscal pathway runs via the interpolar nucleus of the spinal trigeminal nucleus (subnucleus interpolaris; SpVi) and the medial part of

the posterior medial (POm) to layers I and V (both barrel and septa but terminating most densely in the septa) of the PMBSF cortex and the S2 cortex. The secondary projections of this pathway terminate in layers II and III of the septal columns and in layers I and Va of both septal and barrel columns (Alloway, 2008). Finally, the extralemniscal pathway travels through the SpVi and the ventrolateral section of VPM to the septa between barrels in PMBSF cortex as well as to the secondary S2 (Alloway, 2008).

We now elaborate on the neuronal functionality changes in brain neurons after TBI, mainly focusing on the changes that we find in barrel cortex and the effects reported by others in hippocampus, and then suggest a putative mechanism to account for the effects. Our studies on barrel cortex neuronal functionality in the whole animal have provided the greatest amount of information on changes in brain neurons at the systems level, where studies at the level of the hippocampus, conducted in slices, have provided great understanding on changes in brain neurons at the level of synapses and channels. In the context of this review, our main focus will therefore be on the former set of data.

IMMEDIATE AND LONG-TERM TBI EFFECTS ON CORTICAL NEURONAL FUNCTIONALITY

There are no studies of the continuous evolution of changes in neuronal functionality in the same animals and we describe here studies of neuronal functionality changes measured (in different studies) at discrete time points post-injury.

Studies of neuronal functionality in layer IV of barrel cortex after sustained cortical compression, a model of open skull TBI, found immediate suppression of neuronal activity, lasting for 5–20 min, followed by increased cortical activity by 2 h post injury (Ding et al., 2011). In other studies the hypoexcitation appears to be longer-lasting: metabolic studies show significantly reduced somatosensory circuit activation and depressed local cerebral metabolic rates of glucose even at 4 and 24 h after trauma (Dietrich et al., 1994).

We examined the immediate post-TBI neuronal responses at a longer post-injury interval of 24 h post-injury. We examined effects for two major models of TBI with similar levels of severity—the weight-drop impact-acceleration model for severe diffuse injury or closed-head injury, and the lateral FP injury model for severe mixed diffuse and focal injury or open-skull injury. The effects seen in our studies are summarized in Figure 2 which plots the ratio of normalized change in neuronal firing rates in TBI and Sham control animals (Figure 2A: closed skull model TBI; Figure 2B: open skull model TBI). Responses were collected at different amplitudes of two complex whisker waveforms (Johnstone et al., 2014), from all neurons within a cortical layer, from Layer II through to Layer V from the somatosensory barrel cortex using a micro electrode (2-4 MOhm; FHC). Refer **Supplementary Table 1** for sample sizes for each cortical layer for the two different models of TBI (Closed and Open skull) at the two different time points (24 h and 8-10 weeks). We recorded from neuronal clusters, of anesthesized

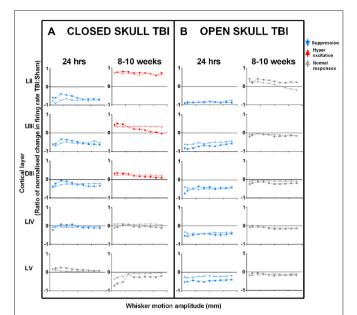


FIGURE 2 | Effects of TBI on sensory cortical neural responses in the short-term (24 h post-TBI) and in the long-term (8-10 weeks post-TBI). Left columns (A): closed skull TBI; (Alwis et al., 2012; Johnstone et al., 2013) Right columns (B): open skull TBI (Johnstone et al., 2014, 2015). For both types of TBI, data are presented for effects seen 24 h post-TBI and 8-10 weeks post-TBI. In both models of TBI, immediate post-injury (24 h) effects of TBI are a suppression of responses, greatest in upper layers and decreasing with cortical depth. However, long-term effects (8-10 weeks) differ: in closed skull TBI there is Hyperexcitation in upper layers with all other layers showing normal responses, whereas mixed TBI results in normal responses in all layers. Data are neural response rates in TBI relative to Sham control, to two complex whisker motion waveforms (Ritt Rough and the Hartmann) as represented by the dotted and dashed lines. Data from top to bottom by depth of cortical layer: Layer II (LII), Upper Layer III (UIII), Deep Layer III (DIII), Layer IV (LIV) and Layer V (LV) (Data pooled across neurons in a layer; error bars omitted for clarity).

animals to which we applied spike-sorting algorithms (Alwis et al., 2012) to extract single neuron data, to find that the effects were identical at single unit and population level, although amplified in the latter.

In both models, at 24 h post TBI there is a suppression of responses in the upper layers (compare first and third data columns in Figure 2: Layer II and Upper III, LII and UIII) and the effect decreases with cortical depth. This was true across simple and complex stimuli, and all types of excitatory cortical neurons (not shown). Changes in cortical activity occurring immediately post trauma are linked to the initial impact of the injury itself (Ordek et al., 2014) and involve elevation in intracranial pressure, damage to blood vessels and tissues, and axotomy, further inducing ionic imbalances (Ding et al., 2011; Johnstone et al., 2013). We have proposed (Johnstone et al., 2014) that this similarity occurs because of effects triggered by a stress wave initiated by the injury process, whether penetrating (in open skull TBI models) or not (in closed skull TBI models). In the open skull TBI model, we found that varying the distance of the injury site from 1-6 mm from barrel cortex did not change the depth-dependency (not shown), confirming that a remote

spreading event caused the neuronal dysfunction. This factor is likely to be a wave of cortical spreading depression (CSD), which is characterized by rapid and almost complete depolarization of large populations of neurons, and propagates in the brain as a regenerating wave (Charles and Brennan, 2009). The wave spreads at a few mm/minute (Haaland et al., 1994; Charles and Brennan, 2009) with its leading edge in the upper layers which contain apical dendrites (Aitken et al., 1998). If depolarization persists, neurons enter an "unresponsive" state and can be rendered hypoactive in the long-term (Basarsky et al., 1998) through synaptic events such as long-term depression (Somjen, 2001). Cortical waves of depression have been observed after injury due to predominantly open skull injury models, including the open skull model (Katayama et al., 1990; Herreras and Somjen, 1993; Basarsky et al., 1998; Theriot et al., 2012) we use, but have yet to be documented after closed skull injury. Overall, our findings suggest CSD may likely be an important factor causing persistent cortical cellular changes after TBI, induced by either closed or open skull injury.

In the longer-term effects, hyperexcitability was reported in neocortical brain slices in layer V at 2 weeks CCI injury, another open skull model TBI (Yang et al., 2010). In our studies we found that the presence or otherwise of long-term hyperexcitation may depend on injury model. Thus, in contrast to the similar short-term effects described above, there were marked differences in long-term effects in the two forms of TBI we have studied. In closed skull TBI (Figure 2 2nd and 4th data columns), there was neuronal hyper-excitation in responses in the upper sensory cortical layers (Layer II, LII, and Upper III, UIII), normal responses in input layer (Deep III, DIII, and Layer IV, LIV; indicating normal sub-cortical inputs) and weak suppression in infra-granular Layer V neurons (LV). The hyper-excitation in upper layers is consistent with the fact that brain injury selectively affects inhibitory neurons (Cantu et al., 2015). This will change the excitation:inhibition (E:I) balance to favor excitation, causing hyper-excitation, and is discussed more fully below. Short term changes in excitatory transmission can also occur (Faden et al., 1989; D'Ambrosio et al., 1998; Sick et al., 1998; Witgen et al., 2005; Norris and Scheff, 2009), but the long-term hyperexcitability (Alwis et al., 2012) is more simply explained by changes in inhibition, of the type recently demonstrated by Cantu et al. (2015), which indicates that excitation is enhanced due to loss of inhibitory control following a CCI injury, due to a loss of inhibitory neurons in the TBI cortex.

As noted above, neurons in LII and LIII receive excitatory input mainly from LIV (Feldmeyer et al., 1999, 2002, 2006; Lübke et al., 2000; Schubert et al., 2001, 2007; Shepherd and Svoboda, 2005; Lübke and Feldmeyer, 2007; Alloway, 2008; Lefort et al., 2009; Hooks et al., 2011). Since they integrate responses across barrel cortex and output to other cortical areas, changes in LII and UIII are likely to contribute significantly in TBI to deficits in cognition and complex behaviors dependent on sensory input. The effect of LII and UIII changes can be seen even within the same column: in diffuse TBI, LV (with inhibition from LII/UIII) shows opposite effects to upper layers, whereas in mixed focal and diffuse TBI, LV shows the same effects as seen in the upper layers, and this likely results in sub-cortical

changes that are different to the intra-cortical effects due to upper layer changes. Also we have previously reported that in the long term post-trauma cases where we find hyper-excitability in Layers II and Upper III, input layer IV of the same cortex shows perfectly normal responses in times of peak firing rates, excitatory responses over the entire stimulus period, the latency to the peak firing rate and the temporal dispersion of responses. Since layer IV predominantly receives the thalamic input to cortex, and its responses reflect these inputs, these data strongly suggest that sub-cortical regions appear to be normal in our diffuse TBI model.

In the open skull model TBI (Johnstone et al., 2015), "normal" long term responses occurred in all cortical layers (Figure 2 4th data column) despite major structural changes (Johnstone et al., 2015) which did not occur in the closed skull TBI model (Yan et al., 2013). We believe that the difference in long-term neuronal and structural changes in the two forms of TBI (closed and open skull) must account for different behavior outcomes (Hallam et al., 2004) as evidenced in the long term after closed skull TBI, diffuse injury in particular, where TBI animals showed persistent sensorimotor deficits up to 6 weeks post trauma and this was particularly evident in tasks related to direct whisker sensory processing (Alwis et al., 2012) while the open skull injury model showed cognitive and motor deficits and heightened anxiety-like behavior even up to 12 weeks after trauma (Johnstone et al., 2015). Open skull injury animals also showed greater impairment in memory tasks as opposed to closed skull injured animals (Hallam et al., 2004). The effect of LII and UIII long term changes can be seen even within the same column after closed skull diffuse TBI, LV (with increased inhibition from LII/UIII) shows opposite effects to upper layers (LII and UIII), whereas in long term open skull mixed diffuse and focal TBI, LV shows the same effects as seen in the upper layers (LII and UIII), and this effect in LV must result in sub-cortical changes that are also different to the intra-cortical effects due to upper layer changes. Our data support the theory that cortical supragranular layers 2/3 maintain their roles as "privileged substrates" (Nichols et al., 2007) for cortical plasticity. This could explain why TBI in both models produces changes in neuronal responsiveness in L2/3 where excitatory feedback is likely to amplify input from granular layer IV. Neuronal activity as indicated by cFos activation was attenuated even a week after closed skull TBI but then increased above sham levels by 4 weeks after trauma (Hall and Lifshitz, 2010). Consistent with this increased cFos activation in the longerterm, our electrophysiological studies showed hyperexcitation in the supragranular barrel cortex at 8-10 weeks post closed skull TBI (Alwis et al., 2012), as noted above. Cortical short-term hypoactivity transitioning to long-term hyperexcitation after closed skull brain injury is suggestive of initial circuit disruption followed by delayed cortical reorganization. Maladaptive circuit reorganization is a putative mechanism that could result in excitation/inhibition imbalance (Alwis et al., 2012, 2013; Greer et al., 2013).

While it is important to recognize that excitation can also be affected in the TBI brain (Faden et al., 1989;

D'Ambrosio et al., 1998; Sick et al., 1998; Witgen et al., 2005; Norris and Scheff, 2009), loss of inhibition is the most likely change accounting for the neuronal hyperexcitability we reported to occur in sensory cortex after diffuse TBI. This review of inhibitory changes in TBI should be viewed therefore in the context that excitatory changes also occur and together with the newer findings of changes in inhibition, lead to a much better picture of the complex and dynamic neuronal functionality changes in the brain. We note that in keeping with these postulates, excitatory activity and connectivity is enhanced while inhibition is compromised after trauma (Bonislawski et al., 2007; Hall and Lifshitz, 2010; Alwis et al., 2012). We propose that in long term after closed skull TBI, hyperexcitation in the supragranular Layer II and Upper III is due to loss of inhibition in these layers either due to reduced local intra-LII/UIII inhibitory inputs to local excitatory neurons or due to a loss of excitatory drive from LII/UIII to deeper infragranular inhibitory neurons such as the Martinotti cells (MC) in LVA which feedback inhibition to LII/UIII excitatory neurons and other interneurons. This hypothesis is elaborated schematically in Figure 3, which shows specifically the barrel cortex circuitry relevant to LII/UIII. This proposal is consistent with the fact that in the short-term, there is a depth-dependency to the electrophysiological changes in barrel cortex in both closed-skull and open-skull models of injury (Alwis et al.,

2012, 2013; Johnstone et al., 2013, 2014, 2015; Yan et al., 2013).

Histologically, neuroplasticity markers such as growth associated protein (GAP-43) and synaptophysin, a pre-synaptic regenerative marker were elevated above sham controls as early as 3 days (Hulsebosch et al., 1998) and at 4 weeks post injury in the hippocampus suggestive of dynamic circuitry reorganization through synaptogenesis and regeneration (Hall and Lifshitz, 2010)

LOSS OF INHIBITION UNDERLIES TBI AND MANY OTHER BRAIN PATHOLOGIES

Under normal conditions, the activity of cortical neurons is a finely-balanced interplay between excitation and inhibition (E and I) and a balance between these two opposing synaptic conductances is essential for proper cortical function. Then, manipulations in animal models of experimental TBI that selectively decrease either excitation or inhibition will shift cortical activity to result in either a hypoexcitatable or hyperexcitable state (Dudek and Sutula, 2007). There is accumulating evidence that changes in inhibition appear to be one of the major changes that modulates the E:I balance in the long-term TBI brain, as in other brain disorders, as we will review here.

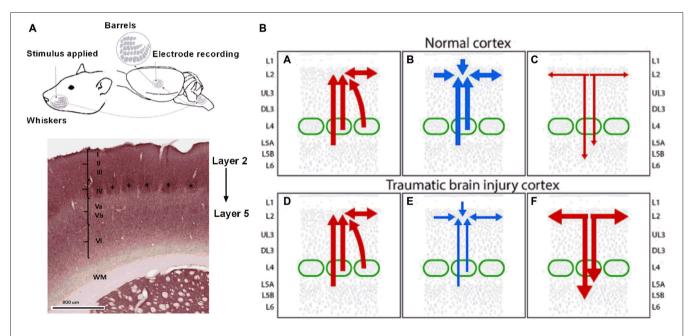


FIGURE 3 | Putative alterations in inhibitory circuits in supragranular layers 2/3 following the long term after diffuse TBI. Panel (A) on the left shows a schematic representation of the rat whisker tactile system and electrophysiology recording from the barrel cortex. Evoked extracellular neuronal responses were recorded from the somatosensory barrel cortical layers 2–5 in response to whisker stimulation. Note the cluster of cells or barrels as denoted by "*" in cortical layer 4. Panel (B) on the right shows hyperexcitation in the supragranular layer 2 and Upper 3 in the long term after diffuse TBI resulting from either reduced local L2/U3 inhibitory inputs or due to a loss of excitatory drive from L2/U3 to deeper infrangranular layers which feed inhibition from L5 to L2/3. Top Row: all panels show circuitry in normal cortex, Lower Row: all panels show circuitry in TBI cortex. Left Panels (A,D) show excitatory inputs (in red) to L2/3 from local L2/3, L4 and L5 excitatory pyramidal neurons within and neighboring cortical columns. Panel (B) shows inhibitory inputs (in blue) to L2/3 from local L2/3 inhibitory neurons, input layer L4 and deeper L5. Panel (E) shows diminished inhibitory input as represented by the thinner blue lines and arrows to L2/3 from L2/3, L4 and L5 in the TBI cortex resulting in hyperexcitabilty in L2 and U3. Right panels show excitatory outflow in normal (C) and hyperexcitable TBI cortex as represented by thicker red lines and arrows (F). Figure details based on information in Xu and Callaway (2009) and Petersen and Crochet (2013).

TABLE 1 | Differential neurochemical expression, innervation type and function of major inhibitory neuronal subtypes.

Inhibitory neuronal subtype	Neurochemical marker	Target membrane domain	Function
Chandelier cells	PV or CB	Axon initial segment	Edit a neuron's output by affecting generation and timing of action potentials (APs)
Basket cells			
• Large	PV, CB and NPY, cholecystokinin (CCK) occasionally SOM and CR	Soma and proximal dendrites	Allows presynaptic neurons to control the gain of summated potentials and thereby control AP discharge of target cells—(phasing and synchronization of neural activity)
 Small 	VIP		
Nested	PV or CB		Affects the generation and propagation of dendritic calcium spikes
Martinotti cells	SOM	Distal dendrites and tufts	Affects the generation and propagation of dendritic calcium spikes
Bitufted cells	CB, CR, NPY, VIP, SOM or CCK	Dendrites	Influences dendritic processing and integration of synaptic inputs.
Bipolar cells	VIP		
Double bouquet cells	CB, CR and CB, VIP or CCK		Influences synaptic plasticity either locally or by integrating with back propagating APs
Neurogliaform cells	nNOS		

Inhibitory neurons can alter a specific activity of the target cell (pyramidal neuron) by selectively innervating a specific membrane domain. (Table derived from information in Markram et al., 2004; Spruston, 2008).

Diversity and Classification of Cortical Inhibitory Neurons

The neocortex contains a diversity of cell types, 20–30% of which are inhibitory neurons (that release the neurotransmitter GABA) with diverse morphological, molecular and electrophysiological properties (DeFelipe, 1993; Thomson and Deuchars, 1994; Cauli et al., 1997; Kawaguchi and Kubota, 1997; Somogyi et al., 1998; Gupta et al., 2000) and therefore it is possible to classify them on the basis of these characteristics. They can be clearly distinguished from excitatory neurons in that most mature inhibitory neurons have aspiny dendrites.

The axonal arborizations of inhibitory neurons are usually confined to their respective cortical columns but can also extend horizontally across columns but do not project to white matter areas (DeFelipe, 2002). Interneurons are arranged in highly ordered circuits within the cortical layers, and also connect to different cortical and subcortical regions, allowing for signal modification throughout the cortical and subcortical network. The interactions between excitatory pyramidal cells and GABAergic interneurons fall into two broad categories namely: "feedback" inhibition, in which an inhibitory neuron synapses back onto the same excitatory neuron which activated it and provides negative feedback and "feedforward" inhibition in which excitatory neurons synapse onto inhibitory neurons which do not synapse back onto those same excitatory neurons. Feedback and feedforward inhibition form the two fundamental building blocks of cortical inhibition (Isaacson and Scanziani, 2011) and both might be altered following injury.

Various methods are used to identify and differentiate between the subtypes of cortical inhibitory neurons including their firing properties, cortical location, morphology and immunochemistry (Kawaguchi and Kubota, 1997; Gupta et al., 2000; Markram et al., 2004). One major means used to discriminate between the different interneuronal subtypes

is by their differential expression of neuromarkers such as calcium binding proteins [calbindin (CB), parvalbumin (PV) and calretinin (CR)] or neuropeptides [neuropeptide Y (NPY), Somatostatin (SOM), vasointestinal peptide (VIP), neuronal nitric oxide synthase (nNOS) and cholecystokinin (CCK)] or combinations of both. The heterogenous GABAergic interneurons have specialized functions in regulating excitatory neuronal activity by innervating a specific subdomain (soma, axon and dendritic regions) of the pyramidal cell (DeFelipe, 1997; Somogyi et al., 1998; Markram et al., 2004; Refer Table 1). For example, Chandelier cells (ChCs) that express the calcium binding protein PV uniquely target the axon initial segment of excitatory pyramidal neurons (Inda et al., 2009) and are capable of suppressing the initiation of action potentials in pyramidal neurons (Miles et al., 1996), acting as "rectifiers" of local circuit activity (Zhu et al., 2004) when network excitability goes out of control.

Basket cells (BCs) that mostly express two calcium binding proteins (DeFelipe, 1997) PV and CB are characterized by their extensive axonal arborizations that exert inhibitory effects on neurons in *supra* and *infra* granular cortical layers in neighboring and distant columns and are therefore considered as a primary source of lateral inhibition(Kisvárday et al., 1993). Large number of interconnections between BCs also suggests their involvement in long range lateral inhibition (Kisvárday et al., 1993). These cells are also thought to be involved in the gamma oscillations which enable fast processing (Fries et al., 2007) required for high levels of cognitive control.

MC, another class of GABAergic interneurons predominantly express the neuropeptide, SOM (Wang et al., 2004; Gentet et al., 2012) and are capable of extensive horizontal axonal projections extending upto millimetres in length within cortical layer I (Kawaguchi and Kubota, 1997; Wang et al., 2002), thereby inhibiting the dendritic tufts of pyramidal cells in nearby and distant columns. MCs are considered to be the only source of

cross columnar inhibition via layer I to layers II-VI (Wang et al., 2004).

The CB and CR immunoreactive (IR) groups of neurons include the dendrite targeting bipolar, bitufted cells (BTCs) and double bouquet cells. Bipolar and double bouquet cells mainly target the basal dendrites of pyramidal neurons (Markram et al., 2004) and are thought to be involved in regulating the activity of other cortical GABAergic neurons through disinhibition (Wang et al., 2002). BTCs on the other hand form inhibitory synaptic contacts by projecting their axon collaterals onto pyramidal cells, neighboring BTCs and multipolar cells within the same layer and influence synaptic plasticity through back propagation of dendritic action potentials (Kaiser et al., 2001) while VIP IR neurons comprise of cells with radially oriented dendrites that extend to several cortical layers and are involved in translaminar inhibition (Kawaguchi and Kubota, 1996; Cauli et al., 1997; Bayraktar et al., 2000) and neuronal communication between pyramidal cells and other interneurons

Other GABAergic neurons include neurogliaform cells (NGFCs) that form local horizontal axonal arborizations in layer I of the cortex (Chu et al., 2003) and are characterized by the expression of nNOS (Kubota et al., 2011). The multipolar Cajal Retzius cells are another class of interneurons found in layer I. A subset of these multipolar cells expresses the calcium binding protein CR in addition to alpha-actinin-2 (Aac; Kubota et al., 2011). The axons of these cells have extensive horizontal trajectories that target the terminal dendrites of pyramidal cells also mediating dendritic inhibition.

Thus owing to their diverse characteristics impairment of specific sub-populations of inhibitory neurons is likely to have different functional consequences and effects on cortical excitability and information processing.

Changes in Inhibition in TBI

The neocortex and hippocampus are brain regions that appear particularly susceptible to TBI. Following TBI, they have been shown to undergo synaptic reorganization consistent with changes in the balance between excitation and inhibition.

Cortex

Spontaneous and evoked burst discharges have been reported in the rat neocortical layer V brain slices 2 weeks after CCI, an open skull injury, and postulated to be due to decreased inhibition (Yang et al., 2010). As noted above, Ding et al. (2011) reported that after open skull injury, there was hyperexcitability in the cortex 2 h post injury, the longest time point they examined. Such increases in cortical excitation following cortical injury (Imbrosci and Mittmann, 2011) are mainly due to loss or reduced activity of inhibitory neurons. Cortical hyperexcitability and elevated glutamate activity are associated with increases in frequency and amplitude of spontaneous excitatory synaptic currents and a decrease in frequency of spontaneous inhibitory synaptic currents 2–6 weeks in the chronically injured epileptogenic neocortex (Li and Prince, 2002). This was also observed in cortex after an open skull CCI brain injury, accompanied by reductions in the number

TABLE 2 | Putative alterations in circuitry balance and function as a consequence of changes in the number of inhibitory neurons following TBI and epileptic seizures.

Interneuron Subtype	Putative alteration in function	
Parvalbumin	Impaired perisomatic inhibition (Huusko and Pitkänen, 2014) and reduction of miniature inhibitory post-synaptic currents (mIPSCs; Knopp et al., 2008)	
	Loss of long range inhibition to adjacent cortical columns (Buriticá et al., 2009)	
Calbindin	Hyperexcitability in Dentate gyral circuits and impaired dendritic inhibition of pyramidal cells (Maglóczky et al., 1997; Carter et al., 2008)	
Calretinin	Impaired columnar inhibition (Buriticá et al., 2009) Impaired synchronization of dendritic inhibitory neurons. Inefficient control of excitatory inputs to pyramidal cells resulting in impaired synaptic plasticity and seizure generation (Toth et al., 2010)	
Neuropeptide Y Somatostatin	Impaired dendritic inhibition (Huusko et al., 2015) Impaired dendritic projections to pyramidal cells resulting in hippocampal hyperexcitability and generation of epileptic seizures (Cossart et al., 2001).	
Cholecystokinin	Impaired perisomatic inhibition (Huusko et al., 2015)	

Functional consequences of loss of particular subsets of Inhibitory neurons following trauma and epilepsy (Table derived from information in Maglóczky et al., 1997; Cossart et al., 2001; Carter et al., 2008; Knopp et al., 2008; Buriticá et al., 2009; Toth et al., 2010; Huusko et al., 2015).

of PV and SOM interneurons at 2–4 weeks following injury (Cantu et al., 2015). Loss of GABAergic control over pyramidal neurons would produce cortical pyramidal cell hyperexcitability but does not have to involve loss of the GABAergic neurons. Indeed, following CCI injury there were no changes in number of NeuN positive cells in the injured cortex of TBI animals compared to sham animals, but the density of PV- and SOM-IR inhibitory neurons was decreased in the injured cortex at 2–4 weeks post-injury (Cantu et al., 2015). In a similar vein we have postulated that in the closed-skull TBI model we have used, the long-term hyperexcitability results from a reduction in certain subsets of GABAergic interneurons or in their activity rather than a complete loss of inhibition (Alwis et al., 2013).

The postulated consequences to circuitry balance and function of loss of different forms of inhibition is summarized in Table 2. Note that the effects can and do differ for different brain regions since GABAergic interneurons likely have specific local functions in each site. Thus, there is a diversity of effects reported, but the overwhelming effect is of hyperexcitation. SOM-IR interneurons appear to be particularly efficient in counteracting increasing levels of cortical excitability and a selective loss of these dendrite targeting cells has been reported in experimental animal epilepsy models (Cossart et al., 2001) and human patients (de Lanerolle et al., 1989). This suggests the involvement of these interneurons in generation of epileptic seizures. Chandelier cells, that predominantly express the calcium binding protein PV and specifically innervate the axon initial segment of pyramidal cells, are reported to be strongly involved in preventing hyperexcitability in the cortex (Zhu et al., 2004) and a selective

loss of these cell types in epileptic loci is indicative of their involvement in epileptic activity (Ribak, 1985). Human TBI studies by Buriticá et al. (2009) reported a decrease in PV-IR inhibitory neurons in layer II, and increases in interneuron-targeting CB-IR in layers III and V and CR-IR in layer II of the cortex. Finally, the functional implication of a loss of PV expressing interneurons following TBI like that reported by Pavlov et al. (2011) is deficits in gamma oscillations and impaired modulation of excitatory signals (Sohal et al., 2009).

Overall, it can be postulated that trauma induced reduction in the activity of certain subsets of interneurons could compromise the excitation/inhibition balance, while a loss or reduction in the functionality of inhibitory cells could have profound effects on cortical information processing.

Hippocampus and Thalamus

In keeping with these postulates, several studies have also reported reductions in the number of GABA receptors and/or GABAergic neurons in hippocampal sub regions of the hilus and dentate gyrus after different forms of TBI and experimental epilepsy (Lowenstein et al., 1992; Toth et al., 1997; Santhakumar et al., 2000; Cossart et al., 2001). Santhakumar et al. (2000) reported a decrease in hippocampal hilar interneuronal populations labeled with GAD67 and PV mRNA probes following an FP injury. Transient reductions were seen in hippocampal GABA_{B1} and GABA_B mRNA while irreversible reductions in GABA_{B1} and GABA_{B2} mRNA expression in thalamus ipsilateral to the injury were seen even up to 4 months post TBI (Drexel et al., 2015). Mild TBI caused no neuronal loss (Eakin and Miller, 2012; Almeida-Suhett et al., 2014). However in moderate TBI the number of GABAergic interneurons was significantly reduced in the CA1 region at 7 days following a CCI (Almeida-Suhett et al., 2015). Finally, in severe TBI, there are reductions in PV-IR interneurons in the ispsilateral and contralateral thalamic VPM-VPL complex (Huusko and Pitkänen, 2014) and substantial reductions in PV, CR, neuropeptide (NPY), CCK and SOM interneurons in hippocampal subfields CA1, CA3 and dentate gyrus (DG), even up to 6 months after open skull TBI (Huusko et al., 2015). A progressive loss in phasic inhibition associated with a loss in PV positive GABAergic neurons was reported in the ipsilateral and contralateral hippocampus following a LFPI, an open skull TBI (Pavlov et al., 2011). These various studies therefore suggest a graded loss of hippocampal inhibition, at least in particular brain regions, dependent on injury

However, other studies report GABA upregulation in the long term after TBI, as a compensatory mechanism to counteract increases in glutamate network activity. Upregulation of GABA_A receptor subunits was seen in hippocampal subregions 10 days and/or 4 months after TBI (Drexel et al., 2015). Increases in GABAergic NPY expressing interneuron fibers have been reported in the injured parietal cortex following a FP model of TBI, and it was suggested this indicated a role of NPY neurons in neuroprotection (McIntosh and Ferriero, 1992).

Functional and circuit reorganization occur in the cortex and hippocampus following TBI. Glutamate excitotoxicity, a secondary injury mechanism of TBI, alters dendritic outgrowths in GABAergic cortical neurons independent of cell death (Bywood and Johnson, 2000). Morphological and biochemical alterations, such as dendritic regression thought to have an effect on neurotransmission, occur in surviving interneurons further impairing cortical functionality (Monnerie and Le Roux, 2007). Other mechanisms that impact on cortical and hippocampul excitability levels include mossy fiber sprouting in hippocampuls following CCI injury (Hunt et al., 2009) and increases in excitatory synapses (Buckmaster et al., 2002) and excitatory input to cortical pyramidal neurons (Brill and Huguenard, 2010).

Changes in Inhibition in other Brain Disorders

Impaired inhibition, shifting the balance towards increased excitation, has been observed in other forms of brain disorders including epilepsy, stroke and schizophrenia. In a kainic acid induced experimental model of epileptogenesis (Fritsch et al., 2009), there was extensive loss in the number of GABAergic interneurons IR for GAD67. There was also a reduction in GluK1 sub unit, which is activated by glutamate to release GABA, and this would further contribute to a reduction of tonic inhibition in the basolateral amygdala (BLA) circuit. This was accompanied by increased protein levels of GAD65/67 expression and α1 subunit of GABAA receptor at 7-10 days after status epilepticus (SE) in surviving interneurons presumably a compensatory effect for inhibitory neuronal loss (Fritsch et al., 2009). In the long-term after SE, ipsilateral reduction in phasic GABAA mediated inhibition have been reported 1 month after TBI and a further significant decrease in synaptic inhibition in both hemispheres 6 months after TBI in a model of posttraumatic epilepsy (Pavlov et al., 2011). Reductions have also been observed in other GABAergic neurons such as CB expressing interneurons in the hippocampal DG of human epileptic patients (Maglóczky et al., 1997) and in animal models (Carter et al., 2008) of long term acquired epilepsy. Thus hippocampal CB neurons may be involved in hyperexcitability in the epileptic DG.

In both trauma and cerebral ischemic stroke, similar processes such as excitotoxicity, oxidative stress, inflammation and apoptosis contribute to loss of cell and tissue integrity. Degeneration of hippocampal GABAergic BCs occurs 12 h after cerebral ischemia (Crain et al., 1988) suggesting the selective vulnerability of inhibitory neurons in stroke just as in trauma. In cortex, accumulating evidence also indicates an excitation/inhibition imbalance in experimental models of stroke (Clarkson and Carmichael, 2009; Clarkson et al., 2010, 2011). Immediately after stroke in the first 30 mins within onset of reperfusion there is a rapid decrease in the expression of GABAA receptor subunit (α 1 and β 2) mRNAs in hippocampal areas CA1, CA3 and DG (Li et al., 1993) accompanied by GABAA receptors down regulation in cortex and hippocampus (Alicke and Schwartz-Bloom, 1995)

which return to normal within 2 h post stroke. Cortical GABA levels are reduced in the short term, 8-18 days and in the long term, 6 weeks after stroke (Liepert et al., 2000; Bütefisch et al., 2008). Blicher et al. (2015) also found GABA levels to be reduced in the primary motor cortex in the long term, 3-12 months after stroke. Such reductions in GABA mediated inhibition facilitate functional recovery during periods of plasticity (Paik and Yang, 2014). Contrary to the above findings, GABAA receptor mediated tonic inhibition increases significantly 3, 7 and 14 days after stroke due to a decrease in the normal uptake of extracellular GABA through neuronal and astrocytic GABA transporters (GAT; Clarkson et al., 2010; Carmichael, 2012). However, Alicke and Schwartz-Bloom found that during transient ischemia prolonged exposure to *in vitro* concentrations of synaptic GABA agonists could downregulate GABAA receptors (Alicke and Schwartz-Bloom, 1995) by restoring the excitation/inhibition balance and promoting functional recovery. The persistent increase in tonic inhibition for up to 2 weeks following stroke is considered to be a compensatory mechanism of the brain to counteract and minimize neuronal

Besides impaired GABA mediated inhibition following stroke, reductions in inhibitory CB expressing interneurons have also been reported in ischemic regions of cerebral cortex after middle cerebral artery occlusion (MCAO; Ouh et al., 2013). Transient cerebral ischemia has been reported to alter dendritic morphology of GABAA receptor alpha 1-subunit-IR interneurons in CA1 of the hippocampus as early as 3 days persisting up to 5 weeks. Similar dendritic abnormalities have also been observed in CA1 interneurons even in long term, 12–14 months after ischemia (Arabadzisz and Freund, 1999) leading to altered GABA neurotransmission.

Like other brain disorders, schizophrenia has also been shown to be associated with multiple abnormalities in pre and post synaptic GABAergic PV expressing BCs weakening their inhibitory control over pyramidal cells (Lewis et al., 2012). Memory function is impaired in schizophrenia owing to diminished gamma-frequency synchronized neuronal activity resulting from altered *perisomatic inhibition* of pyramidal neurons (Lewis et al., 2005). A bilateral reduction in CB neuronal density was also reported in the planum temporale in schizophrenic patients, suggesting a reduction in *columnar/vertical inhibition* provided by double bond conversions (DBCs) that account for the majority of CB expressing interneurons in the brain (Chance et al., 2005).

Given that brain disorders, including TBI can alter inhibitory circuits in a number of ways and that inhibitory neurons play an important role in regulating cortical activity, our working hypothesis is that diffuse TBI can alter intra-cortical inhibition following diffuse TBI. We hypothesize further that altered inhibition, along with DAI, explains the pattern of responses we observed in the closed skull, diffuse injury model, and the open skull, mixed diffuse and focal injury model. Although the lack of detailed information regarding the timing and extent of post TBI changes in cortical cellular physiology make it difficult to speculate precisely, we argue broadly that in moderate-to-

severe TBI, the initial stress wave from head or brain impact results in DAI which reduces responses at 24 h in both models; then over the next 8–10 weeks, additional, cell death from direct focal injury will result in slower death of excitatory and inhibitory neurons, dampening overall activity and returning the E:I balance to normal levels. However structural damage associated with focal injury will produce different deficits in the open skull model (diffuse and focal injury) compared to the closed skull model (diffuse injury only). Further, interplay between the effects of diffuse TBI [e.g., immediate axonal injury and long term interneuron damage] and focal injury (e.g., cell death) causes neuronal and behavior deficits to evolve more slowly in mixed (focal and diffuse) TBI.

CONCLUSIONS FOR FUTURE DIRECTIONS

As we noted at the outset of the review, little has been (and is still) known of the changes in systems-level neuronal processing that cause long-term cognitive, motor and sensory deficits. There is a wealth of data on hippocampal electrophysiology at the slice level, but very little from the cortex, especially at the whole animal level. Our thesis is that prolonged cognitive and motor deficits in people with TBI may be due to sensory cortical deficits, induced by changes in the E:I balance. We have summarized the accumulating evidence that changes specifically in inhibition appear to be one of the major changes modulating the E:I balance in the long-term TBI brain, to favor excitation. The available evidence certainly shows that excitatory activity and connectivity is enhanced while inhibition is compromised after trauma, and may not be restricted to TBI alone amongst brain disorders. The existing data suggest that, at least in cortex, the major change is in the supragranular layers.

Against the relative uniformity of effects in cortex, at the sub-cortical level it has been reported that there is also upregulation of inhibition in long term TBI to counteract increases in glutamatergic activity. Thus, some regions of hippocampus show increased excitation but other regions show increased suppression in long-term TBI. There is sprouting of fibers in hippocampus and increases in excitatory synapses and excitatory input to cortical pyramidal neurons, all of which will impact on the EI balance in the brain.

With respect to neuronal functionality in the intact brain, our studies in the sensory barrel cortex of rats show that, at a systems level:

- 1. Evoked neural activity recorded with intra-cranial microelectrodes from neurons in upper cortical layers (those most accessible for sampling with extra-cranial electrodes) very reliably maps neuronal changes in TBI to behavior deficits [closed skull injury: (Alwis et al., 2012), open skull injury: (Johnstone et al., 2015)].
- 2. These neural changes are detected with microelectrode recordings even after *mild* TBI which shows no histological or molecular changes but results in mild behavior deficits.
- 3. Electrophysiology very well differentiates between differences in the changes in neuronal functionality in different types

of TBI, which produce very similar neural changes in the short-term but very different long-term effects (and different behavior outcomes).

These data provide compelling evidence that electrophysiology can provide the high-precision information needed to monitor and understand the temporal evolution of changes in neuronal functionality in TBI. Non-invasive extra-cranial electrophysiological recording from the brain [using the electroencephalogram, (EEG)] is very attractive as the equipment is portable, the process is much more cost effective compared to any other method of monitoring brain activity, and testing can be done at the patient's bedside. There has been a resurgence of interest in extra-cranial EEG recordings of neuronal activity in brain injury (Abend et al., 2010). However, electrophysiological recordings have been underutilized clinically and poorly studied and there is a dearth of basic and applied information on the evolution of changes in brain neuronal function and processing in different forms of TBI (Bosco et al., 2014). In fact a survey of 330 physicians concluded that "little data exists to allow for evidence based [continuous EEG] implementation or management related to [continuous EEG] findings" (Abend et al., 2010). Studies on the utility of quantitative EEG (qEEG) as a detection tool for mild TBI or concussion concluded that qEEG by itself provided limited diagnostic utility (Nuwer et al., 2005; Arciniegas, 2011; Rapp et al., 2015). Other review studies show the usefulness of continuous EEG and electrophysiological monitoring in the detection of seizures and SE (Schmitt and Dichter, 2015) and in estimating the prognosis of acute brain injury (Claassen and Vespa, 2014). Continuous EEG monitoring also allowed for identification and treatment of subclinical seizures in pediatric acute TBI patients (O'Neill et al., 2015). Other observational studies comparing the performance of quantitative handheld EEG to head CT provide growing evidence that it can be used to predict intercranial lesions in acute mild TBI (Ayaz et al., 2015). We believe that our data also provides a basis for such studies to now commence in humans. Our animal data show that driven activity is consistently altered in TBI, but differently long-term in various types of TBI. Hence, the use of evoked potentials in addition to the EEG will allow us to establish a successful biomarker for use in continuous, bedside monitoring of neuronal functionality in humans. Further, we have securely established that neuronal activity in upper cortical layers is a powerful indicator of cortical changes in TBI, and differentiates between different forms of TBI. The accessibility of these layers for non-invasive recording allows their activity to be monitored for the evolution of neuronal functionality changes with high-precision spatial and temporal definition.

We propose that monitoring of [resting] EEG activity as well as evoked potentials [e.g., visually evoked potentials (VEPs) and/or somatosensory evoked potentials (SEPs)] in human patients with moderate-to-severe TBI, at time periods from 24 h after admission to ICU post-TBI, through to 6 months post-TBI, would be invaluable in defining the evolution of neuronal functionality changes in TBI in each patient. As noted above, this has the potential to ensure therapy is appropriate for brain processes occurring at that time in each patient. Event related

potentials and EEG spectral power have been used to identify deficits in response inhibition (Roche et al., 2004) with TBI causing changes in N2 and P3 waveform components in response to visual stimuli and changes in EEG alpha power (Roche et al., 2004). A recent exciting development in the use of EEG for bedside monitoring of TBI is the finding (Li et al., 2014) that novel EEG analysis technique of symmetrical channel EEG analysis (SESA) combined with sophisticated signal processing and statistical analyses of the approximate entropy (a measure of the regularity and unpredictability of signal fluctuations over time-series data such as the EEG) and the Slow Wave Coefficient (the ratio of the summed power in the delta and theta bands to the summed power in the alpha and beta bands, i.e., the ratio of the power in the low frequency bands to the power in the high frequency bands) well indexes unilateral TBI, the most common form of TBI. Other newer techniques are currently being developed and offer potential to distinguish severe TBI from mild and also TBI from other brain disorders (Tsirka et al., 2011; Prichep et al., 2012; McBride et al., 2013; Teel et al., 2014). We believe that the time is appropriate for clinical trials of the use of non-invasive extra-cranial electrophysiology to monitor and define the evolution of post-TBI changes in cortical neuronal functionality.

AUTHOR CONTRIBUTIONS

SFC—generated some part of the overall structure especially with respect to inhibition, wrote most of the manuscript and generated most of the figures. DSA—generated some part of the overall structure especially with respect to barrel cortex structure, wrote that section of the manuscript, assisted with editing, and generated **Figure 1**. RR—generated the overall structure of the manuscript, wrote and edited some part of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnsys.2016.000 47/abstract

SUPPLEMENTARY TABLE 1 | Table summarizes sample sizes for each cortical layer for the two different models of TBI (Closed and Open skull) at the two different time points (24 h and 8–10 weeks) examined for two complex whisker deflection stimulus (Ritt Rough and the Hartmann).

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Knockout of Cyclophilin-D Provides Partial Amelioration of Intrinsic and Synaptic Properties Altered by Mild Traumatic Brain Injury

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Mitochondria are central to cell survival and Ca²⁺ homeostasis due to their intracellular buffering capabilities. Mitochondrial dysfunction resulting in mitochondrial permeability transition pore (mPTP) opening has been reported after mild traumatic brain injury (mTBI). Cyclosporine A provides protection against the mPTP opening through its interaction with cyclophilin-D (CypD). A recent study has found that the extent of axonal injury after mTBI was diminished in neocortex in cyclophilin-D knockout (CypDKO) mice. Here we tested whether this CypDKO could also provide protection from the increased intrinsic and synaptic neuronal excitability previously described after mTBI in a mild central fluid percussion injury mice model. CypDKO mice were crossed with mice expressing yellow fluorescent protein (YFP) in layer V pyramidal neurons in neocortex to create CypDKO/YFP-H mice. Whole cell patch clamp recordings from axotomized (AX) and intact (IN) YFP+ layer V pyramidal neurons were made 1 and 2 days after sham or mTBI in slices from CypDKO/YFP-H mice. Both excitatory post synaptic currents (EPSCs) recorded in voltage clamp and intrinsic cellular properties, including action potential (AP), afterhyperpolarization (AHP), and depolarizing after potential (DAP) characteristics recorded in current clamp were evaluated. There was no significant difference between sham and mTBI for either spontaneous or miniature EPSC frequency, suggesting that CypDKO ameliorates excitatory synaptic abnormalities. There was a partial amelioration of intrinsic properties altered by mTBI. Alleviated were the increased slope of the AP frequency vs. injected current plot, the increased AP, AHP and DAP amplitudes. Other properties that saw a reversal that became significant in the opposite direction include the current rheobase and AP overshoot. The AP threshold remained depolarized and the input resistance remained increased in mTBI compared to sham. Additional altered properties suggest that the CypDKO likely has a direct effect on membrane properties, rather than producing a selective reduction of the effects of mTBI. These results suggest that inhibiting CypD after TBI is an effective strategy to reduce synaptic hyperexcitation, making it a continued target for potential treatment of network

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INTRODUCTION

Even mild forms of traumatic brain injury (mTBI) can produce significant and long lasting disruption of cognitive function. This has been shown with the Immediate Post-Concussion Assessment and Cognitive Testing battery (ImPACT) given to mTBI patients in the emergency department (Shores et al., 2008; Peterson et al., 2009; Ponsford et al., 2011). While some improvement occurs over time, this tool shows that impairment may last weeks to months after injury (Belanger et al., 2005). More than 15% have a measurable cognitive deficit at 1 year (Kashluba et al., 2008; Lee et al., 2008). Recent studies suggest that this impairment may last for many years or decades in some patients (De Beaumont et al., 2009; Monti et al., 2013; Zhang et al., 2015).

Diffuse axonal injury (DAI) has been commonly been identified as a key neuropathological feature in both TBI patients and animal models of TBI (Oppenheimer, 1968; Povlishock and Katz, 2005; Bazarian et al., 2007; Mayer et al., 2010; Browne et al., 2011; Greer et al., 2011; Orr et al., 2016). In fact, it has been suggested to be predictive of the clinical outcome assessed with the Extended Glasgow Outcome Scale (Lee et al., 2012; Bigler, 2015). The acute pathophysiology begins with the disruption of neuronal and axonal cell membranes, which initiates a pathophysiological process of abnormal intracellular function (Werner and Engelhard, 2007; Spain et al., 2010; Kramer et al., 2016). Membrane defects cause a deregulated flux of ions, including calcium (Takahashi et al., 1981; Giza and Hovda, 2014). These ionic changes result in enhanced release of excitatory neurotransmitters, particularly glutamate (Katayama et al., 1990; Zhou et al., 2013; Giza and Hovda, 2014). To restore ionic balance, membrane pump activity increases, raising glucose consumption and depleting energy stores (Yoshino et al., 1991; Giza and Hovda, 2014; Shijo et al., 2015). This will cause calcium influx to mitochondria, impairing oxidative metabolism which can also cause acidosis and edema (Giza and Hovda, 2001; Barkhoudarian et al., 2011; Blennow et al.,

A number of studies have suggested that cellular metabolism reduction occurs after even mild TBI in patients (Bergsneider et al., 2000; Giza and Hovda, 2001; Pratico et al., 2002; Vagnozzi et al., 2010) as well as in animal models (Foda and Marmarou, 1994; Tavazzi et al., 2005). Experimental evidence has linked the severity of brain injury and recovery with the extent of ATP and N-acetylaspartate (NAA) decrease and recovery (Vagnozzi et al., 2005; Tavazzi et al., 2007). The posttraumatic change in cerebral metabolism relates largely to mitochondria dysfunction and calcium overload (Tavazzi et al., 2005; Vagnozzi et al., 2010).

Mitochondria can adapt and change energy production for mild cellular stress, but when extreme, the mitochondria undergo permeability transition, wherein a permeability pore (the mitochondrial permeability transition pore (mPTP), or mPTP) opens in the inner membrane, allowing in molecules less than 1500 kD (Halestrap, 2009; Brenner and Moulin, 2012). Once this occurs, ATP can become depleted and the cell can then undergo necrotic death (Halestrap, 2009). In fact, the mPTP is a key effector of cell death associated with

many neurological diseases, including TBI, ischemia, stroke and neurodegenerative disorders (Griffiths and Halestrap, 1993; Lifshitz et al., 2004; Baines et al., 2005; Schinzel et al., 2005; Osman et al., 2011; Rao et al., 2014). Cyclosporine A interacts with cyclophilin-D (CypD) to block the mPTP (Nicolli et al., 1996). Blocking the mPTP with cyclosporine A or its analog NIM811 given either pre- (Büki et al., 1999; Okonkwo and Povlishock, 1999) or post- TBI has been shown to be neuroprotective in animal models of TBI (Mbye et al., 2009; Kilbaugh et al., 2011; Readnower et al., 2011). CypD induces the opening of the mitochondrial pore by sensitizing it to calcium (Bernardi and Di Lisa, 2015). Thus manipulation of CypD represents a target for controlling this pathway to energy-depletion and cell damage. This role of CypD in affecting mitochondrial permeability transition has been confirmed in studies utilizing CypD knockout mice (Baines et al., 2005; Gainutdinov et al., 2015). Studies have also shown that the absence of CypD attenuates mitochondrial and neuronal perturbation, and ameliorates learning and memory in Alzheimer's disease (AD; Du et al., 2008). CypD inactivation also protects axonal damage in experimental autoimmune encephalomyelitis (Forte et al., 2007). A recent study noted that axonal injury was reduced after mTBI in cyclophilin-D knockout (CypDKO) mice (Hånell et al., 2015b). What has not yet been explored is whether CypDKO affects physiological neuronal function. Previously, we reported altered intrinsic and synaptic properties of layer V pyramidal neurons in somatosensory cortex 1 and 2 days after mTBI in yellow fluorescent protein (YFP)-H mice (Greer et al., 2012; Hånell et al., 2015a). This "H" mouse strain has YFP in a subset of layer V pyramidal neurons (Feng et al., 2000), allowing visualization of the full morphology, including the status of the axon in live (or fixed) tissue. By using CypD knockout mice crossed with YFP-H mice, here we evaluated whether CypD knockout ameliorates the intrinsic and synaptic abnormalities induced by mild fluid percussion injury 1 and 2 days after injury.

MATERIALS AND METHODS

Experimental Animals

We crossed two existing mouse lines to study the effect of inhibition of the mPTP opening in neural function after mTBI. The first mouse-line expresses YFP (homozygous) under control of the Thy1-promoter (YFP-H, Jackson Labs, Bar Harbor, ME, USA). The second line consisted of CypD knock-out mice. The gene encoding CypD is ppif and thus these mice are also known as ppif^{-/-}. CypD genotyping, crossing and mouseline maintaining were managed by Dr. Povlishock's laboratory (Hånell et al., 2015b). The two lines were crossed and followed by identification of YFP expressing ppif^{-/-} mice. The offspring of this cross that were positive for YFP expression were used for this study and are referred to here as CypDKO/YFP-H. The age of the mice used in the study was 6-8 weeks old. The mice were grouped in 12 h/12 h non-reversed light cycle on corn cob bedding with continuous free access to food and water. All animal procedures were approved by the institutional animal

care and use committee (IACUC) of Virginia Commonwealth University.

Central Fluid Percussion Injury

Mild central fluid percussion injury was induced as described previously (Greer et al., 2011). Animals were anesthetized with 4% isoflurane in 100% O2. Anesthesia was maintained with 2% isoflurane during the surgery. The body temperature was maintained at 37°C by a thermostatically controlled heating pad (Harvard Apparatus, Holliston, MA, USA). Pulse rate, respiratory rate, and blood oxygenation were monitored intraoperatively via a pulse oximetry sensor (STARR Life Sciences, Oakmont, PA, USA). A 3.0 mm circular craniotomy was made along the sagittal suture midway between Bregma and lambda with dura intact (IN). This location consistently produces DAI throughout primary somatosensory cortex (see Greer et al., 2011). A sterile Leur-Loc syringe hub made from a 20 gauge needle was affixed to the craniotomy site using cyanoacrylate and dental acrylic, then filled with saline to keep dura moisture. After the dental acrylic hardened, topical bacitracin and lidocaine ointment were applied to the incision site. This surgery required 45-75 min. The animal was then removed from anesthesia and monitored in a warmed cage until fully ambulatory (60-90 min of recovery). Injury or sham procedure was applied immediately after recovery.

For the injury induction, each animal was re-anesthetized with 4% isoflurane in 100% O2, and the hub was attached on to a fluid percussion apparatus (Custom Design and Fabrication; Virginia Commonwealth University; Richmond, VA, USA). A mild severity injury (1.7 \pm 0.06 atmospheres) was induced by a brief fluid pressure pulse upon the IN dura. The peak pressure was measured by the transducer (Tektronix 5111). After injury, the animals were visually monitored for recovery of spontaneous respiration. The duration of transient unconsciousness was determined by measuring the time of the following reflexes recovery: toe pinch, tail pinch, pinna, and righting. The injury was considered effectively mild when righting occurred in less than 6 min. For these experiments, the mean righting time was 1.2 ± 0.2 min for sham and 5.3 ± 0.2 min for injured mice. After recovery of the righting reflex, animals were placed in a warmed holding cage and monitored during recovery (typically \sim 60 min) before being returned to the vivarium. For sham injury, all of the above steps were followed with the exception of the release of the pendulum to induce the injury.

Acute Slice Preparation and Patch-Clamp Recording

Mice were anesthetized with isoflurane and decapitated for quick brain removal 1 and 2 days after surgery. The brain was immediately chilled in ice-cold oxygenated sucrose-modified artificial cerebral spinal fluid (aCSF) containing: (in mM) 2.5 KCl, 10 MgSO₄, 0.5 CaCl₂, 1.25 NaH₂PO₄, 234 sucrose, 11 glucose, and 26 NaHCO₃. Coronal slices, 300 μ m thick were cut with a Leica VT 1200 slicer (Leica Microsystems, Wetzlar, Germany). The slices were incubated for 30–45 min at 34°C in an oxygenated aCSF containing: (in mM) 126 NaCl,

3 KCl, 2 MgCl₂, 2 CaCl₂, 1.25 NaH₂PO₄, 10 glucose, and 26 NaHCO₃. Then the slices were remained at room temperature thereafter until placed in the recording chamber, which was maintained at 28 \pm 0.5°C. Whole-cell patch-clamp recordings were performed under infrared Dodt contrast microscopy (Zeiss AxioExaminer); a 60× water-immersion objective was used to visually identify YFP+ layer V pyramidal neurons of primary somatosensory cortex (beneath the injury site) with axon descending into the white matter (IN) or ending with an axonal swelling (axotomized (AX)) deep to the surface of the slice to avoid those AX by the vibratome. We have shown previously that these morphologies are easily identified in the living slice for YFP+ layer V pyramidal neurons (Greer et al., 2012). Only YFP+ neurons were chosen for recording. All layer V YFP+ neurons in the YFP-H line are expected to be pyramidal neurons and in all cases an apical dendrite was apparent, confirming that they were pyramidal neurons. The slices were continuously perfused with aCSF solution that was saturated with 95% O2 and 5% CO2. Patch electrodes (final resistances, 2–4 M Ω) were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL, USA) on a horizontal Flaming-Brown microelectrode puller (Model P-97, Sutter Instruments). The intracellular solution contained (in mM): 130 K-gluconate, 10 Hepes, 11 EGTA, 2.0 MgCl2, 2.0 CaCl2, 4 Na-ATP, and 0.2 Na-GTP. The liquid junction potential was 13.7 mV and was uncorrected. Electrode capacitance was electronically compensated. For spontaneous and miniature excitatory postsynaptic current (sEPSC and mEPSC) recording, neurons were voltage clamped at -70 mV using MultiClamp 700B (Molecular Devices, Sunnyvale, CA, USA) and digitized with a Digidata 1440A and pClamp software (Molecular Devices, at 20 kHz). Tetrodotoxin (TTX, 1 µM) was included in the aCSF for mEPSC recordings. Currents were filtered at 1 kHz. Action potentials (AP) were recorded in current-clamp mode while neurons were maintained at -60 mV, filtered at 10 kHz. To obtain intrinsic and cellular property measurements, a series of hyperpolarizing and depolarizing steps were applied in current-clamp, beginning with a −200 pA step (400 ms) and then increasing by 10 pA for a total of 70 sweeps (individual depolarizing or hyperpolarizing presentation) per cell (last sweep was 490 pA). APs were present on 16 or more of the 70 sweeps. Individual sweeps had between 1 and 30 APs. Access resistance was continuously monitored. If the series resistance increased by 20% at any time, the recording was terminated.

Data Analysis

For these experiments in CypDKO/YFP-H mice, 1–4 neurons were recorded per slice and 4–5 slices were used from each animal. A total of nine sham injured mice (71 neurons from \sim 40 slices) and 10 injured mice (88 neurons from \sim 45 slices) were used here. EPSC data analysis was performed by MiniAnalysis (Synaptosoft). For the intrinsic properties, here we show additional measures not previously reported for the YFP-H mice. In our previous publication (Greer et al., 2012), for measures of AP amplitude, threshold, and after hyperpolarization (AHP)

duration, we measured only two APs on only a single sweep in response to a mid-level depolarizing current. We have since developed an analysis program that allows us to measure all intrinsic properties shown in this report for every AP on every sweep. The AHP and ADP were also determined from all recorded APs on all sweeps. We use the term rheobase here as others have used it to indicate the lowest current level that elicits an AP. Measures shown here are the mean per cell which reflects the mean for all APs in the file. We therefore used this analysis procedure for both the original YFP-H (re-analyzed with this new program) and CypDKO/YFP-H mice reported here. To compare the current CypDKO/YFP-H data to that of YFP-H mice that we have previously reported, in Figures 2, 8 only, we normalized all data to the mean of their respective control group. For the YFP-H group, we previously showed that there was no significant difference between the results for naïve and sham animals (Greer et al., 2012). Thus the YFP-H controls contain a combination of sham and naïve data. For the CypDKO/YFP-H group, only shams were studied and thus the control groups is made up of only shams. For the EPSC data reported here, we compared the data from 1 and 2 days CypDKO/YFP-H shams and found that there was no significant difference (*t*-tests, p > 0.05, see Supplementary Figure 1), and thus these two control groups were combined into a single CypDKO/YFP-H sham control group. For all EPSC measures, significance was tested with a 1-way analysis of variance (ANOVA). Also for EPSC data, all cell types are included together, as we have also previously shown that cell type does not affect these measures (Hånell et al., 2015a). Since the data for intrinsic properties did vary between 1 and 2 days shams for CypDKO/YFP-H, these remain separate groups for both the direct comparison with controls (Figures 4-7) as, well as for the normalization (Figure 8). Data originally published in Greer et al. (2012) was re-analyzed and normalized for the YFP-H group shown in Figure 8 and Table 1. Data originally published in Hånell et al. (2015a) was normalized for the YFP-H group in Figure 2. For each cell type identified with the pattern of AP firing, a z-test was used to determine if there was a significantly different percentage present in any of the subject groups (Figure 3F). For all other measures, significance was tested using 2-way ANOVAs, with a Bonferroni post hoc test (SPSS software from IBM), with normality assumed. Results are reported as Mean \pm SEM. In the figures with data normalized to the mean of the control group (Figures 2, 8), significance shown is for comparison between the original experimental group and controls and not tested on the normalized data. It is shown in this way simply to identify which abnormalities induced by TBI were ameliorated in the CypDKO/YFP-H mice.

RESULTS

CypDKO Eliminated the Increase of Excitatory Synaptic Activity After mTBI

We previously observed an increase in the frequency of excitatory synaptic currents recorded from layer V pyramidal neurons after this mild central fluid percussion injury in YFP-H mice (Hånell

et al., 2015a). Here in the CypDKO/YFP-H, EPSCs appeared qualitatively similar to those in YFP-H mice (Figure 1A). In the YFP-H mice, our previous findings showed a significant increase in sEPSCs for both AX and IT neurons at both 1 and 2 days survival times. After the same injury in the CypDKO/YFP-H, this trauma-induced increase did not occur (Figure 1B). The sEPSC amplitude was also unaffected by the mTBI in CypDKO/YFP-H mice (Figure 1C). In YFP-H mice, we previously showed that the mEPSC frequency was increased at 1 day in AX and at 2 days in IN neurons (Hånell et al., 2015a). In the CypDKO/YFP-H, there was no significant difference in mEPSC frequency for any of the injured groups compared to sham (Figure 1D). The mEPSC amplitude was also unaffected by the injury (Figure 1E) in CypDKO/YFP-H mice. These results suggested that the CypDKO/YFP-H did in fact ameliorate the increased excitatory synaptic activity observed after injury in YFP-H mice. For comparison between YFP-H and CypDKO/YFP-H mice, the values for each cell in injured cortex were normalized to their respective control mean (Figure 2). In Figures 2A,C the CypDKO/YFP-H-induced amelioration of increased excitatory synaptic activity after mTBI can be seen.

CypDKO Ameliorated Some of the Intrinsic Properties Altered After mTBI

Cell Types

In order to compare intrinsic properties of pyramidal neurons in different conditions, the neurons must first be divided into different subgroups based on AP firing patterns during depolarizing current steps. This is necessary since measures such as AHP amplitude and duration, rheobase, and certainly frequency per depolarization level will be consistently different depending on the firing pattern subtype. We have previously identified three subtypes for pyramidal neurons in layer V of this YFP-H tissue that were also observed here (Figure 3): (1) intrinsically-bursting (IB); (2) regular-spiking with an initial doublet (RS_D); and (3) regular-spiking without doublet (RS). Here, firing pattern subtypes were quantitatively identified from depolarizing steps for sweeps containing 5-8 APs in the following manner. Neurons with an IB subtype had 3 or more APs per sweep with an interval of less than 10 ms (Figure 3A) or had two or more "doublets" per sweep (Figure 3B) with a doublet defined as two APs of less than 15 ms interval. The RS_D neurons had a single doublet per sweep (Figure 3C), while RS neurons had no APs with an interval of less than 15 ms (Figure 3D). This strategy clearly separated the three firing types (**Figure 3E**). All three types were observed in sham and mTBI conditions. In addition, in the CypDKO/YFP-H, the percentage of each type was similar between sham and AX as well as IN TBI groups at both 1 and 2 days survival times (Figure 3F). The previously observed loss of IB neurons for the IN 2-days after injury (IN-2D) group in YFP-H mice (Greer et al., 2012) was not present here in the CypDKO/YFP-H.

AP Firing Frequency Adaption

AP firing frequency adaptation is an important characteristic for each neuron's contribution to network function, particularly in

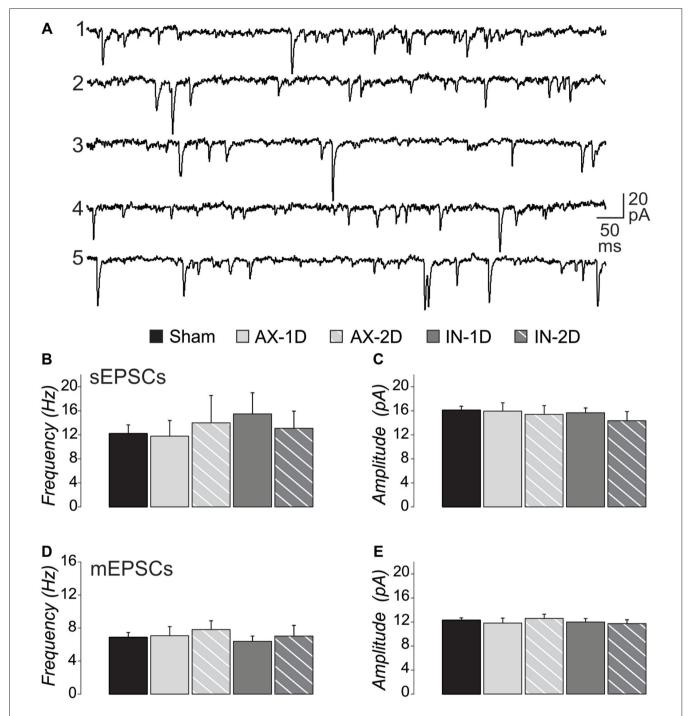


FIGURE 1 | When applied in cyclophilin-D knockout (CypDKO)/yellow fluorescent protein (YFP)-H mice, mild traumatic brain injury (mTBI) does not increase excitatory post synaptic current (EPSC) frequency. (A) Examples of spontaneous excitatory post synaptic currents (sEPSCs) recorded from the following groups of CypDKO/YFP-H mice: (1) Sham; (2) Axotomized (AX)-ID; (3) AX-2D; (4) IN-ID; and (5) IN-2D. For this EPSC data, there was no significant difference between the ID and 2D shams and thus these were combined to form a single control sham group. The sEPSC frequency (B) and amplitude (C) were not significantly different between sham and injured groups (analysis of variance (ANOVA), p > 0.05, N = 33 sham, 9 AX-ID, 9 AX-2D, 12 IN-1D, and 8 IN-2D neurons). The miniature excitatory post synaptic current (mEPSC) frequency (D) and amplitude (E) were not significantly different between sham and injured groups (1-way ANOVA, p > 0.05, N = 32 sham, 14 AX-ID, 9 AX-2D, 12 IN-1D, and 8 IN-2D neurons).

instances of heightened activity levels. The RS and RS_D neuron firing types could also be separated based on the change in total adaptation with level of injected current. Total adaptation

was calculated as the frequency of first two APs divided by the frequency of the last two APs in the sweep. Since RS_D neurons fire a doublet even with low levels of depolarization,

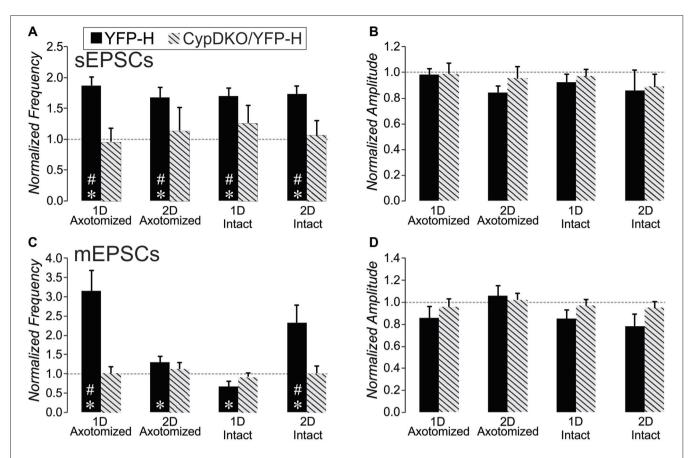


FIGURE 2 | Comparison of the effect of mTBI in YFP-H to that in CypDKO/YFP-H mice. For each neuron from injured cortex, the values were normalized to the mean of their respective controls. For the YFP-H group, the non-normalized data was reported in Hånell et al. (2015a). For the CypDKO/YFP-H group the non-normalized data is shown in Figure 1. (A) The sEPSC frequency increases observed in YFP-H are ameliorated in the CypDKO/YFP-H. (B) The sEPSC amplitude was unaffected in either YFP-H or CypDKO/YFP-H. (C) The mEPSC increased frequency observed at 1 day in AX and 2 days in IN was also ameliorated in the CypDKO/YFP-H. (D) The mEPSC amplitude was unaffected by injury in either the YFP-H or CypDKO/YFP-H. *Significant effect of subject group (2-way ANOVA, p < 0.05); #significantly different from control with *post hoc* analysis. Number of neurons for the CypDKO/YFP-H groups are given in the previous figure. For the YFP-H groups, for sEPSCs, N = 48 control, 27 AX-ID, 15 AX-2D, 16 IN-1D, and 15 IN-2D neurons, and for spontaneous mEPSCs, N = 15 control, 5 AX-ID, 10 AX-2D, 5 IN- 1D, and 4 IN-2D neurons.

their adaptation at that level is high. The firing rate occurring after the doublet increases with greater levels of injected current (see Figure 4C) while the doublet frequency is maintained. This means that the total adaptation will be increasingly lower with greater levels of depolarizing current injection (see RSD cell, crosses plotted against right axis in Figure 4A). In RS neurons in contrast, while the overall frequency increases with greater levels of depolarization, the frequency increases most for the first two APs (Figure 4B). This means that total adaptation will increase with increasing levels of depolarization (see RS, filled squares plotted against left axis in Figure 4A). Calculation of the correlation between total adaptation and depolarizing current level typically produced a positive value for RS but a negative value for RS_D neurons (Figures 4D,E). With the exception of a single RS and a single RS_D neuron, this was true for all layer V pyramidal neurons from all subject groups. This further confirms our quantitative method of separating pyramidal neuron firing types. For measures such as total adaptation that were affected by the beginning doublet, population means were then compared between subject groups only for the RS neurons (since there were a greater number of these than RS_D). The mean total adaptation for RS neurons was not significantly different between injured and sham conditions (**Figure 4J**, 2-way ANOVA, no significant effects, p > 0.05).

Late adaptation was calculated as the frequency of the 4th and 5th AP divided by the frequency of the last two APs in sweeps with 8 or more APs. There was no consistent correlation between late adaptation and level of injected depolarizing current for any subject group whether examined in RS or RS_D neurons (**Figures 4F,G**). When the population means were examined for late adaptation, there was no significant difference between injured and controls in YFP-H mice (see **Figure 8C**). However, in the CypDKO/YFP-H, a 2-way ANOVA showed a significant effect of subject group and Bonferroni *post hoc* analysis showed that there was a greater adaptation for IN mTBI neurons compared to sham (**Figure 4K**, p < 0.05).

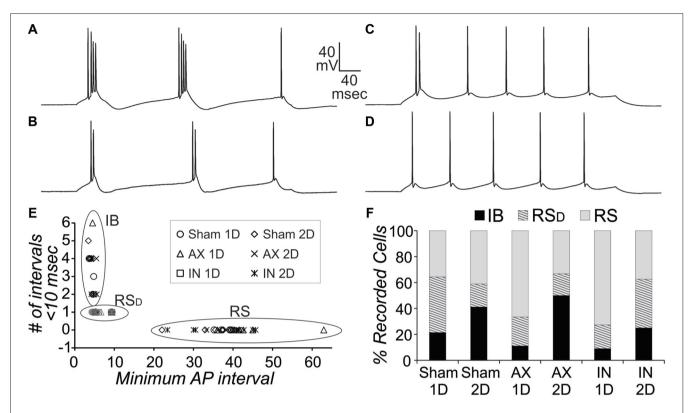


FIGURE 3 | Action potential (AP) firing pattern subtypes of YFP+ pyramidal neurons from CypDKO/YFP-H mice. (A,B) Examples of intrinsically-bursting (IB) neurons, with high frequency bursts of APs. (C) Example of an regular spiking (RS_D) neuron with doublet only at start of sweep. (D) Example of an RS neuron. (E) These three AP firing types were distinguished using the minimum AP interval and the number of AP intervals less than 10 ms. RS neurons had no AP intervals less than 15 ms. RS_D neurons had only one interval less than 10 ms, while IB neurons had at least two intervals of 10 ms or less. (F) The percent of these three types for each subject group. Percentages were not significantly different among groups (z-tests, ρ > 0.05). N = 14, 17, 9, 6, 11, and 8 neurons for sham 1D, sham 2D, AX 1D, AX 2D, IN 1D, and IN 2D, respectively.

Slope of the Frequency vs. Injected Depolarizing Current

We previously described a significant increase in the slope of the frequency (calculated across the full 400 ms depolarization) vs. injected depolarizing current (F-I Slope) for IN-2D compared to controls in YFP-H mice (Greer et al., 2012). Here, in the CypDKO/YFP-H cortex, this effect was not observed (**Figure 4I**). Both RS and RS_D cells were included in this measure. In fact a 2-way ANOVA showed an effect of day only, with no difference between subject groups. Examples of the plot of frequency vs. injected current for individual cells from each subject group are shown in **Figure 4H**.

Basic Membrane Properties

Basic membrane properties of input resistance, rheobase and time to first AP at rheobase were compared between sham and injured animals for the combination of RS and RS_D neurons. Input resistance was significantly greater in YFP-H mice and was not corrected in the CypDKO/YFP-H cortex (see **Figure 8D**). In the CypDKO/YFP-H, a 2-way ANOVA with Bonferroni *post hoc* showed a significantly greater input resistance in AX neurons compared to sham (**Figure 5A**). The IN neurons were not significantly different from sham. Time to first AP at rheobase was variable

between neurons within a group and not significantly different between subject groups in CypDKO/YFP-H (**Figure 5B**). In YFP-H mice rheobase was significantly increased specifically in the AX 1-day after injury (AX-1D) group (see **Figure 8F**). In the CypDKO/YFP-H cortex this effect was reversed, such that rheobase was significantly decreased compared to sham for both AX-1D and IN 1-day after injury (IN-1D) (**Figure 5C**).

Action Potential Properties

We previously found significant differences in AP properties in YFP-H mice after injury, which included an increased AP amplitude and overshoot (Greer et al., 2012). While we found no difference in AP threshold originally, when measured for only a few APs per cell, here we re-evaluated the YFP-H mice data. When the AP threshold was measured for all APs in the file and then averaged per subject group, a 2-way ANOVA showed a significant effect of subject group and Bonferroni post hoc showed that AP threshold for both AX 2-day after injury (AX-2D) and IN-2D was significantly more depolarized (less than 1.0 when normalized to YFP-H controls, **Figure 8G**). In CypDKO/YFP-H mice this effect was ameliorated only for the IN-2D group (**Figure 6A**). A 2-way ANOVA showed a significant effect of day (p < 0.005) and a significant interaction between

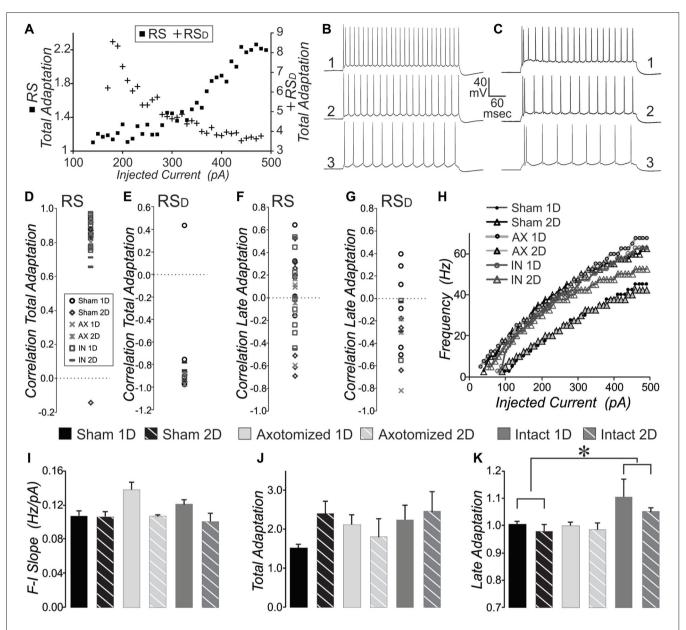


FIGURE 4 | Measures of intrinsic properties of adaptation and F-I slope in layer V pyramidal neurons from CypDKO/YFP-H mice. (A) Plot of total adaptation vs. injected current for one example RS (squares, left axis) and one example RS_D (crosses, right axis) neuron, where total adaptation = frequency of first two APs in the sweep divided by the frequency of the last two APs in the sweep. (B,C) AP firing pattern at 450 (1), 300 (2) and 200 (3) pA for the RS (B) and RS_D (C) neurons plotted in (A). (D) The correlation between total adaptation and injected current plotted for each RS neuron for all subject groups. All except one sham neuron show significantly positive correlations. (E) The correlation between total adaptation and injected current for all RS_D neurons from all groups. All except one sham neuron have a significantly negative correlation. (F) Correlation between late adaptation and injected current for all RS neurons. (G) Correlation between late adaptation and injected current for all RS_D neurons. (H) Example plots for typical individual neurons of sweep frequency (across the 400 ms depolarizing step) vs. injected current. (I) Mean F-I Slope for RS neurons. A 2-way ANOVA showed an effect of survival day, but no effect of subject group (significance at p < 0.05). (J) Total adaptation for RS neurons. A 2-way ANOVA showed no significant effects. (K) Late adaptation for combined RS and RS_D neurons. A 2-way ANOVA with Bonferroni post hoc showed a significant greater late adaptation for intact (IN) compared to sham neurons (p < 0.05). N for (D,F) = 5 Sham 1D, 7 Sham 2D, 6 AX ID, 3 AX 2D, 8 IN 1D, and 4 IN 2D neurons. N for (E,G) = 6 Sham 1D, 3 Sham 2D, 2 AX 1D, 2 AX 2D, 2 IN 1D, and 2 IN 2D neurons. N for (I-K) = 11 Sham 1D, 10 Sham 2D, 8 AX 1D, 5 AX 2D, 10 IN 1D, and 6 IN 2D neurons. *Significantly different, p < 0.05.

subject group and day (p < 0.005). Bonferroni *post hoc* analysis showed that AP threshold was significantly more depolarized for the AX-2D group compared to both the sham-2D and IN-2D. in the CypDKO/YFP-H mice. We previously found an increase

in AP amplitude and overshoot in YFP-H injured mice. We have also since found that these effects are likely to due to an increase in the amplitude of the sodium conductance in these cells. These effects were ameliorated in the CypDKO/YFP-H

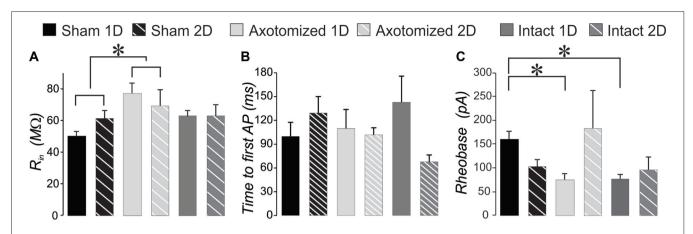


FIGURE 5 | Intrinsic properties that reflect basic neuronal membrane properties for injured compared to sham controls in CypDKO/YFP-H mice. (A) A 2-way ANOVA showed a significant effect of subject group for input resistance (p < 0.02). Bonferroni *post hoc* analysis showed that the AX group had a significantly greater input resistance compared to shams. (B) Time to the peak of the first AP measured from beginning of depolarizing current for the rheobase sweep. A 2-way ANOVA showed no significant differences (p > 0.05). (C) Rheobase is the lowest current level to generate an AP. A 2-way ANOVA showed no effect of subject group or survival time, but there was a significant interaction of these two. *Post hoc* analyses including Bonferroni method showed that both AX and IN neurons had a significantly lower rheobase at 1 day post injury. N = 11 Sham ID, 10 Sham 2D, 8 AX ID, 5 AX 2D, 10 IN ID, and 6 IN 2D neurons. *Significantly different, p < 0.05.

mice. There was no significant difference between subject groups for AP amplitude and the increased overshoot was significantly reduced in AX-1D and not significantly different from sham in the other subject groups (Figures 6B,C, 8H,I).

AHP and Depolarizing After Potential (DAP)

In YFP-H mice both the AHP and DAP are affected by the injury (Figures 8J,N). Note that AHP was calculated by subtracting the minimum value following the AP from the baseline period prior to the depolarizing step. Thus larger values represent ones that were more depolarized and likely reflect a smaller K+ current. For the same reason, normalized values greater than 1.0 reflect AHPs that were more depolarized than controls, again likely due

to smaller K+ currents. In the CypDKO/YFP-H, 2-way ANOVAs showed effect of survival day, but no effect of subject group for either AHP amplitude or AHP time to peak (**Figures 7A,B**, p > 0.05). Thus the general effect of more depolarized AHPs found in YFP-H mice was ameliorated in the CypDKO/YFP-H. For the AHP rise time there were no significant effects in the CypDKO/YFP-H (**Figure 7C**). For DAPs, there were also no significant effects in the CypDKO/YFP-H for percent of APs with a DAP nor for the time to peak of the DAP measured relative to the preceding AP (**Figures 7D,F**). For the DAP amplitude measured as a percentage of the AP amplitude, there was a significant effect of survival day only in the CypDKO/YFP-H (**Figure 7E**, 2-way ANOVA, p < 0.05).

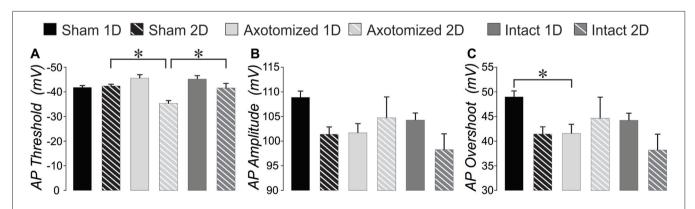


FIGURE 6 | AP measures: injured compared to shams in CypDKO/YFP-H mice. (A) AP threshold averaged across all APs evoked per neuron. A 2-way ANOVA showed a significant effect of survival day (p < 0.005) and the interaction of subject group and survival day (p < 0.005). Bonferroni *post hoc* analyses showed that the AX-2D group had a significantly more depolarized AP threshold compared to sham-1D and IN-1D. **(B)** For the measure of AP amplitude, a 2-way ANOVA showed a significant effect for day only (p < 0.05). **(C)** For AP overshoot, a 2-way ANOVA showed a significant effect of day (p < 0.05) and a significant interaction between subject group and day (p < 0.05). Bonferroni *post hoc* analyses showed that AX-ID had a significantly smaller overshoot compared to sham-ID (p < 0.05). N = 11 Sham ID, 10 Sham 2D, 8 AX 1D, 5 AX 2D, 10 IN ID, and 6 IN 2D neurons. *Significantly different, p < 0.05.

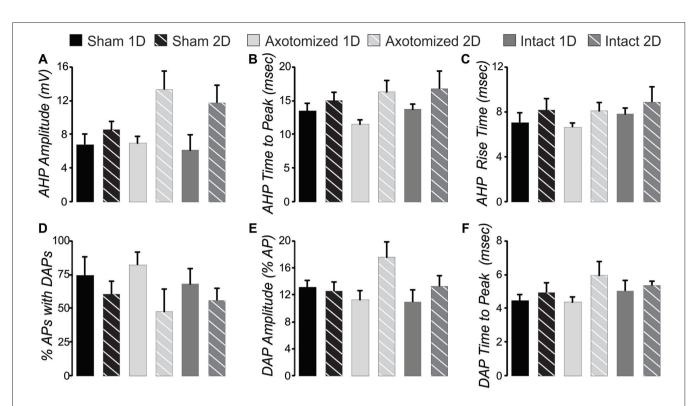


FIGURE 7 | **After hyperpolarization (AHP) and depolarizing after potential (DAP) measures in the CypDKO/YFP-H. (A)** AHP amplitude calculated as the minimum voltage following the AP minus the baseline voltage prior to the depolarizing step. Thus, larger values represent more depolarized AHPs and likely lower levels of K+ current. A 2-way ANOVA showed an effect of survival day but no effect of subject group. (**B)** AHP time to peak measured relative to the AP peak. A 2-way ANOVA showed an effect of survival day but no effect of subject group (significance < 0.05). (**C)** There were no significant effects for AHP rise time (2-way ANOVA, p > 0.05). (**D)** The percent of APs with DAPs was calculated within individual neurons for all APs produced from that neuron. There were no significant effects for this measure (2-way ANOVA, p > 0.05). (**E)** DAP amplitude was calculated as a percentage of the AP amplitude. A 2-way ANOVA showed an effect of survival day but no effect of subject group (p < 0.05). (**F)** DAP time to peak was measured relative to the time of the preceding AP. A 2-way ANOVA showed an effect of survival day but no effect of subject group (p < 0.05). (**F)** DAP time to peak was measured relative to the time of the preceding AP. A 2-way ANOVA showed an effect of survival day but no effect of subject group (p < 0.05). (**F)** DAP time to peak was measured relative to the time of the preceding AP. A 2-way ANOVA showed an effect of survival day but no effect of subject group (p < 0.05). (**F)** DAP time to peak was measured relative to the time of the preceding AP. A 2-way ANOVA showed an effect of survival day but no effect of subject group (p < 0.05). (**F)** DAP time to peak was measured.

For a full comparison of the injury-induced effects in YFP-H vs. CypDKO/YFP-H mice, all intrinsic property data were normalized to their controls and YFP-H measures were plotted adjacent to those for the CypDKO/YFP-H (Figure 8). Indicators of significance in this figure are as measured against the raw data (shown in Table 1 for the YFP-H mice, and in Figures 4-7 for the CypDKO/YFP-H mice. This shows in the CypDKO/YFP-H complete or partial amelioration of injuryinduced effects on F-I slope, input resistance, rheobase, AP threshold, amplitude and overshoot, AHP amplitude and DAP amplitude occurred. The more depolarized AP threshold for the AX-2D group failed to be ameliorated by the CypDKO/YFP-H. In addition, there was some alteration of intrinsic properties in the CypDKO/YFP-H that were not originally observed after injury in YFP-H mice. These included an increase in late adaptation, a decrease in rheobase for the IN-1D group, and a decrease in the AP overshoot for the AX-1D group.

DISCUSSION

Here we investigated whether CypDKO would ameliorate the intrinsic and synaptic functional alterations produced by mTBI at 1-2 day survival times. Our results suggest a partial amelioration. The increase in excitatory synaptic connections (Hånell et al., 2015a) was ameliorated, as the sEPSC and mEPSC frequencies for mTBI were similar to those for sham in CypDKO/YFP-H mice. The intrinsic properties that were ameliorated were the increased F-I slope, the increased input resistance, and the increased AP, AHP, and DAP amplitudes. Some intrinsic properties were reversed, but became significant in the opposite direction. This was true for the Rheobase and the AP overshoot. In the YFP-H mice with CypD IN, the rheobase was significantly increased only for the AX-1D group compared to controls. The increased rheobase reflects a decreased excitability, as more current is required to produce an AP. In the CypDKO/YFP-H mice, this was reversed to the point that the rheobase was significantly decreased in AX-1D compared to controls for CypDKO/YFP-H mice. There was also a significant reduction in the rheobase for the IN-1D group compared to controls for the CypDKO/YFP-H mice. These suggest an increased membrane excitability in the CypDKO/YFP-H mice selectively after mTBI. Not returned to normal levels were the AP threshold and input resistance. In addition, for the measure of late adaptation, there were significant differences with mTBI

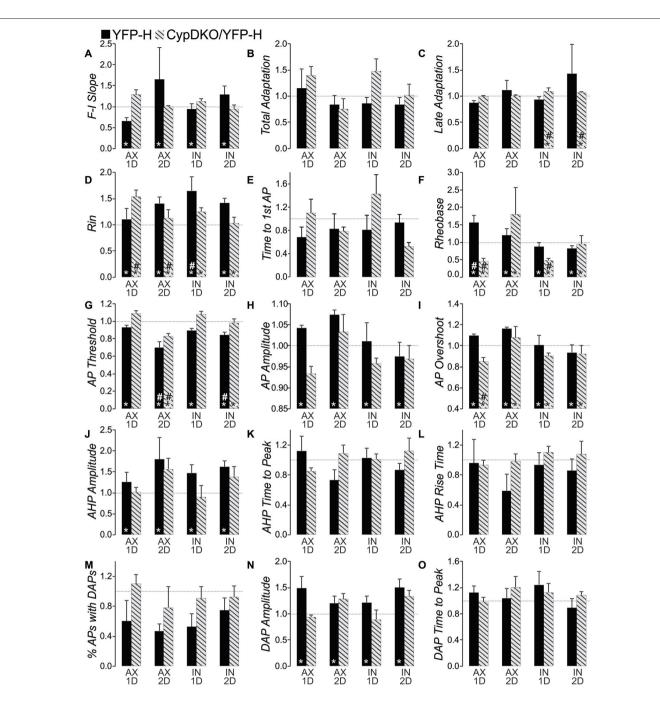


FIGURE 8 | **Comparison of effects in YFP-H to those in the CypDKO/YFP-H. (A–O)** Measure indicated by vertical axis label. All values were normalized to the mean of their respective controls. Non-normalized data for the YFP-H group is reported in **Table 1**. Non-normalized data for the CypDKO/YFP-H group is shown in **Figures 4–7**. YFP-H shown in black bars, and CypDKO/YFP-H shown in gray bars with black stripes, with survival time and axonal status indicated on the x-axis. In all cases 2-way ANOVAs were used to compare the raw (non-normalized data) subject groups of AX and IN neurons at 1 and 2 days survival times to their respective controls. *Significant effect of subject group, #significant difference of that subject group from control on Bonferroni *post hoc*. In all cases level of significance was considered p < 0.05. Numbers of neurons for each CypDKO/YFP-H group shown in previous figures. For YFP-H mice, N = 30 control, 23 AX ID, 7 AX 2D, 13 IN ID, and 8 IN 2D.

only for the CypDKO/YFP-H and not in YFP-H mice. These results suggest that the CypDKO/YFP-H does not produce a simple and complete amelioration of hyperexcitability induced by mTBI.

Synaptic Effects

DAI is a hallmark of mTBI and is clearly observed within hours of a medial fluid percussion injury in mice (Greer et al., 2011). Sprouting of the axonal trunk of AX pyramidal neurons can

TABLE 1 | Intrinsic property measurements for YFP-H group.

Measure	Control	AX 1D	AX 2D	IN 1D	IN 2D	Group p value
F-I Slope (Hz/pA)	0.14 ± 0.005	0.10 ± 0.01	0.24 ± 0.11	0.14 ± 0.02	0.19 ± 0.03	0.03
Total adaptation	2.5 ± 0.3	2.8 ± 0.9	2.0 ± 0.4	2.1 ± 0.3	2.1 ± 0.3	0.80
Late adaptation	1.2 ± 0.1	1.0 ± 0.05	1.3 ± 0.2	1.1 ± 0.1	1.7 ± 0.7	0.49
Rin (M Ω)	83.2 ± 6.8	92.1 ± 17.7	117.0 ± 10.7	$137.3 \pm 21.9^*$	118.0 ± 7.8	0.02
Time to 1st AP (ms)	113.7 ± 11.0	77.8 ± 19.6	93.9 ± 29.3	91.9 ± 28.7	106.2 ± 16.0	0.76
Rheobase (pA)	114.6 ± 11.0	$180.0 \pm 24.0^{\circ}$	136.7 ± 22.3	100.0 ± 17.1	93.3 ± 9.9	0.01
AP threshold (mV)	-38.9 ± 0.9	-36.0 ± 1.1	-27.2 ± 2.6 *	-34.5 ± 1.0	$-32.7 \pm 1.5^*$	0.00
AP amplitude (mV)	107.5 ± 0.8	112.1 ± 0.7	115.4 ± 1.3	108.6 ± 4.7	104.7 ± 3.7	0.02
AP overshoot (mV)	46.8 ± 0.8	51.2 ± 0.8	54.3 ± 0.9	47.1 ± 4.3	43.6 ± 3.6	0.01
AHP amplitude (mV)	13.1 ± 1.1	14.9 ± 1.5	22.5 ± 3.2	18.4 ± 1.9	20.1 ± 1.8	0.03
AHP time to peak (ms)	13.3 ± 0.7	12.8 ± 1.4	13.9 ± 2.5	13.7 ± 1.7	10.3 ± 1.1	0.38
AHP rise time (ms)	6.9 ± 0.4	6.0 ± 1.1	6.7 ± 1.6	6.6 ± 1.2	5.2 ± 1.0	0.63
% APs with DAPs	61 ± 5	40 ± 10	39 ± 9	37 ± 9	48 ± 11	0.07
DAP amplitude (mV)	16.5 ± 0.9	20.6 ± 2.0	23.7 ± 2.4	19.0 ± 0.9	22.3 ± 2.5	0.03
DAP time to peak (ms)	4.9 ± 0.4	5.5 ± 0.5	5.1 ± 0.7	6.1 ± 1.0	4.4 ± 0.7	0.50

Note that for this dataset we have shown that sham-injured did not differ from naïve and thus these two groups were combined for the single control group here. Shown are mean \pm SEM. N = 30 control, 23 AX 1D, 7 AX 2D, 13 IN 1D, and 8 IN 2D. 1-way ANOVA used to assess group significance and p value shown at right. *Significantly different from control, based on Bonferroni post hoc test, p < 0.05.

be observed 1 day after the injury (Greer et al., 2011), and we have shown that this sprouting produces an increase in functional excitatory synapses in YFP-H mice (Hånell et al., 2015a). In slices from these mice, mEPSC frequency is increased after mTBI, and repeated stimulation suggests this is not due to increased probability of release (Hånell et al., 2015a). The current data suggest that in the absence of CypD, the mTBI no longer induces the increase in functional excitatory synapses (Figures 1, 2).

An effect of CypD knockout on the general function of synapses is not surprising, since CypD is concentrated at synapses and mPTP opening is more likely to occur there (Naga et al., 2007). Some work has suggested that CypD may act to sensitize the mitochondria to calcium (Gainutdinov et al., 2015). Synaptic mitochondria may also be more susceptible to calcium overload than nonsynaptic mitochrondria (Brown et al., 2006). Importantly, while mPTP opening can lead to cell death, CypD likely also acts during normal cellular processes (Barsukova et al., 2011), particularly in energy homeostasis (Elrod et al., 2010; Elrod and Molkentin, 2013; Menazza et al., 2013). Studies utilizing CypD knockout mice or reduction of CypD effectiveness with Cyclosporin A have shown altered cellular or whole animal behavioral function, although it is currently unknown whether these actions occur via CypD's role in mPTP opening or by some other mechanism. Reducing the effectiveness of CypD with Cyclosporin A increases the resting calcium concentration, increasing normal synaptic transmission and reducing hippocampal long term plasticity levels (Levy et al., 2003). Mitochondrial uptake of calcium has also been shown to be involved in the phenomenon of post-tetanic potentiation that is due to increased transmitter release probability dependent on residual calcium in the presynaptic terminal (Dittman et al., 2000). CypD knockout also produces increased anxiety and an enhanced response in avoidance tests (Luvisetto et al., 2008). Mouri et al. (2010) have also shown that the CypDKO mice have a reduction in short term memory, using a battery of behavioral tests. After TBI, sprouting may also be considered a "normal" response to axotomy and certainly not an indicator of mPTP-induced cell death. In fact, no cell death occurs in this model of mild TBI (Singleton et al., 2002; Greer et al., 2011). The sprouting axon likely requires changes in cellular energy, such as an altered membrane potential of the mitochondria located in the growth cone (Verburg and Hollenbeck, 2008). Loss of CypD may reduce the ability of the axons to sprout.

Since CypD likely does participate in normal cellular functions, any plan for treatment must balance the potential risk of altering normal activity with preventing abnormalities after injury. Interestingly, the EPSC frequency and amplitudes were at sham levels and not below these controls, thus selectively correcting the mTBI-induced abnormality. This also occurred in the model of Alzheimer's disease that involves overexpression of human amyloid precursor protein (Du et al., 2008). In those studies, CypDKO corrected the lack of learning in a radial water maze test. Treatment of a moderate lateral fluid percussion injury with Cyclosporine A, that effectively inhibits CypD, also improved behavioral deficits in a motor task as well as spatial learning and memory in the Morris water maze (Alessandri et al., 2002). Thus these selective corrections back to normal levels should make CypD and the mPTP a continued area of focus for potential treatments.

Intrinsic Property Effects

In the YFP-H mice, the AX-1D mTBI group showed an increase in rheobase, suggesting a less excitable membrane. In addition, the AP threshold was more depolarized for both AX and IN groups at 2 days post-injury. The AHP amplitude was also larger for mTBI compared to controls. In contrast to this, other measures suggest increases in excitability for mTBI groups compared to controls, including the increase in input resistance, increase in AP amplitude and AP overshoot, increase in DAP amplitude and increase in F-I Slope at 2 days. The increased input

resistance allows less decay of synaptic currents if the membrane capacitance is unchanged, potentially rendering the neuron more excitable (Mozzachiodi et al., 2008). Increases in DAP amplitude can enhance somatic excitability by shortening the interspike interval (ISI) and converting the somatic spike pattern into bursts terminated by high-frequency doublets (Fernandez et al., 2005). The changes in AP and AHP amplitude are likely due to altered sodium and potassium channel densities and/or current amplitudes. Other models of TBI have been shown to alter current amplitude and expression of sodium and potassium channels (D'Ambrosio et al., 1999; Hains et al., 2005; Lampert et al., 2006; Mao et al., 2010; Lei et al., 2012; Huang et al., 2013; Takahashi et al., 2013). The CypDKO reversed much of the increased membrane excitability induced by mTBI, including the AP and AHP amplitudes. Intracellular calcium levels have been shown to regulate the mRNA for certain sodium channel subunits (Vega et al., 2003) and the cell surface expression of sodium channels (Monjaraz et al., 2000). A very early response to TBI is an excessive release of glutamate (Globus et al., 1995; Koura et al., 1998), likely causing hyper-activity of NMDA receptors (Sun and Faden, 1994; Andriessen et al., 2010) and large increases in intracellular calcium (Weber, 2012) that may overwhelm the mitochondrial calcium sequestration when cyclophilin D is present. Thus the increased calcium may cause a subsequent increase in sodium channel expression. Increased sodium channel and compensatory increases in potassium channels may then directly contribute to the increased AP and AHP amplitudes. The fact that the CypDKO eliminated both the AP and AHP increased amplitude is support for the idea that the AHP increase may reflect a compensatory process. Controlling these likely effects on sodium channel expression are important, as it has been shown that blockade of sodium channels can reduce neuronal loss in models of more severe TBI (Sun and Faden, 1995).

Although the CypDKO generally caused decreases in excitability, the one exception to this was the significantly decreased rheobase at 1D for both AX and IN CypDKO/YFP-H mTBI groups compared to CypDKO/YFP-H controls (Figure 8F). Surprisingly, in these groups the AP threshold was normal relative to the CypDKO/YFP-H controls. This is a

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further example of the complexity of effects produced with the CypDKO. A full blockade of CypD is probably not advisable, given the effects on learning and memory (cited above) as well as the novel significant changes in late adaptation and rheobase induced by CypDKO. Nevertheless, the effectiveness of modulating the mPTP and CypD in reducing axonal injury and cell death (Matsumoto et al., 1999; Sullivan et al., 2000; Hånell et al., 2015b) as well as selectively correcting motor and learning and memory deficits suggest this type of treatment deserves continued examination. This is particularly true since, studies of TBI patients have shown that inhibiting CypD with Cyclosporine A is clinically safe and effectively increases glucose and other metabolites in extracellular fluids (Hatton et al., 2008; Mazzeo et al., 2009). In conclusion, our study suggests that in addition to the previously demonstrated structure preservation in this model (Hånell et al., 2015b), that CypDKO also provides functional protection and a substantial return to normal of many neuronal properties altered by mTBI, making continued exploration of TBI treatment via inhibition of mPTP pore opening worthwhile.

AUTHOR CONTRIBUTIONS

Both JS and KMJ contributed to the design of the experiments. All data from CypDKO animals was collected by JS, while control data was collected by KMJ. Both JS and KMJ contributed to data analysis and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnsys. 2016.00063

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Altered Mitochondrial Dynamics and TBI Pathophysiology

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Mitochondrial function is intimately linked to cellular survival, growth, and death. Mitochondria not only generate ATP from oxidative phosphorylation, but also mediate intracellular calcium buffering, generation of reactive oxygen species (ROS), and apoptosis. Electron leakage from the electron transport chain, especially from damaged or depolarized mitochondria, can generate excess free radicals that damage cellular proteins, DNA, and lipids. Furthermore, mitochondrial damage releases pro-apoptotic factors to initiate cell death. Previous studies have reported that traumatic brain iniury (TBI) reduces mitochondrial respiration, enhances production of ROS, and triggers apoptotic cell death, suggesting a prominent role of mitochondria in TBI pathophysiology. Mitochondria maintain cellular energy homeostasis and health via balanced processes of fusion and fission, continuously dividing and fusing to form an interconnected network throughout the cell. An imbalance of these processes, particularly an excess of fission, can be detrimental to mitochondrial function, causing decreased respiration, ROS production, and apoptosis. Mitochondrial fission is regulated by the cytosolic GTPase, dynamin-related protein 1 (Drp1), which translocates to the mitochondrial outer membrane (MOM) to initiate fission. Aberrant Drp1 activity has been linked to excessive mitochondrial fission and neurodegeneration. Measurement of Drp1 levels in purified hippocampal mitochondria showed an increase in TBI animals as compared to sham controls. Analysis of cryo-electron micrographs of these mitochondria also showed that TBI caused an initial increase in the length of hippocampal mitochondria at 24 h post-injury, followed by a significant decrease in length at 72 h. Post-TBI administration of Mitochondrial division inhibitor-1 (Mdivi-1), a pharmacological inhibitor of Drp1, prevented this decrease in mitochondria length. Mdivi-1 treatment also reduced the loss of newborn neurons in the hippocampus and improved novel object recognition (NOR) memory and context-specific fear memory. Taken together, our results show that TBI increases mitochondrial fission and that inhibition of fission improves hippocampal-dependent learning and memory, suggesting

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that strategies to reduce fission may have translational value after injury.

INTRODUCTION

Mitochondria are at the crossroad between cellular health, survival, and death. Mitochondria not only provide cellular energy through ATP synthesis, but also play an important role in intracellular calcium buffering, reactive oxygen species (ROS) production, and apoptosis. A growing body of literature from both clinical and experimental brain injury research has shown that structural and functional damage of mitochondria is an early event after traumatic brain injury (TBI) that contributes to cell death and poor cognitive outcome (Vink et al., 1990; Okonkwo and Povlishock, 1999; Sullivan et al., 1999; Lifshitz et al., 2003, 2004; Singh et al., 2006; Cheng et al., 2012; Gajavelli et al., 2015). Decreased respiration and reduced ATP production in cortical and hippocampal mitochondria occurs within 24 h post-injury and can last up to 14 days in experimental models of TBI (Xiong et al., 1997; Lifshitz et al., 2003; Singh et al., 2006; Gilmer et al., 2009). Moreover, mitochondrial damage can result in the release of pro-apoptotic factors, such as cytochrome C, that activate cell death pathways and initiate apoptosis (Raghupathi et al., 2000; Brustovetsky et al., 2002). As neurons have high metabolic needs and do not store excess energy, continuous energy production and metabolic maintenance by functional mitochondria is critical for survival, supporting the premise that improving mitochondrial function can offer neuroprotection and improve cognition following TBI (Cheng et al., 2012; Gajavelli et al., 2015).

Mitochondria are dynamic organelles that continuously undergo fusion and fission to form a highly interconnected network throughout the cell (Bereiter-Hahn and Vöth, 1994; Chan, 2006; van der Bliek et al., 2013). These balanced processes alter mitochondrial morphology and allow mitochondria to efficiently respond to cellular energy needs (Bereiter-Hahn and Vöth, 1994; Chan, 2006; Westermann, 2012; van der Bliek et al., 2013). Fusion allows for an increase in cristae density and maximization of ATP production during high metabolic activity and stress (Westermann, 2012; Youle and van der Bliek, 2012). In contrast, fission allows for proliferation and transportation of mitochondria to areas with energy demands, in addition to segregation of damaged mitochondria from the network for subsequent degradation through mitophagy (Youle and van der Bliek, 2012; Otera et al., 2013). An imbalance between fusion and fission, particularly an excess of fission, can be detrimental for energy homeostasis and has been implicated in neurodegenerative diseases (Detmer and Chan, 2007; Knott and Bossy-Wetzel, 2008; Knott et al., 2008; Archer, 2013; Burté et al., 2015). More specifically, excessive fission can lead to reduced mitochondrial respiration and ATP production, increased ROS generation, and release of apoptogenic factors, changes similar to those seen after TBI (Figure 1; Rintoul et al., 2003; Chen et al., 2005; Cribbs and Strack, 2007; Detmer and Chan, 2007; Yu et al., 2008a; Chen and Chan, 2009; Costa et al., 2010; Jahani-Asl et al., 2011; Jheng et al., 2012). Dynamin-related protein 1 (Drp1) is a key regulator of mitochondrial fission, through its interactions with the mitochondrial outer membrane (MOM; van der Bliek et al., 2013). Prior to a fission event, Drp1 translocates to the MOM where it self-assembles and forms an oligomeric structure around the mitochondrion. Hydrolysis of Drp1-bound GTP then drives the subsequent mitochondrial membrane division. Mitochondrial division inhibitor-1 (Mdivi-1) is an allosteric inhibitor of Drp1 that inhibits its oligomeric assembly thereby reducing its GTP binding affinity (Cassidy-Stone et al., 2008). Mdivi-1 has been shown to reduce cell death by attenuating mitochondrial fission in yeast, and in animals models (Cassidy-Stone et al., 2008; Jahani-Asl et al., 2011; Grohm et al., 2012; Rappold et al., 2014; Zhao et al., 2014). Recently, a study has reported that Mdivi-1 reduces cortical cell loss and improves spatial memory after TBI in mice (Wu et al., 2016). However, it is unknown if TBI alters Drp1 translocation to the MOM and mitochondrial dynamics.

In the present study, we measured Drp1 levels in purified mitochondria from the hippocampi of brain injured animals and found increased translocation. CryoEM analysis of images of mitochondria also showed a reduction in their lengths 72 h after TBI that was blocked by Mdivi-1 treatment. Further, we show that injured animals receiving Mdivi-1 have reduced loss of newborn neurons in the hippocampus and improved novel object recognition (NOR) memory and contextual fear memory. These results indicate that TBI enhances mitochondrial fission which contributes to poor cognitive outcome. Thus, strategies aimed at reducing mitochondrial fission can reduce pathology and may have translational value to treat TBI.

MATERIALS AND METHODS

Materials

Adult, male Sprague Dawley rats (300–400 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). Mdivi-1 was purchased from Tocris Bioscience (Bristol, UK). Antibodies for Drp1 (westerns) and Doublecortin (DCX) were purchased from Cell Signaling Technology (Danvers, MA, USA) whereas Drp1 (immuno-gold), TOMM20 and GAPDH antibodies were purchased from Abcam (Cambridge, MA, USA).

Controlled Cortical Impact (CCI) Injury

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) prior to initiating these studies. An electromagnetic Controlled Cortical Impact (CCI) device was used for experimental TBI as previously described (Dixon et al., 1991; Brody et al., 2007; Hoskison et al., 2009). Animals were anesthetized using 5% isofluorane with a 1:1 O2/N2O mixture and then mounted on a stereotaxic frame with anesthesia maintained with 2.5% isofluorane in 1:1 O₂/air. Bilateral 6 mm craniectomies were produced midway between the bregma and lambda (offset 0.5 mm from midline) and a single impact (2.5 mm deformation) was given at a velocity of 5 m/s to the right parietal cortex. Sham-operated animals received all surgical procedures described above excluding the craniectomies and impact. Recovery of pain reflexes and restoration of the righting response were recorded immediately after surgery to ascertain consistency in the injury. For mitochondrial isolation experiments, animals underwent all described procedures, except

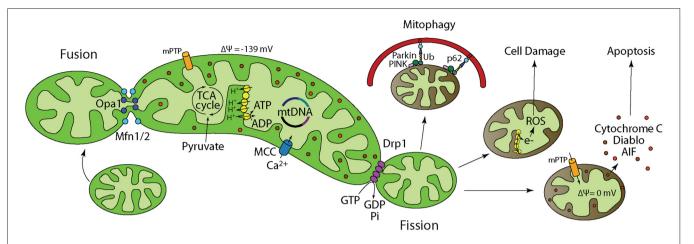


FIGURE 1 | Mitochondrial dynamics and function regulate cellular energy homeostasis, cell survival, growth, and death. Inside of a cell, mitochondria are in a dynamic flux, continuously undergoing fusion and fission to meet cellular energy needs. (1) Fusion is regulated by Optic Atrophy 1 (Opa1) and Mitofusin1/2 (Mfn1/2) and allows for functional complementation and repair of damaged mitochondria. (2) Pyruvate from glycolysis is transported into the mitochondria where pyruvate dehydrogenase catalyzes the decarboxylation of Pyruvate to form Acetyl CoA. Acetyl CoA then enters the TCA cycle. (3) Electromotive force generated by the electron transport chain allows ATP synthesis by the F1-Fo ATP synthase. (4) Mitochondria buffer excess intracellular calcium. When calcium buffering capacity is compromised as a result of mitochondrial damage/depolarization, calcium accumulates in the cytosol and activates degradative enzymes such as calpain and phospholipases that break down cellular proteins and membrane leading to death. (5) Fission is regulated by dynamin-related protein 1 (Drp1) and allows for mitochondria that cannot be repaired to be isolated followed by degradation through mitophagy. Fission is also important for subcellular distribution and transportation of mitochondria based on energy needs in certain areas of the cell. (6) Electrons leaked from the electron transport chain interact with molecular oxygen to generate reactive oxygen species (ROS) that not only damage mitochondrial membrane, mitochondrial DNA (mtDNA), and proteins, but also their cellular counter parts. Neurons have limited defense against oxidative damage and are highly vulnerable to ROS. (7) Mitochondria play a prominent role in apoptotic cell death. Damaged/depolarized mitochondria release cytochrome c and apoptosis inducing factor (AIF) that trigger cell death by activating caspases.

the TBI was delivered using an Impact OneTM Leica Biosystems CCI device with a deformation of 2.8 mm.

Drug Preparation and Administration

Mdivi-1 was dissolved in DMSO to a concentration of 25 mg/ml, after which it was diluted to a working concentration of 1.5 mg/ml. For testing the influence of Mdivi-1 on cognitive function and histopathology after TBI, injured animals were randomly assigned to either vehicle (40% DMSO) or 3 mg/kg Mdivi-1 groups. This dosage of Mdivi-1 was based on previous studies that demonstrated neuroprotection with Mdivi-1 treatment (Grohm et al., 2012). For behavioral testing, injured animals were randomly assigned to two groups and i.p. injected at 30 min, 24 h, and 48 h post-injury with Mdivi-1 (3 mg/kg) or an equivalent volume of vehicle. For immunohistochemistry, animals were injected at 30 min post-injury and again at 8 h post-injury.

Mitochondrial Isolation

To isolate mitochondria from brain tissues, Percoll density gradient centrifugation was used as previously described (Sims and Anderson, 2008). Hippocampi from two animals per experimental group were pooled together to increase the quantity of starting material. Hippocampi from sham animals and ipsilateral (to the injury) hippocampi from injured animals were rapidly removed. Tissue was homogenized in ice-cold isolation buffer (100 mM Tris pH 7.4, 10 mM EDTA, 12% Percoll solution, 1 mM Sodium Fluoride, 1 mM Sodium Molybdate, 100 nM

Okadaic Acid, 1 mM PMSF and 10 μ g/ml leupeptin). The tissue was then homogenized (4 strokes) in a Dounce homogenizer using the loose pestle followed by 8 strokes using the tight pestle. A small fraction of each homogenate was removed for determination of protein content. The remaining homogenate was then layered onto a discontinuous Percoll gradient (26% and 40% Percoll) and centrifuged for 8 min (30,700 g at 4°C). The enriched mitochondrial fraction was removed from the 26/40% interface, transferred to individual centrifuge tubes, and diluted (1:4) with isolation buffer. Fractions were then pelletized by centrifugation (16,700 g at 4°C) for 10 min. The supernatant was discarded and the samples were either immediately prepared for electron microscopy analysis or frozen and stored at -80°C . Samples underwent one freeze-thaw cycle prior to western analysis.

Western Blotting

Protein concentrations of the total homogenate and the mitochondrial fractions (n=3 samples/group, each sample was pooled from two animals) were determined using a Bicinchoninic Acid (BCA) protein assay (Thermo ScientificTM Protein Biology) with BSA as the standard. Equal amounts of protein for each sample were resolved using a SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Membranes were blocked overnight at 4°C with SuperBlockTM (TBS; ThermoFisher Scientific, Grand Island, NY, USA) and then incubated in primary antibody solutions (Drp1, 1:1000; TOMM20, 0.5 μ g/ml; GAPDH, 1 μ g/ml) for

3 h at room temperature. The membrane was then washed and incubated with species-specific, horseradish peroxidase-conjugated, secondary antibodies for 1 h. Immunoreactivity was detected using SuperSignalTM West Pico chemiluminescent substrate (ThermoFisher Scientific; Grand Island, NY, USA) and exposure to Kodak XAR5 film (Rochester, NY, USA). The relative optical density of each band was analyzed using ImageJ (NIH).

Transmission Electron Microscopy and Gold Immunolabeling

Freshly isolated mitochondria from rat hippocampi were applied to freshly glow-discharged (30 s) carbon-coated copper grids, blotted, and then fixed with 4% paraformaldehyde for 15 min on a chilled plate. Excess sample was blotted away and grids were blocked sample-side down on a 50 μ L drop of blocking buffer (5% BSA, 1× HBS). Grids were then floated on a drop of primary antibody (Drp1, 0.02 mg/ml or TOMM20, 0.01 mg/ml) for 30 min and washed before incubation in 12-nm gold-conjugated secondary antibody. The grids were washed and stained in methylamine vanadate (Nanoprodes, Nanovan), blotted, and air dried. CCD images of isolated mitochondria were taken on a JEOL1400 transmission electron microscope running at 120 kV with a Gatan Orius SC1000 camera.

Cryo-Electron Microscopy and Mitochondrial Length Measurements

Freshly isolated mitochondria from rat hippocampi (n = 1)sample/group, each sample was pooled from two animals) were immediately applied to freshly glow-discharged (30 s) 2/2 Quantifoil on 200 mesh copper grids. After 30 s, excess buffer was blotted and the sample was immediately plunged into ethane cooled to liquid N2 temperature. Cryo-preserved grids were stored in liquid N2 until use. Cryo-electron microscopy was performed on a FEI Polara G2 equipped with a Gatan K2 Summit direct electron detector. Multiple areas of the grid were chosen at random and 8×8 montages were collected at $4700 \times$ in low dose/photon counting mode using SerialEM. To quantify the length of mitochondria, individual montages were displayed in IMOD and a line along the long axis of each mitochondrion was drawn and stored in a model for each montage. Lengths were extracted for 200 mitochondria from each model table, imported into Excel and the data displayed by separating the lengths into 500 nm bins. To remove potential bias, the person collecting the primary data and the person quantifying the length of individual mitochondria were both blinded as to the sample identities.

Immunohistochemistry

For immunohistochemistry, groups of animals were injured, and were randomly assigned to receive either vehicle or 3 mg/kg Mdivi-1 (n=5/group). Animals were killed at 24 h post-CCI by an overdose of sodium pentobarbital and exsanguination with ice cold phosphate-buffered-saline (PBS) followed by 4% paraformaldehyde. Brains were collected and post-fixed in 4% PFA for 24 h before step-wise cryopreservation in sucrose (15%)

to 30% in PBS) at 4°C. Brains were cut into 40 µm-thick coronal sections using a cryostat. Tissue sections containing the dorsal hippocampus were selected, permeabilized in PBS containing 0.25% Triton-X100, blocked for 1 h in 2.5% normal goat serum, then incubated in primary antibody solutions overnight. After extensive washing, tissues were incubated in a speciesspecific biotinylated secondary antibody followed by incubation in ABC solution (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). Tissues were then developed in 3,3'diaminobenzidine (DAB) solution, washed and mounted on gelatin-subbed slides and allowed to dry overnight. Tissues were dehydrated in an alcohol series, delipidated in xylenes, and coverslipped with Permount. DCX-positive cells were then counted and the dentate gyrus measured (mm) using a Zeiss Axiovert brightfield microscope. Measurements were performed by an experimenter blind experimenter to sample identity.

Behavioral Tasks

Separate groups of animals were used for behavioral analysis. Animals were injured and randomly assigned to receive either vehicle (n = 6) or 3 mg/kg Mdivi-1 (n = 8). A group of shaminjured animals was prepared and used as baseline controls (n = 8). All behavioral tasks were carried out by an experimenter that was unaware of the treatment groups.

Novel Object Recognition (NOR)

The NOR task is a hippocampal/entorhinal cortex-dependent learning and memory task that assesses novel vs. familiar object recognition (Ennaceur and Delacour, 1988). Animals were placed in the testing chamber (100 × 100 cm box) and allowed to habituate for 10 min per day for 2 days. On day three, two identical objects were placed in the box and the animal was allowed to explore the objects for 10 min. Twenty-four hours later, a new object of the same color and size, but different shape replaced one of the objects and the animal was allowed to again explore the objects for 10 min. The time spent exploring each object was recorded by a blind experimenter on both the familiarization and testing days. The difference in percent time exploring the novel vs. the familiar object [(time spent exploring object/total time exploring) × 100] on the testing day is used as a measure of recognition memory.

Contextual Fear Conditioning

The one-trial contextual fear conditioning (FC) procedure was adapted from Wiltgen et al. (2006) and Drew et al. (2010). The training chamber included only visual contextual cues, but no auditory stimuli (i.e., a tone). The protocol did not include an acclimation exposure before training to ensure no prior association with the context. On the first day, the animal was placed in the training chamber and allowed to familiarize itself with the context for 150 s before receiving a mild foot shock (2 s, 0.7 mA). Thirty seconds later, the animals were removed from the training chamber and returned to their home cage. Twenty-four hours later, the animal was placed back in the training chamber for 3 min (without shock). Freezing behavior (defined

as the absence of movement except that needed for respiration) was recorded for both the training and the testing day every 2 s within the 3 min time period by an experimenter who was unaware of the treatment groups. The percent time the animal remained frozen was calculated and used as an index of fear memory.

Statistical Analysis

For mitochondrial morphology, data were analyzed using the Kruskal-Wallis Analysis of Ranks with Tukey's method for post hoc comparisons and the Mann-Whitney Rank Sum Test. For changes of immunoreactivity across time in western analysis, data were analyzed using a one-way analysis of variance (ANOVA), with a Bonferroni method for post hoc analysis. For NOR, Student's t-tests were used to determine any significant difference in time spent between each object pair during familiarization and testing. For contextual FC, a Student's t-test was used to determine differences between vehicle-treated and Mdivi-1 treated freezing behavior on the testing day. Results were considered significant at p < 0.05. Data are presented as the mean \pm standard error of the mean (SEM).

RESULTS

TBI Increases Drp1 Association with Mitochondria

Translocation of Drp1 to the MOM is critical for fission (Chang and Blackstone, 2007; Cereghetti et al., 2008; Santel and Frank, 2008). Employing protein extracts from purified mitochondrial (n = 3 samples/group, each sample was pooled from two)animals), western blots were performed to determine if TBI alters mitochondrial-associated Drp1 levels. Figure 2A shows representative western blot images indicating an immunoreactive band at approximately 80 kDa [corresponding to the expected migration of Drp1; Cell Signaling Technology (Danvers, MA, USA)] that appears to increase at both 24 h and 72 h after injury. TOMM20 immunoreactivity was used to normalize loading. Quantification of Drp1 immunoreactivity (Figure 2B; n = 3/group) revealed that Drp1 levels are increased in mitochondrial fractions after TBI ($F_{(2,6)} = 13.059$, p = 0.007), reaching statistical significance by 72 h post-injury (p = 0.007). To determine if TBI alters total Drp1 levels (both cytosolic and mitochondria-associated), hippocampal homogenates from sham and injured animals (n = 3/group) were also compared by western blotting. Drp1 optical density was normalized with the optical density for the cytoplasmic protein, GAPDH. Results presented in Figures 2C,D indicate no significant changes in total hippocampal Drp1 levels as a result of CCI injury. In order to assess if the translocated Drp1 is present on the MOM, we performed immuno-gold labeling and electron microscopy analysis. We first performed immuno-gold labeling using antibodies for the outer membrane translocase (TOMM20). A representative electron micrograph is shown in Figure 2E indicates positive TOMM20 staining with localization of gold particles (black dots) on the outer surface of the mitochondrion. **Figure 2F** shows a representative picture for Drp1 immuno-gold labeling (Abcam; Cambridge, MA, USA) of a mitochondrion from the injured hippocampus. Gold signal (black dots) can be seen clustered on the outer membrane of this mitochondrion.

TBI Decreases Mitochondrial Length that is Blocked by Mdivi-1 Treatment

To determine if TBI alters mitochondrial length, mitochondria were isolated from sham and injured hippocampi. CrvoEM micrographs were captured and used for measuring mitochondrial length (200 mitochondria/sample; each sample was pooled from two animals; n = 1 sample/group). Figure 3A shows representative cryoEM micrographs of mitochondria isolated from sham and injured hippocampal tissues. Mitochondria of different lengths can be seen in all groups. However, longer mitochondria are more present in the 24 h sample. In contrast, smaller mitochondria are more present in the 72 h sample. The summary results shown in Figure 3B indicate that TBI significantly alters mitochondrial length (H = 71.413, p = < 0.001) with a significant increase in average length observed at 24 h postinjury (diff. of ranks = 28.922, p = < 0.05), followed by a significant decrease at 72 h post-injury (diff. of ranks = 18.514, p < 0.05), compared to sham injured controls. In order to examine if an inhibitor of Drp1 can attenuate the reduction in mitochondrial length detected 72 h after TBI, a group of injured animals was administered 3 mg/kg (i.p) Mdivi-1 starting at 30 min post-injury, and again at 24, 48, and 72 h postinjury. Mitochondria were isolated from injured hippocampi as described in the "Materials and Methods" Section. The representative cryoEM micrographs shown in Figure 3A indicates longer mitochondria in Mdivi-1 treated animals as compared to untreated injured animals. Quantification of mitochondrial lengths (from 200 mitochondria; n = 1 sample pooled from two animals) revealed that Mdivi-1 significantly increased mitochondrial length at 72 h post-injury (U = 13.097, p = < 0.001; Figure 3B).

To examine differences in distribution of mitochondrial size, lengths were binned (bin sizes of 500 nm) and the number of mitochondria per bin was recorded. Figure 3C shows that in sham animals, 63.5% of the mitochondria are less than 1.5 μm in length. Twenty-four hours after TBI, there is a modest decrease in the number of mitochondria less than 1.5 μm (49.5%) with a shift in the distribution in favor of mitochondria with lengths between 2 μm and 4 μm . By 72 h post-injury, 86% of mitochondria are between 200 nm and 1.5 μm , with very few mitochondria longer than 2 μm . In the Mdivi-1 treated group, the distribution of mitochondria was similar to that seen in sham controls, with 61.5% of mitochondria less than 1.5 μm in length.

Mdivi-1 Treatment Reduces Newborn Neuronal Loss Following TBI

Recently, it has been reported that Mdivi-1 treatment reduces cortical cell loss following TBI (Wu et al., 2016). In addition

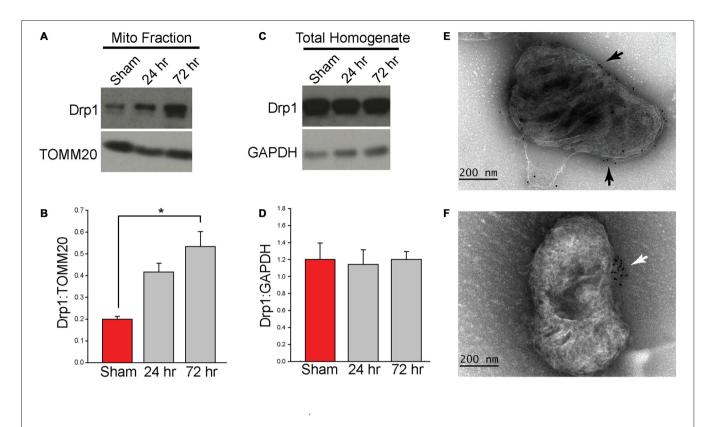


FIGURE 2 | Controlled cortical impact (CCI) injury increases the level of Drp1 in isolated hippocampal mitochondrial fractions. Animals were injured by CCI and mitochondria were isolated from injured hippocampal tissues at 24 h and 72 h post-injury. (A) Representative western blots of isolated ipsilateral hippocampal mitochondrial fractions from sham and injured (24 and 72 h) animals. (B) Relative immunoreactivity of each band was quantified and Drp1 optical density values were normalized to TOMM20. Drp1 levels increase marginally at 24 h and significantly at 72 h (p = 0.007) post-injury. (C) Representative western blots from ipsilateral hippocampal homogenate from sham and injured (24 and 72 h) animals. (D) Immunoreactivity was quantified and optical density values for Drp1 were normalized to GAPDH. Results indicate no Drp1 level changes in the ipsilateral hippocampus. Immuno-gold labeling was also performed using a primary antibody for TOMM20, a mitochondrial outer membrane (MOM) translocase, and Drp1 with a gold-conjugated secondary antibody. (E) Representative image of TOMM20 immuno-gold labeling on the MOM. Gold particles indicated by black arrows. (F) Representative image of Drp1 immuno-gold labeling on a mitochondrion from the injured hippocampus. Gold labeling for Drp1 was found to be associated with the isolated mitochondria and associated with the MOM (indicated by white arrow). Data are presented as the mean \pm standard error of the mean (SEM). *p < 0.05.

to cortical cell loss, both experimental and clinical TBI causes loss of hippocampal neurons, including DCX-positive newborn neurons in the dentate gyrus (Hall et al., 2005; Gao et al., 2008; Yu et al., 2008b; Christidi et al., 2011). We therefore examined if post-injury Mdivi-1 treatment can attenuate the loss of these newborn neurons. Groups of animals were injured, and were randomly assigned to receive either vehicle or 3 mg/kg Mdivi-1 (n = 5/group) 30 min post-injury. A second injection was administered 8 h later. Animals were euthanized 24 h post-injury for examination of DCX-positive neurons. Figure 4A shows representative photomicrographs of DCX-positive cells in the subgranular zone of the ipsilateral hippocampus from sham and injured (vehicle-treated and Mdivi-1 treated) animals. Consistent with previous studies, CCI injury caused a marked reduction of DCX-positive neurons in the ipsilateral hippocampus ($t_{(8)} = 4.817$, p = 0.001), compared to sham animals, with no significant changes observed on the contralateral side. Mdivi-1 treatment significantly attenuated the loss of DCX-positive cells in the ipsilateral hippocampus after injury ($t_{(8)} = -2.381$, p = 0.044; **Figure 4B**) as compared to injured animals treated with vehicle.

Mdivi-1 Treatment Improves Recognition Memory and Contextual Fear Memory

As we observed that Mdivi-1 inhibits changes in mitochondrial length in the injured hippocampus and reduces the loss of newborn neurons after TBI, we questioned if this would result in improved learning and memory performance on the hippocampal-dependent NOR and context-specific fear memory tasks (Hernández-Rabaza et al., 2009; Drew et al., 2010; Denny et al., 2012; Kheirbek et al., 2012; Nakashiba et al., 2012; Suárez-Pereira et al., 2015). Sham (n = 8) and injured animals (vehicle-treated, n = 6; Mdivi-1 treated, n = 8) were used for this experiment according to the timeline shown in **Figure 5A**. During the familiarization phase of the NOR task all three groups equally explored both objects, suggesting no pre-existing biases or asymmetries. Long-term memory was tested 24 h

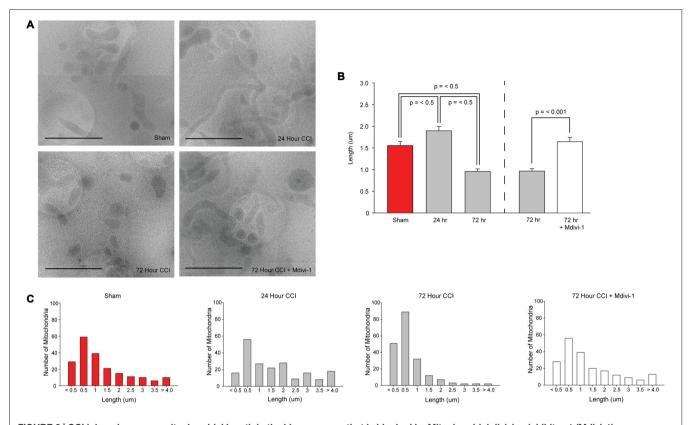


FIGURE 3 | CCI injury decreases mitochondrial length in the hippocampus that is blocked by Mitochondrial division inhibitor-1 (Mdivi-1).

(A) Representative cryoEM micrographs of isolated hippocampal mitochondria from sham, 24 h post-CCI, 72 h post-CCI, and 72 h post-CCI + Mdivi-1.

(B) Summary results for average mitochondrial length for each group. Please note, the results for the 72 h injured group are presented twice to represent the statistical comparisons to assess mitochondria length over time after injury, and to assess the effect of Mdivi-1 at 72 h. (C) Population distributions of hippocampal mitochondrial lengths from sham, 24 h post-CCI, 72 h post-CCI, and 72 h post-CCI + Mdivi-1 animals. Lengths were binned (bin sizes of 500 nm) and the number of mitochondria per bin are shown. X-axis labels indicate the lower bin value for each bin range. Data are presented as the mean ± SEM.

later by replacing one of the familiar objects with a novel one. Sham animals spent significantly more time exploring the new object (**Figure 5B**; $t_{(14)}=7.270$, p=<0.001), indicating intact recognition memory. In contrast, injured animals receiving vehicle-treatment had impaired recognition memory, spending equivalent times exploring both the familiar and the novel object ($t_{(10)}=-1.225$, p=0.249). Interestingly, Mdivi-1 treated injured animals show a significant preference for the novel object vs. the familiar object ($t_{(14)}=9.408$, p=<0.001; **Figure 5B**), indicating preserved recognition memory.

The same animals were then tested 28 days post-injury on a one-trial context-specific FC task. **Figure 6** shows that prior to delivery of a mild foot shock all animals were mobile and showed minimal freezing behavior in the training context. Twenty-four hours later, the memory for the training context was tested. Sham animals froze approximately 60% of the observation time. By comparison to the sham group, injured animals with vehicle treatment showed reduced freezing behavior. On the other hand, Mdivi-1 treated group froze at a level comparable to shams and significantly more than the vehicle-treated controls $(t_{(12)} = -2.839, p = 0.015)$, indicating improved contextual fear memory.

DISCUSSION

Growing evidence in the literature indicates that mitochondria exist in a dynamic flux, undergoing continuous fission and fusion to meet cellular energy needs and an imbalance of these processes can be detrimental to cell survival (Rintoul et al., 2003; Chen et al., 2005; Cribbs and Strack, 2007; Detmer and Chan, 2007; Knott and Bossy-Wetzel, 2008; Knott et al., 2008; Yu et al., 2008a; Chen and Chan, 2009; Costa et al., 2010; Jahani-Asl et al., 2011; Jheng et al., 2012; Archer, 2013; Burté et al., 2015). In the present study, we investigated whether TBI alters mitochondrial dynamics. The results of this study reveal four key findings: (1) TBI reduces mitochondrial length 72 h after injury that is blocked by Mdivi-1, a pharmacological inhibitor of Drp1; (2) TBI does not alter total hippocampal Drp1, but rather increases the translocation of Drp1 to the mitochondria to initiate fission; (3) Mdivi-1 attenuates TBI-triggered death of newborn neurons; and (4) post-injury treatment with Mdivi-1 improves longterm memory in hippocampal-dependent tasks, namely NOR memory and context-specific fear memory. Our findings, along with recently published data, indicate that excessive mitochondrial fission may contribute to hippocampal neuronal

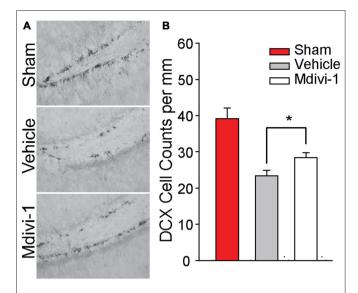


FIGURE 4 | Mdivi-1 treatment reduces loss of newborn neurons in the dentate gyrus of the hippocampus after CCI injury. (A) Representative photomicrographs of Doublecortin (DCX) positive staining in the dentate gyrus of the ipsilateral hippocampus in a sham and 24 h post-injury animals (vehicle-treated and Mdivi-1 treated). (B) Quantification of DCX+ cells in the ipsilateral and contralateral dentate gyrus for each group revealed that animals treated with Mdivi-1 had increased DCX+ cells in the ipsilateral hippocampus compared to vehicle-treated controls, suggesting reduced loss of newly born neurons. Data are presented as the mean \pm SEM. *p < 0.05.

death and neurocognitive impairments after TBI (Wu et al., 2016).

The dynamic processes of fusion and fission maintain metabolic homeostasis and allow mitochondria to efficiently respond to fluctuating energy needs (Bereiter-Hahn and Vöth, 1994; Chan, 2006; Westermann, 2012; van der Bliek et al., 2013). Mitochondrial fusion is regulated by large GTPases associated with the inner (Optic Atrophy 1, Opa1) and outer mitochondrial membranes (Mitofusins 1/2, Mfn1/2; Figure 1). Fusion promotes mixing of contents, thereby allowing for functional complementation of mitochondrial DNA (mtDNA) needed for repair of damaged mitochondria (Westermann, 2012; Youle and van der Bliek, 2012). Mitochondria also undergo hyperfusion in states of high energy demand to increase their cristae density to maximize ATP production (Westermann, 2012; Youle and van der Bliek, 2012). Mitochondrial fission is primarily regulated by the GTPase Drp1 and also serves a number of cellular functions (Bereiter-Hahn and Vöth, 1994; Chan, 2006; Westermann, 2012; Youle and van der Bliek, 2012; Otera et al., 2013; van der Bliek et al., 2013). By subdividing the network, mitochondria can proliferate and be transported to areas that require metabolic activity (Westermann, 2012; Youle and van der Bliek, 2012). Additionally, when mitochondria are damaged, fission allows for segregation from the network and subsequent degradation of the damaged portion of the mitochondrion through mitophagy (Youle and van der Bliek, 2012; Otera et al., 2013). Excessive fission events can cause small, fragmented mitochondria and have been implicated in mechanisms of neuronal death and dysfunction in many

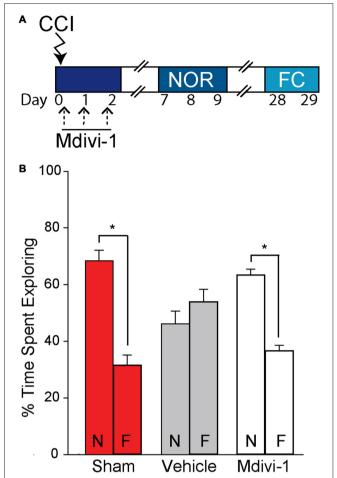


FIGURE 5 | Post-CCI mdivi-1 treatment rescues recognition memory after CCI injury. CCI animals were treated with either 3.0 mg/kg of Mdivi-1 (n=8) or vehicle (n=6) starting 30 min post-injury, and subsequently at 24 and 48 h post-injury. (**A**) Representative timeline for Mdivi-1 treatment and behavioral experiments. Rats were familiarized with two identical objects during the training period and percent of time spent exploring each object was quantified to control for any pre-existing biases. No significant biases to either object were observed. (**B**) Twenty-four hours later, one of the objects was replaced by a novel object and percent of time spent exploring both the novel (N) and familiar (F) objects were recorded. Mdivi-1 treated animals demonstrated improved recognition memory in the Novel object recognition (NOR) task indicated by a significant increase in exploration of the novel object vs. the familiar object. Data are presented as the mean \pm SEM. *p < 0.05. CCI, controlled cortical impact; FC, fear conditioning; NOR, novel object recognition.

neurodegenerative conditions including Parkinson's disease and Alzheimer's disease (Knott and Bossy-Wetzel, 2008; Knott et al., 2008). Results of the current study show that length of hippocampal mitochondria is significantly decreased by 3 days post-injury, a finding consistent with excessive fission. Additionally, administration of the Drp1 inhibitor Mdivi-1 blocked TBI-induced decreases in mitochondrial length. In addition to a significant reduction in mitochondrial length at 72 h post-injury, we observed a transient, but significant, increase in mitochondrial length at 24 h post-injury. An increase in mitochondrial length may indicate

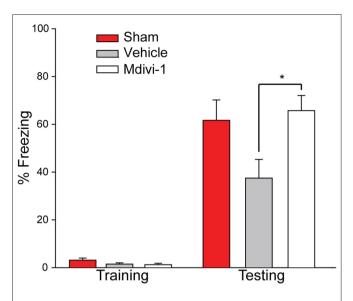


FIGURE 6 | Post-CCI mdivi-1 treatment rescues contextual memory after CCI injury. CCI animals were treated with either 3.0 mg/kg of Mdivi-1 (n=8) or vehicle (n=6) starting 30 min post-injury, and subsequently at 24 and 48 h post-injury. Four weeks post-injury, rats were exposed to the FC training chamber, before receiving a mild foot shock (2 s, 7.0 mV). Prior to foot shock, animal in all three groups explored the context equally (training). Twenty-four hours later (one-trial) the rats were replaced back into the training chamber and their freezing behavior was recorded for a total of 3 min. Rats treated with Mdivi-1 demonstrated improved contextual fear memory in a one-trial context-specific FC task indicated by a significant increase in freezing behavior compared to vehicle-treated controls. Data are presented as the mean \pm SEM. *p < 0.05.

an acute period of increased mitochondrial fusion. Both clinical and experimental studies have shown there is an acute period of increased glucose utilization after TBI (Giza and Hovda, 2001; Prins et al., 2013; Gajavelli et al., 2015). As mitochondrial fusion has been shown to increase ATP production, the acute increase in mitochondrial length we observed may be consistent with increased glucose utilization after TBI. Alternatively, as increased fusion has been shown to rescue dysfunctional mitochondria through complementation of contents with healthy mitochondria, an acute increase in fusion after TBI may be indicative of such a mechanism as well (Westermann, 2012; Youle and van der Bliek, 2012). Future investigations are required to further characterize changes in the balance of fusion and fission and the contribution of these mechanisms to TBI pathology.

Approximately 97% of cellular Drp1 is cytosolic. In response to stimuli, Drp1 translocates to, and interacts with, the MOM protein Fis1 to initiate fission (Smirnova et al., 2001; van der Bliek et al., 2013). Consistent with this, the Drp1 immunogold labeling results show gold particles association with the MOM (Figure 2E). After translocation to the MOM, Drp1 self-assembles and forms an oligomeric structure around the mitochondrion. Hydrolysis of bound GTP then drives the subsequent membrane division. Dysregulation of Drp1 function has been linked to aberrant mitochondrial fission causing

mitochondrial dysfunction and cell damage (Rintoul et al., 2003; Chen et al., 2005; Cribbs and Strack, 2007; Detmer and Chan, 2007; Yu et al., 2008a; Chen and Chan, 2009; Costa et al., 2010; Jahani-Asl et al., 2011; Jheng et al., 2012). Dysregulation of Drp1 can result from: (1) changes in Drp1 protein levels (e.g., transcription/translation); (2) changes in activity (e.g., post-translational modifications/phosphorylation); and/or (3) changes in translocation to the MOM (Frank et al., 2001; Reddy et al., 2011; Wang et al., 2011; Manczak and Reddy, 2012; Zhao et al., 2013). Our results reveal that although total Drp1 levels in hippocampal homogenates do not change after injury, mitochondrial association of Drp1 significantly increases 72 h after injury (Figure 2), indicating an increase in translocation from the cytosol. These results are consistent with the decrease in mitochondrial size also observed at 72 h after injury, indicating an increase in mitochondrial fission. Drp1 is highly responsive to environmental signaling and appears to be an important link in communicating changes in energy demands and allowing for appropriate changes in mitochondrial dynamics (Detmer and Chan, 2007; Santel and Frank, 2008; Otera et al., 2013). A number of intracellular signaling pathways have been shown to regulate mitochondrial fission by modifying Drp1, including phosphorylation, ubiquitination, sumolyation, and nitrosylation (Chang and Blackstone, 2007; Taguchi et al., 2007; Cereghetti et al., 2008; Knott et al., 2008; Santel and Frank, 2008; Kanamaru et al., 2012). However, whether these modifications of Drp1 occur after TBI are yet to be determined.

Recently, a study has shown that administration of Mdivi-1 decreases cortical cell loss and improves performance in the Morris water maze task after TBI (Wu et al., 2016). In addition to causing the loss of mature neurons, TBI causes the death of newborn neurons, with previous studies showing that approximately 50% of DCX-positive newborn neurons die within 24 h to 3 days of the injury (Hall et al., 2005; Gao et al., 2008; Yu et al., 2008b). Consistent with the neuroprotective effect of Mdivi-1, a significant protection of DCX-positive newborn neurons in the dentate gyrus of the hippocampus was observed after Mdivi-1 treatment. These results suggest that inhibiting mitochondrial fission may improve the survivability of newborn cells after TBI. This protection was associated with improved performance in behavioral tasks known to be influenced by treatments which impair ongoing neurogenesis (one-trial context-specific fear memory, Figure 6; Hernández-Rabaza et al., 2009; Drew et al., 2010; Denny et al., 2012; Kheirbek et al., 2012; Nakashiba et al., 2012; Suárez-Pereira et al., 2015). Although this improvement is consistent with the protection of newborn neurons, we cannot rule out the possibility that adult neurons protected by Mdivi-1 treatment could have contributed to the improved memory we observed.

While results of the current study indicate that TBI causes excessive mitochondrial fission, and that strategies to reduce fission can offer neuroprotection and improve outcome, a number of limitations exist that constrain interpretation of the results: (1) the morphological results were obtained from isolated hippocampal mitochondria. It is possible that the morphology of isolated mitochondria may not reflect the morphology of mitochondria in vivo; (2) as mitochondrial

isolation was carried out from the entire hippocampus, the cell-type or regional specificity of fission events cannot be determined; (3) although Mdivi-1 has been shown to be a specific inhibitor of Drp1 *in vitro*, the *in vivo* specificity of this chemical can be questioned (Cassidy-Stone et al., 2008). Additionally, only one dosage of Mdivi-1 (3 mg/kg) was used in this study and whether higher or lower doses would yield similar results is yet to be determined. A dose response curve would be needed to determine the optimal dose for improvements in pathological and cognitive outcomes after injury.

SUMMARY

As mitochondrial dynamics play an integral role in cellular health, its dysregulation may affect neuronal survival and plasticity. The current results reveal that TBI causes a decrease in mitochondrial size and increased Drp1 translocation to mitochondria, indicating an increase in fission events. Inhibition of Drp1 with Mdivi-1 treatment restores mitochondrial length, reduces loss of newborn hippocampal neurons, and improves hippocampal-dependent learning and memory after injury. Overall, these results indicate a role for Drp1 and excessive mitochondrial fission in neuronal death and cognitive dysfunction after TBI. By investigating pathological changes in mitochondrial fission, these studies provide an innovative

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perspective on mechanisms of metabolic dysfunction and may lead to novel mitochondrial-targeted therapeutic approaches to improve outcome after brain injury.

AUTHOR CONTRIBUTIONS

TDF, MJH, JZ, ANM, MNW, and PKD have made substantial contributions to the design, acquisition, analysis, and interpretation of the original research described in this manuscript. All have played a role in the drafting and revision of the manuscript, and have approved of the final version. All authors agree to be accountable for all aspects of the work and ensure that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Traumatic Brain Injury Upregulates Phosphodiesterase Expression in the Hippocampus

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Traumatic brain injury (TBI) results in significant impairments in hippocampal synaptic plasticity. A molecule critically involved in hippocampal synaptic plasticity, 3',5'-cyclic adenosine monophosphate, is downregulated in the hippocampus after TBI, but the mechanism that underlies this decrease is unknown. To address this question, we determined whether phosphodiesterase (PDE) expression in the hippocampus is altered by TBI. Young adult male Sprague Dawley rats received sham surgery or moderate parasagittal fluid-percussion brain injury. Animals were analyzed by western blotting for changes in PDE expression levels in the hippocampus. We found that PDE1A levels were significantly increased at 30 min, 1 h and 6 h after TBI. PDE4B2 and 4D2 were also significantly increased at 1, 6, and 24 h after TBI. Additionally, phosphorylation of PDE4A was significantly increased at 6 and 24 h after TBI. No significant changes were observed in levels of PDE1B, 1C, 3A, 8A, or 8B between 30 min to 7 days after TBI. To determine the spatial profile of these increases, we used immunohistochemistry and flow cytometry at 24 h after TBI. PDE1A and phospho-PDE4A localized to neuronal cell bodies. PDE4B2 was expressed in neuronal dendrites, microglia and infiltrating CD11b⁺ immune cells. PDE4D was predominantly found in microglia and infiltrating CD11b+ immune cells. To determine if inhibition of PDE4 would improve hippocampal synaptic plasticity deficits after TBI, we treated hippocampal slices with rolipram, a pan-PDE4 inhibitor. Rolipram partially rescued the depression in basal synaptic transmission and converted a decaying form of long-term potentiation (LTP) into long-lasting LTP. Overall, these results identify several possible PDE targets for reducing hippocampal synaptic plasticity deficits and improving cognitive function acutely after TBI.

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INTRODUCTION

Every year in the United States an estimated 1.7 million people sustain a traumatic brain injury (TBI) and nearly 70–80% of those who survive report chronic learning and memory deficits (Lew et al., 2006; Faul et al., 2010). The hippocampus is critically involved in learning and memory and is highly susceptible to damage after TBI. Even when not directly damaged, hippocampal atrophy and neuronal loss are often observed in TBI patients (Bigler et al., 1997;

Tomaiuolo et al., 2004). This progressive hippocampal atrophy is also observed in experimental models of TBI, and is accompanied by a loss of dentate hilar cells and neurons in the CA3 region as well as synaptic loss (Maxwell et al., 2003; Hall et al., 2005; Scheff et al., 2005; Witgen et al., 2005). In addition to these gross morphological changes, in experimental models of TBI there are deficits in hippocampal basal synaptic transmission and long-term potentiation (LTP; Miyazaki et al., 1992; Titus et al., 2013b). Finding molecular targets to attenuate the damage caused by TBI and improve hippocampal synaptic plasticity is of critical importance to assist the estimated 3–5 million people currently living with cognitive disabilities from TBI in the United States (Langlois et al., 2006; Zaloshnja et al., 2008).

Targeting TBI-induced LTP deficits is a promising strategy for improving learning and memory (Atkins, 2011). During hippocampal LTP and long-term memory formation, 3',5'cyclic adenosine monophosphate (cAMP) signaling is critical to activate the transcription factor cAMP-response element binding protein (CREB) and mediate gene transcription required for long-term memory formation (Frey et al., 1993; Bourtchuladze et al., 1994). We have found that in an experimental model of TBI, cAMP levels are depressed and CREB activation is impaired during a learning task (Titus et al., 2013a,b). The exact molecular mechanisms leading to the decrease in cAMP levels and impairments in CREB activation after TBI are unknown. cAMP is synthesized from ATP by adenylyl cyclases (ACs) and cAMP signaling is tightly regulated in discrete spatial-temporal microdomains through hydrolysis by phosphodiesterases (PDEs; Houslay, 2010). Whether cAMP signaling in the hippocampus is decreased after TBI by either changes in ACs or PDEs is unknown and this knowledge could guide the development of therapeutic strategies for treating the cognitive consequences of TBI.

One strategy for improving cAMP signaling is to inhibit PDEs. However, an important consideration is that the PDE superfamily is a large family of enzymes with 11 different PDE families (Bender and Beavo, 2006; Omori and Kotera, 2007). Within each of the PDE families there are multiple subfamilies encoded by individual genes, with several splice variants within each subfamily. Eight of the PDE families hydrolyze cAMP: PDEs 1, 2, 3, 10, and 11 hydrolyze both cAMP and cGMP, and PDEs 4, 7, and 8 hydrolyze only cAMP (Maurice et al., 2014). Of these PDEs, PDE1, 3, 4, 8, and 10 have been found in the hippocampus (Johansson et al., 2012). The PDE1 family consists of three subfamilies, PDE1A, 1B, and 1C, which are encoded by separate genes. PDE1 is commonly referred to as a Ca²⁺/calmodulin-stimulated PDE, and the activity of PDE1 can be enhanced through increases in calcium and calmodulin signaling (Omori and Kotera, 2007; Heckman et al., 2015). The PDE3 family consists of two subfamilies, PDE3A and 3B, which are encoded by two separate genes. While PDE3 hydrolyzes cAMP and cGMP, the ability for PDE3 to hydrolyze cAMP is inhibited in the presence of cGMP (Bender and Beavo, 2006). The PDE8 family is a cAMP-specific PDE that consists of two subfamilies, PDE8A and 8B (Martinez and Gil, 2014). The PDE10 family is another dual-specific PDE which is encoded by one gene, PDE10A (Kelly and Brandon, 2009). Given the wide array of inhibitors that target each of these families, it is important to

understand how TBI affects the expression and activity of each these enzymes to target the relevant PDE (Menniti et al., 2006; Spina, 2008; Titus et al., 2015b).

Of the cAMP-degrading PDEs, the most notable one in the context of learning and memory is the PDE4 family (Li et al., 2011; Heckman et al., 2015). The PDE4 family is encoded by four genes, PDE4A, 4B, 4C, and 4D. With the exception of PDE4C, all of these subfamilies are expressed in the brain, immune system and cardiovascular system (Henkel-Tigges and Davis, 1990; Reneerkens et al., 2009). A unique feature of the PDE4 family is that they are further classified into four groups: long, short, super-short, and dead-short isoforms (Titus et al., 2015a). This classification is based on the presence, or absence, of upstream conserved regions (UCR) near the N-terminus, which allow for differential post-translational regulation of these isoforms (Hansen and Zhang, 2015). The PDE4A family has six isoforms: PDE4A1, 4A4/5, 4A7, 4A8, 4A10, and 4A11. The PDE4B family has five isoforms: PDE4B1-5. The PDE4D family has 10 isoforms: 4D1-7, 4D9, 4D10, and 4D11. Identifying the spatial and temporal expression pattern of these PDEs in the hippocampus after brain injury could facilitate the development of a more targeted approach for reducing TBI-induced cognitive deficits.

Targeting cAMP-degrading PDEs acutely after trauma has yielded promising results in experimental models of spinal cord injury and cerebral ischemia (Kato et al., 1995; Schaal et al., 2012). However, pan-PDE inhibitors have broad effects given the widespread expression of PDE isoforms. In the context of experimental TBI, the anti-inflammatory and neuroprotective effects of pan-PDE4 inhibitors are overshadowed by the vasodilation and hemorrhagic effects when administered acutely after trauma (Atkins et al., 2012, 2013). This emphasizes the need for more specific targets when treating the acute phase of TBI. In a previous study, we reported that TBI alters PDE expression in the ipsilateral parietal cortex acutely after trauma, but whether similar changes occur in the hippocampus is unknown (Oliva et al., 2012). Determining which PDEs are upregulated after trauma could provide more specific drug targets for reducing neuronal death, inflammation and atrophy in the hippocampus after TBI. In this study we investigated changes in PDE1, 3, 4, 8, and 10 after TBI using western blot analysis, immunohistochemistry and flow cytometry analysis of the hippocampus.

MATERIALS AND METHODS

Fluid-Percussion Injury Surgery

One hundred adult male Sprague Dawley rats were used in this study (2–3 mos, 300–350 gm, Charles River Laboratories). All experimental procedures were performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and with approval from the University of Miami Animal Care and Use Committee. Animals were anesthetized (3% isoflurane, 70% N₂O, 30% O₂, 5 min) and received a 4.8 mm diameter craniotomy at –3.8 mm posterior to bregma and 2.5 mm lateral to the midline over the right parietal cortex. A plastic female Luer Lock adapter (18 gage) was affixed at the craniotomy site

with cyanoacrylate and dental cement. Animals were allowed to recover for 12-16 h while fasting with water ad libitum. Animals were re-anesthetized (induction for 5 min with 3% isoflurane, 70% N₂O, 30% O₂, maintenance during surgery with 1% isoflurane, 70% N₂O, 30% O₂), then intubated, mechanically ventilated (Stoelting) and given rocuronium (10 mg/kg, intraarterial) and penicillin/benzathine (20,000 IU/kg, intramuscular). Head and body temperature were maintained between 36.6 and 37.2°C using rectal and temporalis muscle thermistors connected to feedback-regulated heating lamps. Physiological parameters (blood pO_2 and pCO_2 , blood pH, and mean arterial blood pressure) were monitored via a tail artery catheter and maintained at normal levels throughout the surgery. Animals were prospectively randomized into sham or TBI surgery groups. Brain trauma was produced with a fluid-pulse (16 ms duration, 2.0 ± 0.2 atmospheric pressure) at the craniotomy site. Shamoperated animals received all surgical procedures identical to the TBI animals with the exception of the fluid-pulse. At the end of the surgery, animals received buprenorphine (0.01 mg/kg, subcutaneously). Criteria for exclusion from the study were: mortality, >15% loss of body weight, non-resolving infection at the surgical site, inability to feed or drink, motor paralysis, listlessness, self-mutilation, excessive grooming leading to loss of dermal layers, spontaneous vocalization when touched or poor grooming habits. No animals were removed from the study. To determine the number of animals needed for the study, a power analysis was prospectively performed to detect a 50% difference in PDE protein expression between groups with western blot analysis at 80% power and significance level of 0.05 (Oliva et al., 2012). An n value of 6 animals/group was obtained. Investigators were blind to the animal surgery treatment for the electrophysiology analyses.

Western Blot Analysis

At 30 min, 1 h, 3 h, 6 h, 24 h, or 7 days after TBI or sham surgery, animals were deeply anesthetized (3% isoflurane, 70% N_2O , 30% O_2 , 5 min) and decapitated (n = 6/time point for TBI animals, n = 3/time point for sham animals). The ipsilateral hippocampus was rapidly dissected on ice, snap frozen with liquid nitrogen and stored at -80°C. Tissue was homogenized with a Dounce homogenizer (15 s, 4°C) in: 15 mM Tris pH 7.6, 250 mM sucrose, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 0.1 mM Na₃VO₄, 50 mM NaF, 2 mM Na₄P₂O₇, 1.25 μg/ml pepstatin A, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 1x phosphatase inhibitor cocktail set II (EMD Millipore). Each hippocampus was homogenized in 750 µl of buffer. Total protein was determined using Coomassie Plus assay (Bio-Rad Laboratories). Homogenates were boiled with sample buffer (9 min, 95°C). Equal amounts of protein per lane (60 µg/sample) were electrophoresed on 12.5% SDS-PAGE gels. Proteins were transferred to Immobilon-P membranes (EMD Millipore) and membranes were incubated with the following primary antibodies: β-actin (AC-15, 1:10,000, Sigma-Aldrich), PDE1A (sc-50480, 1:4,000, Santa Cruz Biotechnology), PDE1B (ab14600, 1:500, Abcam; Giachini et al., 2011), PDE1C (sc-67323, 1:500, Santa Cruz Biotechnology; Haering et al., 2015), PDE3A (sc-20792, 1:250, Santa Cruz Biotechnology; Soler

et al., 2015), phospho-PDE4A (GTX14610, 1:2,000, GeneTex), PDE4A5 (ab42094, 1:2,000, Abcam; Carito et al., 2012), PDE4A8 (GTX14606, 1:1,000, GeneTex), PDE4B (sc-25812, 1:500, Santa Cruz Biotechnology; Suhasini et al., 2015), PDE4D (sc-25814, 1:500, Santa Cruz Biotechnology; Kunal et al., 2012), phospho-PDE4D (ab59212, 1:1,000, Abcam), PDE8A (sc-30059, 1:500, Santa Cruz Biotechnology; Dong et al., 2010), PDE8B (sc-17234, 1:500, Santa Cruz Biotechnology; Shimizu-Albergine et al., 2012), and PDE10A (sc-67298, 1:250, Santa Cruz Biotechnology; Giralt et al., 2013). These antibodies were chosen based on previously published studies and resulted in bands that corresponded to the appropriate, apparent molecular weights. Identification of specific PDE isoforms was based on known molecular weights. Secondary antibodies conjugated to horseradish peroxidase were used for detection (1:1,000, Cell Signaling Technology). Epitopes were visualized with enhanced chemiluminescence or enhanced chemiluminescence plus (GE Healthcare) and x-ray film (Phenix Research Products). Quantification of films was performed using ImageJ 1.48v (NIH). Levels of each protein were normalized to β-actin within each sample and then to the average of sham levels. No significant differences in PDE levels were observed in sham animals at the different survival time points (data not shown), so the three sham animals at each recovery time point were pooled into one sample for analysis to reduce animal numbers. Representative western blots show sham animals taken from 3 and 24 h post-recovery.

Immunohistochemistry

Animals were deeply anesthetized (3% isoflurane, 70% N₂O, 30% O_2 , 5 min) at 24 h after sham surgery (n = 3) or TBI (n = 3) and perfused with saline (75 mL) and then with 4% paraformaldehyde in PBS (350 mL, 4°C). Brains were sectioned with a vibratome (50 µm thick) and free floating sections were blocked with PBS containing 5% normal goat serum, 0.2% fish skin gelatin and 0.3% TX-100. Sections were incubated with the following primary antibodies: PDE1A (sc-50480, 1 μg/ml, Santa Cruz Biotechnology), phospho-PDE4A (GTX14610, 1 µg/ml, GeneTex), PDE4B2 (ABS181, 2 µg/ml, Millipore; Ghosh et al., 2012), PDE4D (ABS22, 2 μg/ml, Millipore; Kuroiwa et al., 2012), MAP2 (M9942, 2 μg/ml, Sigma-Aldrich) and NeuN (MAB377, 4 μg/ml, Millipore). Primary PDE antibodies were selected based on our western blot data demonstrating that these antibodies recognized proteins of the appropriate known molecular weights. Secondary antibodies used were conjugated to Alexa 488, Alexa 546 or Alexa 647 (Invitrogen). Cell nuclei were visualized using Hoechst 33342 (Invitrogen).

Images were obtained with a FluoView FV1000 laser scanning confocal microscope (Olympus America) equipped with a 10X 0.4 NA air objective, 20X 0.85 NA oil-immersion objective and 60X 1.42 NA oil-immersion objective, and an LD laser (405 nm), multi-line argon laser and HeNe(G) laser. Sections from different animals were processed in parallel and at least three sections from each animal were imaged.

Flow Cytometry

At 24 h after sham surgery (n = 6) or TBI (n = 6), animals were deeply anesthetized (3% isoflurane, 70% N₂O, 30% O₂,

5 min) and transcardially perfused with PBS (120 mL, 4°C). The ipsilateral hippocampus was dissected at 4°C. Tissue was mechanically dissociated into single cell suspension and cells were labeled with CD45 Alexa 647 (202212, 1.25 µg/ml, BioLegend) and CD11b v450 (53-4321-80, 1 μg/ml, eBioscience). Dead cells were excluded using LIVE/DEAD Fixable Near-IR dead cell stain (L10119, 1 µl/ml, Life Technologies). Cells were fixed and permeabilized with BD Cytofix/Cytoperm Fixation/Permeabilization kit (554714, BD Biosciences). Cells were intracellularly labeled with phospho-PDE4A (GTX14610, 2 μg/ml, GeneTex), PDE4B2 (ABS181, 2 μg/ml, EMD Millipore) or PDE4D (sc-25814, 2 μg/ml, Santa Cruz Biotechnology). PDE staining was detected with PE-conjugated secondary antibodies (12-4739-81, 10 µg/ml, eBioscience). Flow cytometry data was acquired on a BD LSR II flow cytometer with four emission lasers at 407, 488, 532, and 640 nm. Data collection was performed using BD FACSDiva 8.0.1 (BD Biosciences) and analyzed with Kaluza 1.2 software (Beckman Coulter).

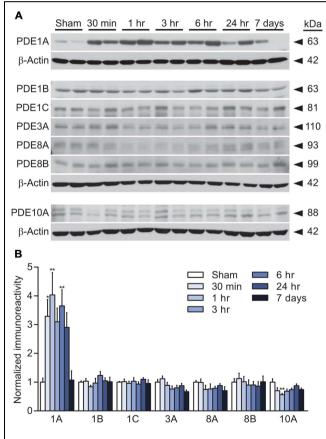


FIGURE 1 | PDE expression changes after TBI in the hippocampus. (A,C,E) Representative western blots of the ipsilateral hippocampus analyzed for PDE1A, 1B, 1C, 3A, 8A, 8B, and 10A levels. Each of the corresponding β-actin western blots are shown below the PDE western blots. **(B,D,F)** Densitometry results. Significant increases in PDE1A were observed at 30 min, 1 h and 6 h post-injury. PDE10A decreased at 1 h post-injury. n = 6/group, p < 0.05, p < 0.01 vs. Sham; one-way ANOVA with Tukey's HSD correction for multiple comparisons.

Electrophysiology

At 24 h after sham surgery (n = 13) or TBI (n = 15), animals were deeply anesthetized (3% isoflurane, 70% N₂O, 30% O₂, 5 min) and then decapitated. The ipsilateral hippocampus was dissected and sliced with a vibratome at 4°C (Leica Microsystems). Slices from the middle third of the hippocampus were collected (400 µm thick) in artificial cerebral spinal fluid (aCSF): 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 10 mM D-glucose, 2 mM CaCl₂, 1 mM MgCl₂ saturated with 95% O₂/5% CO₂. Slices recovered at room temperature for at least 60 min. Slices were transferred to a submerged recording chamber and perfused at 2.5-3 ml/min with aCSF at 31°C (Warner Instruments). Field excitatory postsynaptic potentials (fEPSPs) were recorded from the stratum radiatum of area CA1 with glass electrodes filled with 2 M NaCl (1-3 M Ω). The Schaffer collateral pathway was stimulated with a platinumiridium concentric bipolar electrode (tip diameter 25 µm, FHC). Electrophysiological responses were recorded using a Multiclamp 700B amplifier (Axon Instruments) and pClamp 10.4 software (Axon Instruments). Recordings were low-pass filtered at 2 kHz and digitized at 20 kHz (Digidata 1440A, Molecular Devices). Input-output (I–O) curves were generated with stepwise current increases from 20 to 180 µA. Paired-pulse facilitation (PPF) was measured with 50-250 ms stimulation intervals, delivered at a current intensity of 40-50% of the maximum fEPSP. For baseline responses prior to LTP induction, fEPSPs were recorded at a current intensity of 40-50% of the maximum fEPSP, delivered at 0.033 Hz for at least 20 min. Long-term potentiation (LTP) was induced with high frequency stimulation (HFS) of 100 Hz for 1 s at the current intensity used for baseline stimulation. Rolipram (3 μM) or vehicle (0.3% DMSO) were bath applied in aCSF beginning 10 min prior to LTP induction and for 30 min after tetanization. The amount of depolarization during tetanization was analyzed by integrating the entire HFS response (total) or integrating the last 50 ms of depolarization (steady-state; Klann et al., 1998). Synaptic fatigue was calculated by normalizing each fEPSP during tetanization to the first fEPSP of the HFS (Rutten et al., 2008b).

Data Analysis

Statistical comparisons were made using GraphPad Prism 6.05 or SigmaPlot 12.0 software. Western blot data and tetanization responses were analyzed using a one-way ANOVA and Tukey's HSD correction for multiple comparisons. Flow cytometry data was analyzed using an unpaired Student's t-test. I–O responses, PPF, and LTP data were analyzed using a repeated measures two-way ANOVA and Tukey's HSD correction for multiple comparisons. Significance was designated at p < 0.05. Results presented are mean \pm SEM.

RESULTS

We have previously reported that cAMP levels are decreased in the hippocampus from 15 min to 4 h after TBI (Atkins et al., 2007). To determine whether this decrease is associated with changes in levels of PDE, the enzyme that degrades cAMP, we

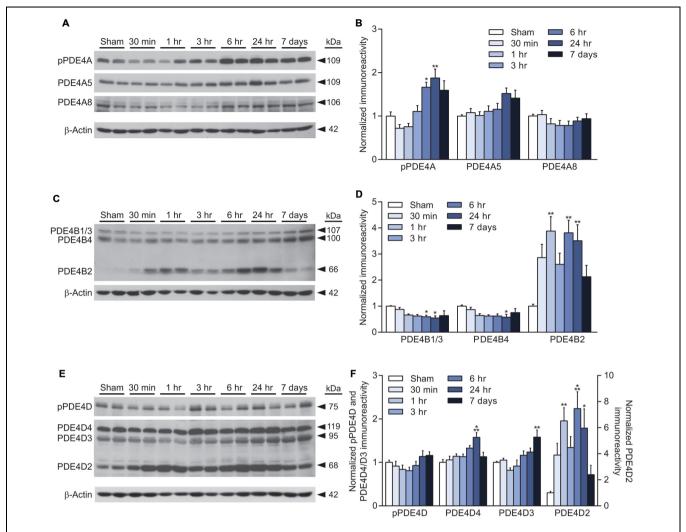


FIGURE 2 | Increased PDE4B2 and 4D2 expression in the hippocampus after TBI. (A,C,E) Representative western blots of the ipsilateral hippocampus analyzed for changes in PDE4A, 4B, and 4D. (B,D,F) Densitometric analysis. Significant increases in phospho-PDE4A, and total levels of PDE4B2, 4D4, 4D3, and 4D2 were observed. Levels of PDE4B1/3 and 4B4 decreased after TBI. n = 6/group, *p < 0.05, **p < 0.01, ***p < 0.001 vs. Sham; one-way ANOVA with Tukey's HSD correction for multiple comparisons.

evaluated the hippocampus by western blot analysis for PDE expression. We chose to evaluate PDE1, 3, 4, 8, and 10 since these are either cAMP-specific or degrade both cAMP and cGMP and are found in the brain or inflammatory cells that could infiltrate the brain after injury (Boswell-Smith et al., 2006). At 30 min, 1 h, 3 h, 6 h, 24 h, or 7 days after moderate parasagittal fluid-percussion brain injury or sham surgery, animals were analyzed by western blotting for changes in PDE expression in the ipsilateral, injured hippocampus (**Figure 1**). We found that PDE1A was significantly increased as early as 30 min after TBI, and remained elevated at 1 and 6 h post-injury. PDE1B and 1C levels were unchanged after TBI between 30 min to 7 days post-injury. No significant changes in expression were also observed for PDE3A, 8A, or 8B. PDE10A levels were unchanged with the exception of a small decrease at 1 h post-injury.

PDE4 is the major cAMP-degrading enzyme in the brain and the predominant PDE in inflammatory cells (Boswell-Smith et al.,

2006). PDE4A, 4B, and 4D are present in the brain, whereas PDE4C is expressed only at very low levels in the brain (Perez-Torres et al., 2000; Lakics et al., 2010; Johansson et al., 2012). PDE4A and 4D are regulated by phosphorylation, and we found that phosphorylation of PDE4A, but not 4D, was significantly increased at 6 and 24 h after TBI (Figures 2A,E). Total levels of both PDE4A5 and 4A8 were unaltered after TBI (Figures 2A,B). Levels of PDE4B1/3 were modestly, but significantly decreased at 6 and 24 h after TBI (Figures 2C,D). Similarly, PDE4B4 was decreased at 24 h after injury. In contrast, both PDE4B2 and 4D2 were significantly increased at 1, 6, and 24 h after TBI (Figures 2D,F). PDE4D4 and 4D3 were also elevated at 24 h and 7 days post-injury, respectively (Figures 2E,F).

To determine the localization of these changes, we performed immunohistochemistry at 24 h after TBI for PDE1A, phospho-PDE4A, PDE4B2, and PDE4D4, the isoforms that showed the largest changes in expression after TBI. PDE1A was found in

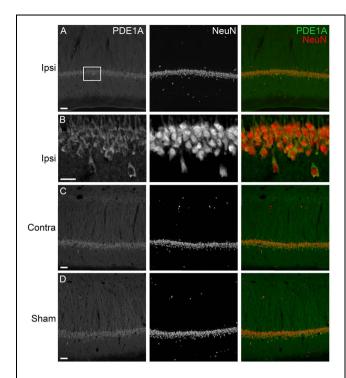


FIGURE 3 | Immunohistochemistry of PDE1A in the hippocampus. **(A)** The ipsilateral hippocampus was immunostained for PDE1A at 24 h after TBI. PDE1A (*green*) colocalized to NeuN-positive cells (*red*) in area CA1. Scale bar 50 μ m. **(B)** Higher magnification of the ipsilateral CA1 pyramidal cell layer (*box*). Scale bar 20 μ m. **(C)** Expression of PDE1A in the contralateral hippocampus in area CA1. Scale bar 50 μ m. **(D)** PDE1A immunohistochemistry in area CA1 from a sham animal. Scale bar 50 μ m.

nuclei and neurites of neurons (Figure 3). Phospho-PDE4A localized predominately to neuronal nuclei (Figure 4). In contrast, PDE4B2 was expressed in dendrites as determined by co-localization with MAP2, but not in the cell bodies or nuclei (Figure 5). PDE4D immunoreactivity was present in scattered cells throughout the hippocampus, suggestive of immune cell infiltration (Figure 6).

To more definitively determine the cellular expression of PDE4D, we performed flow cytometry (**Figure 7**). We also selected PDE4B2 and phospho-PDE4A for analysis since these were also significantly elevated in the injured hippocampus at 24 h post-TBI and have been identified in immune cells (Shepherd et al., 2004; Ghosh et al., 2012). Phospho-PDE4A, PDE4B2, and 4D were localized to microglia and infiltrating CD11b⁺/CD45⁺ immune cells in the hippocampus at 24 h postinjury.

Next, to determine if upregulation of PDE4 expression is involved in hippocampal synaptic plasticity deficits after TBI, we assessed whether administration of a pan-PDE4 inhibitor would rescue the deficits in hippocampal LTP induced by TBI. Hippocampal LTP in area CA1 induced with a single tetanus was significantly impaired in slices from TBI animals (**Figures 8A,B**). Rolipram (3 μ M) applied to the hippocampal slices rescued the deficits in LTP expression [time \times animal treatment interaction $F_{(177,2115)}=1.60,\ p<0.001;$ **Figures 8A,B**]. Both

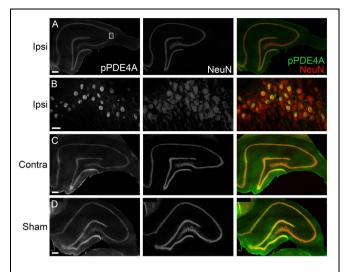


FIGURE 4 | Immunohistochemistry of phospho-PDE4A in the hippocampus. (A) At 24 h after TBI, phosphorylated PDE4A (*green*) was found throughout all subregions of the ipsilateral hippocampus and colocalized with NeuN-positive cells (*red*). Scale bar 100 μm. (B) Higher magnification of the ipsilateral CA3 pyramidal cell layer (*box*). Scale bar 20 μm. (C) Expression of phospho-PDE4A in the contralateral hippocampus. Scale bar 100 μm. (D) Phospho-PDE4A immunohistochemistry in the sham, non-injured hippocampus. Scale bar 100 μm.

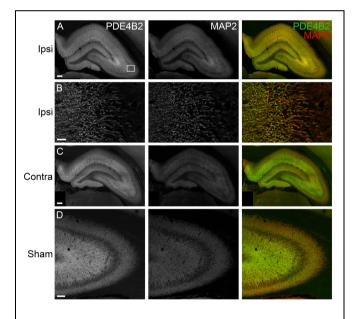


FIGURE 5 | Immunohistochemistry of PDE4B2 in the hippocampus at 24 h after TBI. (A) PDE4B2 (green) colocalized to MAP2-positive dendrites (red) throughout all subregions of the ipsilateral hippocampus. Scale bar 200 μ m. (B) Higher magnification of the ipsilateral CA3 region (box). Scale bar 20 μ m. (C) Expression of PDE4B2 in dendrites in the contralateral hippocampus. Scale bar 200 μ m. (D) PDE4B2 immunohistochemistry in area CA3 from a sham animal. Scale bar 100 μ m.

total and steady-state depolarization levels as well as synaptic fatigue during tetanization were comparable between all groups, suggesting that the impairment in LTP after TBI was not due to

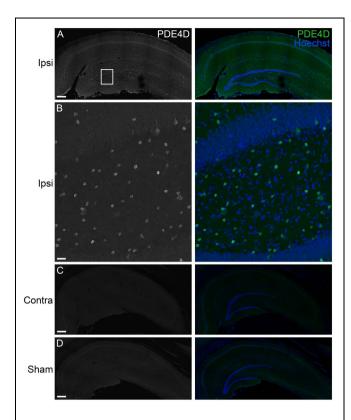


FIGURE 6 | Immunohistochemistry of PDE4D in the hippocampus at 24 h after TBI. (A) PDE4D (green) was found in scattered cells throughout all regions of the ipsilateral hippocampus. Hoescht counterstaining was used to identify the hippocampal pyramidal cell layers (blue). Scale bar 250 μm . (B) Higher magnification of the ipsilateral dentate gyrus (box). Scale bar 20 μm . (C) Expression of PDE4D was at very low levels in the contralateral hippocampus. Scale bar 250 μm . (D) PDE4D was present at very low levels in the sham, non-injured hippocampus. Scale bar 250 μm .

differences in depolarization during the tetanus (**Figures 8C,D**). To evaluate whether changes in basal synaptic transmission were involved in the rescue of hippcampal LTP, we analyzed input–output (I–O) responses. I–O curves were significantly depressed in slices from TBI animals as compared to sham animals [stimulation intensity \times animal treatment interaction $F_{(24,288)}=2.76$, p<0.001]. This depression in basal synaptic transmission was partially rescued with rolipram [main effect of animal treatment $F_{(3,288)}=8.12$, p<0.001; **Figures 9A,B**]. PPF was depressed after TBI and also partially rescued with rolipram treatment [main effect of animal treatment $F_{(3,144)}=3.35$, p<0.05; **Figure 9C**]. These results indicate that hippocampal basal synaptic transmission and LTP expression in area CA1 were impaired acutely after TBI and rescued with the pan-PDE4 inhibitor, rolipram.

DISCUSSION

Downregulation of the cAMP signaling pathway following TBI significantly alters hippocampal synaptic plasticity, however, the underlying mechanisms are unknown. The PDE enzymes play

a critical role in the regulation of cAMP levels, and alterations in PDE expression and phosphorylation may be involved in the depression of cAMP levels following TBI. Therefore, in the present study we evaluated whether expression of PDEs such as PDE1, 3, 4, 8, and 10, which are present in the central nervous system and hydrolyze cAMP, were altered acutely after TBI (Bender and Beavo, 2006; Omori and Kotera, 2007; Lakics et al., 2010). While not all PDE isoforms showed altered expression levels, several were significantly changed. PDE1A, 4B2, and 4D2, and phosphorylation of PDE4A were significantly upregulated, whereas PDE4B1/3, 4B4, and 10A levels decreased acutely after TBI. The PDEs 1B, 1C, 3A, 4A5, 4A8, 8A, and 8B, and phospho-PDE4D were unchanged acutely after TBI. Although an a priori power analysis was conducted to ensure the sample sizes were sufficient to detect significant changes in PDE levels after TBI, for these particular PDEs the negative results should be interpreted cautiously.

Phosphodiesterases are key enzymes involved in regulating cAMP levels in the central nervous system. Understanding the spatio-temporal alterations in PDE isoform expression levels after injury is important for targeting the relevant isoform as well as treating within the appropriate therapeutic time window. In a previous study, we found that there were significant temporal changes in PDE expression within the ipsilateral cortex acutely after TBI (Oliva et al., 2012). Like the present results found in the injured hippocampus, TBI induced upregulation of PDE1A, 4B2, and 4D2 in the ipsilateral cortex. However, not all PDEs were regulated similarly between the cortex and hippocampus. For example, PDE10A was upregulated in the cortex, but downregulated in the hippocampus acutely after TBI. PDE4A5 and 4A8 were downregulated in the cortex, but unaltered in the hippocampus. Furthermore, both PDE4D4 and 4D3 were upregulated at 24 h after TBI in the hippocampus, but unchanged in the cortex. These discrepancies suggest that developing a therapeutic to target a specific PDE and particular functional outcome requires consideration of both the temporal and spatial regulation of that particular PDE isoform.

The upregulation of PDE1A in the hippocampus after TBI suggests that beyond decreasing cAMP levels, cGMP levels may also be affected by TBI. PDE1 is a family of Ca²⁺/calmodulindependent PDEs encoded by three genes (A-C) and involved in the regulation of both cGMP and cAMP (Bender and Beavo, 2006). In the human brain, PDE1A is distributed in the parietal cortex and hippocampus, but at lower levels than PDE1B or 1C (Lakics et al., 2010). In the present study, expression of PDE1A was significantly increased, while PDE1B and 1C were unchanged after TBI. Although the PDE1 family uses both cyclic nucleotides as a substrate, PDE1A displays a higher affinity toward cGMP than cAMP (Bender and Beavo, 2006). Conversely, PDE10A which is also a dual-substrate PDE, was downregulated in the hippocampus. These opposing changes may be involved in the lack of changes in basal cGMP levels reported in the hippocampus after TBI (Temple et al., 2001). However, NMDAstimulated levels of cGMP are actually increased in the ipsilateral hippocampus after TBI and further studies are required to determine if cGMP-selective PDEs expressed in the brain such as PDE9 are altered by TBI (Temple et al., 2001). Together these

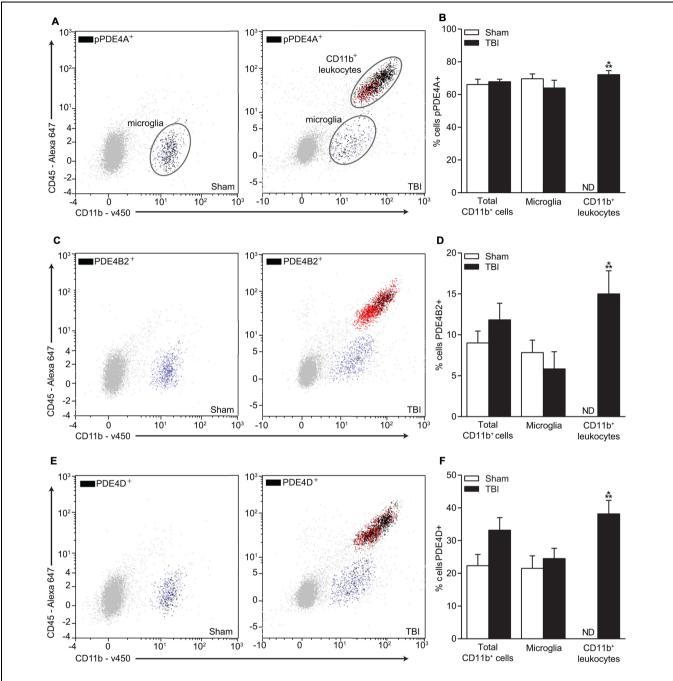


FIGURE 7 | Flow cytometry of microglia and infiltrating CD11b⁺ immune cells and co-localization with phospho-PDE4A, PDE4B2, and 4D. (A) Phospho-PDE4A, (C) PDE4B2, and (E) PDE4D expression were localized to microglia and infiltrating immune cells in the injured hippocampus at 24 h post-injury. There was a significant increase in (B) phospho-PDE4A, (D) PDE4B2 and (F) PDE4D-positive infiltrating CD11b⁺/CD45⁺ immune cells. n = 6/group, ***p < 0.001 vs. Sham; unpaired Student's t-test.

results support the potential therapeutic use of PDE1 inhibitors such as vinpocetine, amantadine and caffeine, which have shown promise to improve cognitive impairments in preclinical models of TBI and in humans (Dixon et al., 1999; Li et al., 2008; Amen et al., 2011).

In particular, the PDE4 family was highly regulated in the hippocampus after TBI. PDE4B2, 4D2, 4D3, and 4D4

expression levels and phosphorylation of PDE4A were increased following TBI, while PDE4B1/3 and 4B4 levels were decreased. Due to the similar molecular weights of PDEB1 and B3, we were unable to differentiate which particular isoform decreased after TBI. The changes in PDE4 isoforms were transient with most returning to sham, non-injured levels by 7 days postinjury. The upregulation of PDE4 isoforms correlates loosely

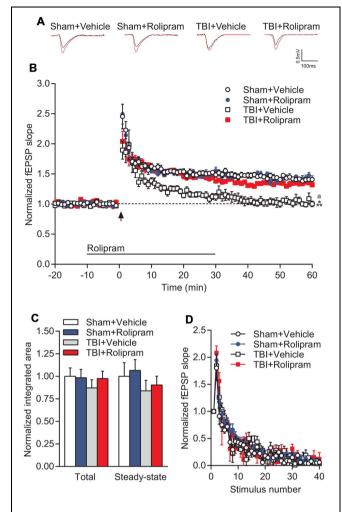


FIGURE 8 | PDE4 inhibition significantly rescued TBI-induced LTP deficits in area CA1 of the hippocampus. (A) Representative traces of the fEPSP before (black) and 60 min after (red) tetanization. (B) fEPSP slopes were normalized to baseline prior to LTP induction. Rolipram (3 μ M) or vehicle (0.3% DMSO) were bath applied 10 min prior to and for 30 min after tetanization (bar). Hippocampal LTP was induced with 1 × 100 Hz tetanization, 1 s long (arrow). (C) Total and steady-state depolarization levels during tetanization. (D) Synaptic fatigue during tetanization. Sham + Vehicle n=10 slices/9 animals, Sham + Rolipram n=14 slices/12 animals, TBI+Vehicle n=8 slices/7 animals, TBI+Rolipram n=8 slices/8 animals; ***p<0.001 vs. Sham + Vehicle or Sham + Rolipram, p=0.005 vs. TBI + Rolipram; repeated-measures two-way ANOVA with Tukey's HSD correction for multiple comparisons.

with the decrease in cAMP levels and PKA activation after TBI (Atkins et al., 2007). However, a causal link between an upregulation of PDE4 expression and decreased cAMP levels has not been established. Alternatively, changes in AC activity or expression could be involved in the decrease in cAMP levels after TBI. Accordingly, reduction in type 1 AC enzyme levels have been reported within the hippocampus of Alzheimer's disease patients, aged rodents and after ischemia, and are correlated with impairments in cAMP signal transduction (Araki et al., 1995; Yamamoto et al., 2000; Nagakura et al., 2002; Ramos et al., 2003; Mons et al., 2004). Further studies are needed to

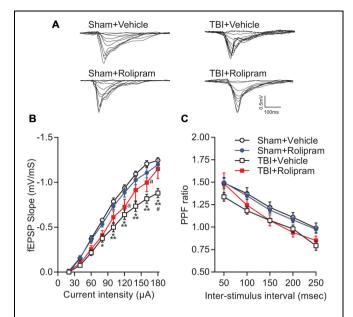


FIGURE 9 | Effects of rolipram on hippocampal synaptic transmission at 24 h post-TBI. (A) Representative traces of the fEPSP evoked during input–output (I–O) responses in stratum radiatum of area CA1 with Schaffer collateral stimulation. (B) The I–O curve was significantly shifted downward in hippocampal slices from TBI animals as compared to sham animals. Rolipram (3 μ M) significantly enhanced the fEPSP slope in slices from TBI animals. *p<0.05, ***p<0.05, ***p<0.001 vs. Sham + Vehicle, *p<0.01 vs. TBI + Rolipram, *p<0.05 vs. Sham + Vehicle. (C) A decrease in PPF was observed in TBI + Vehicle-treated slices (p<0.05 TBI + Vehicle vs. Sham + Vehicle) that was not significant in TBI + Rolipram-treated slices. Data represent the ratio of the second fEPSP slope to the first fEPSP slope. Sham + Vehicle n=10 slices/9 animals, Sham + Rolipram n=14 slices/12 animals, TBI+Vehicle n=8 slices/7 animals, TBI + Rolipram n=8 slices/8 animals; repeated-measures two-way ANOVA with Tukey's HSD correction for multiple comparisons.

determine if AC levels are also altered by TBI or whether the increase in PDE expression after TBI also results in increased PDE activity.

The PDE4 subfamily, like other PDEs, is subject to posttranslational modification to allow for rapid feedback in controlling the spatial and temporal dynamics of cAMP signaling (Houslay, 2010). Unlike the other PDE4 isoforms, the long isoforms have a UCR1 domain that contains a PKA phosphorylation site that enhances PDE4 activity (Mackenzie et al., 2002). The PKA phosphorylation site allows for cAMP signaling to negatively feedback on itself through enhanced activation of long PDE4A isoforms. PDE4A5 and 4A8 are both long isoforms with similar molecular weights, which precluded definitively identifying which isoform was increased in phosphorylation after TBI. Phospho-PDE4A localized to both hippocampal neurons and infiltrating CD11b⁺ leukocytes. While this increase in phospho-PDE4A is indicative of an increase in PKA-mediated PDE4A activity, the timing of this change does not correlate with changes seen in cAMP and phospho-PKA, which are depressed in the hippocampus during this time frame (Atkins et al., 2007). However, it is possible that there could be changes in cAMP and PKA signaling

in microdomains mediating this effect that were missed with the western blot analysis or immunohistochemistry. Subcellular, compartmentalized signaling changes have been reported when assessing differences between PDE4B and 4D signaling (Blackman et al., 2011). An alternative possibility was that another protein kinase mediated this phosphorylation, such as MAPK-activated protein kinase 2, which has been reported to phosphorylate a serine near the PKA phosphorylation site of PDE4A5 (Mackenzie et al., 2011).

In contrast to the results with phospho-PDE4A, the increase in PDE4B2 and 4D2 expression correlated with the decrease in cAMP levels after TBI (Atkins et al., 2007). Within 1 h after trauma, PDE4B2 and 4D2 were significantly elevated in the injured hippocampus and remained elevated for up to 24 h postinjury. Although the immunohistochemistry results indicated that PDE4B2 and phospho-PDE4A localized to neurons, flow cytometry revealed expression of PDE4B2 and phospho-PDE4A in microglia and infiltrating CD11b+/CD45+ leukocytes as well. The absence of obvious expression in microglia or infiltrating immune cells with immunohistochemistry suggests that phospho-PDE4A- and PDE4B2-positive immune cells may be present near the contusion site between the parietal cortex and hippocampus which was likely sampled in the western blot analysis. Immunohistochemistry of this damaged area can be hindered by autofluorescence of red blood cells present at the contusion site. These findings are supported by other studies which have found that both isoforms are differentially distributed throughout the brain and also found in the immune system. PDE4B and 4D are present in monocytes, macrophages and neutrophils and regulate pro-inflammatory mediators such as tumor necrosis factor and neutrophil infiltration (Jin and Conti, 2002; Ariga et al., 2004; Shepherd et al., 2004). Interestingly, phospho-PDE4A and PDE4D were expressed in a larger proportion of infiltrating CD11b+/CD45+ cells as compared to PDE4B2. While the increase in phospho-PDE4A, PDE4B2, and PDE4D in immune cells may contribute to inflammatory signaling after TBI, the upregulation of PDE4B2 in hippocampal dendrites suggests that this molecule may have multiple roles in TBI and may also be involved in learning and memory deficits after TBI.

PDE4 is well known to be involved in hippocampal LTP and long-term memory formation (Navakkode et al., 2004; Rutten et al., 2008a,b; Li et al., 2011). General pan-PDE4 inhibitors such as rolipram reverse learning and memory impairments in animal models of Alzheimer's disease, psychosis, stress, and ischemia (Henkel-Tigges and Davis, 1990; Imanishi et al., 1997; Bach et al., 1999; Gong et al., 2004; Chen et al., 2010; Wiescholleck and Manahan-Vaughan, 2012; Sierksma et al., 2014). In accordance with these previous studies, we found that bath application of rolipram to hippocampal slices from TBI animals reversed the decay of LTP (Navakkode et al., 2004). However, the dose of rolipram required to rescue hippocampal LTP (3 µM) at 24 h post-injury was threefold greater than the dose sufficient to rescue hippocampal LTP deficits at 2 weeks post-injury (1 µM; Titus et al., 2013a,b). Indeed, 1 µM rolipram did not rescue the deficits in hippocampal LTP at 24 h post-injury (data not

shown). Given that expression of most PDE4 isoforms returned to sham, non-injured levels by 7 days post-injury, we speculate that the acute, transient upregulation of PDE4 isoforms at 24 h post-injury resulted in the necessity for a higher dose of rolipram to rescue hippocampal LTP deficits at this acute time point.

Interestingly, rolipram also partially improved the depression of basal synaptic transmission in area CA1 of the hippocampus. Basal synaptic transmission in the hippocampus is mediated primarily through AMPA-type glutamate receptors, which are well known to be regulated by PKA (Man et al., 2007). Trafficking of AMPA-type glutamate receptors into the postsynaptic membrane is increased by PKA phosphorylation of serine 845 on the GluA1 subunit (Oh et al., 2006; Middei et al., 2013). GluA1 levels have been found to be altered in some experimental models of TBI (Schumann et al., 2008; Kharlamov et al., 2011). The observed partial rescue in basal synaptic transmission may possibly be through regulation of AMPA receptor trafficking although further studies are needed to evaluate this potential mechanism.

In summary, we found that in an experimental model of TBI there was an acute upregulation of PDE1A, 4B2, 4D4, 4D3, 4D2, and phosphorylated PDE4A in the hippocampus after injury. Furthermore, we found that PDE4B2 and phospho-PDE4A were localized to neurons, microglia and infiltrating CD11b⁺ leukocytes in the injured hippocampus early after trauma. PDE4D was found predominantly in microglia and infiltrating CD11b⁺/CD45⁺ leukocytes. Rolipram, a pan-PDE4 inhibitor, rescued synaptic plasticity deficits in the hippocampus at 24 h after TBI. Given the role of these PDE4 subfamilies in learning, memory and inflammation, this study highlights new potential targets for reducing damage and improving hippocampal synaptic plasticity after TBI.

AUTHOR CONTRIBUTIONS

NW contributed in experimental design, data collection and analysis, and writing of the manuscript. DT contributed in experimental design, data collection and analysis, and writing of the manuscript. AO contributed in experimental design, data collection and analysis, and writing of the manuscript. CF contributed in experimental design, data collection, and writing of the manuscript. CA contributed in experimental design, data analysis, and writing of the manuscript.

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Traumatic Brain Injury Alters Methionine Metabolism: Implications for Pathophysiology

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Methionine is an essential proteinogenic amino acid that is obtained from the diet. In addition to its requirement for protein biosynthesis, methionine is metabolized to generate metabolites that play key roles in a number of cellular functions. Metabolism of methionine via the transmethylation pathway generates S-adenosylmethionine (SAM) that serves as the principal methyl (-CH₃) donor for DNA and histone methyltransferases (MTs) to regulate epigenetic changes in gene expression. SAM is also required for methylation of other cellular proteins that serve various functions and phosphatidylcholine synthesis that participate in cellular signaling. Under conditions of oxidative stress, homocysteine (which is derived from SAM) enters the transsulfuration pathway to generate glutathione, an important cytoprotective molecule against oxidative damage. As both experimental and clinical studies have shown that traumatic brain injury (TBI) alters DNA and histone methylation and causes oxidative stress, we examined if TBI alters the plasma levels of methionine and its metabolites in human patients. Blood samples were collected from healthy volunteers (HV; n = 20) and patients with mild TBI (mTBI; GCS > 12; n = 20) or severe TBI (sTBI; GCS < 8; n = 20) within the first 24 h of injury. The levels of methionine and its metabolites in the plasma samples were analyzed by either liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry (LC-MS or GC-MS). sTBI decreased the levels of methionine, SAM, betaine and 2-methylglycine as compared to HV, indicating a decrease in metabolism through the transmethylation cycle. In addition, precursors for the generation of glutathione, cysteine and glycine were also found to be decreased as were intermediate metabolites of the gamma-glutamyl cycle (gamma-glutamyl amino acids and 5-oxoproline). mTBI also decreased the levels of methionine, α -ketobutyrate, 2 hydroxybutyrate and glycine, albeit to lesser degrees than detected in the sTBI group.

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Abbreviations: ANOVA, analysis of variance; BCAAs, branched-chain amino acids; BHMT, betaine homocysteine methyltransferase; CT, computed tomography; DRS, Disability Rating Scale; GC-MS, gas chromatography-mass spectrometry; GCL, glutamate cysteine ligase; GCS, Glasgow coma scale; GGT, gamma-glutamyl transpeptidase; GGCT, gamma-glutamyl cyclotransferase; HAM-D, Hamilton depression rating scale; HV, healthy volunteers; LC-MS, liquid chromatography-mass spectrometry; MAT, L-methionine S-adenosyltransferase; mTBI, mild TBI; ROS, reactive oxygen species; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SSRIs, serotonin reuptake inhibitors; TBI, traumatic brain injury; TMB, tetramethylbenzidine.

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Taken together, these results suggest that decreased levels of methionine and its metabolic products are likely to alter cellular function in multiple organs at a systems level.

Keywords: concussion, epigenetic changes, metabolomics, protein methylation, S-adenosylmethionine, transsulfuration

INTRODUCTION

It has been appreciated for more than 30 years that the resting metabolic expenditure of the severely injured brain is almost 40% higher than that of the non-injured brain, and is associated with a negative nitrogen balance (the difference between nitrogen uptake and nitrogen excretion), suggesting increased protein catabolism (Clifton et al., 1985). Increasing enteral nutrition to compensate for the enhanced nitrogen excretion has shown only partial success (Clifton et al., 1985), possibly due to poor gut absorption and/or impaired gut motility. In particular, insufficient availability of essential amino acids (primarily obtained from the diet) can not only negatively influence cell survival and function in the injured brain, but also can impair the function of other organs as well. In addition to serving as the building blocks for proteins, amino acids and their metabolites play critical roles in various cellular and physiological functions (e.g., regulation of cerebral perfusion by the arginine metabolite nitric oxide). Although a few studies have measured amino acid levels in traumatic brain injury (TBI) patients (Aquilani et al., 2000; Jeter et al., 2012, 2013; Vuille-Dit-Bille et al., 2012), whether the metabolic products of these amino acids are altered in TBI patients is largely unknown.

Methionine is an essential amino acid for protein synthesis and is often incorporated as the first amino acid. Metabolism of methionine occurs by two primary pathways: the transmethylation and the transsulfuration (Figures 1A, 3A, 4A). The transmethylation pathway generates S-adenosylmethionine (SAM), an important methyl donor for the methylation of lipids, proteins, and nucleotides. For example, SAM-dependent DNA and histone methyltransferases (MTs) have been found to be key enzymes in the epigenetic regulation of gene expression (Cantoni, 1975; Bird, 2007). SAM also donates methyl groups for the synthesis of phosphatidylcholine, a major phospholipid component of cell membranes and an important signaling molecule for both intra- and inter-cellular communication (Hirata and Axelrod, 1980). In addition to generating SAM, methionine is required for the synthesis of glutathione via the transsulfuration pathway. Under conditions of stress, cells use glutathione to scavenge reactive oxygen species (ROS) in order to reduce oxidative damage. Both experimental and clinical studies have shown that TBI causes oxidative damage to the injured brain, which may be related to decreases in glutathione availability (Povlishock and Kontos, 1992; Bayir et al., 2002; Singh et al., 2006; Bains and Hall, 2012). Thus, decreases in the levels of methionine and/or its metabolic products may underlie oxidative damage and the progression of TBI pathology and outcome.

In the present study, we measured the levels of methionine and several of its metabolites in plasma samples collected within the first 24 h of their injury from patients who experienced either a severe (GCS \leq 8) or mild (GCS > 12) TBI (sTBI or mTBI). Plasma samples from healthy volunteers (HV) were used as controls. An acute time point for sample collection was chosen as experimental studies have shown that robust oxidative damage and cell death occurs during this period. Our results indicate that both mild and severe TBI cause significant reductions in plasma methionine levels. A decrease in the transmethylation product SAM was observed in sTBI patients, as were the plasma levels of choline, betaine, and dimethylglycine. The levels of the transsulfuration metabolite cysteine, as well as the gammaglutamyl cycle metabolites (gamma-glutamyl amino acids and 5-oxoproline), were also found to be reduced after sTBI. Taken together, these results demonstrate that TBI decreases methionine and its key metabolites, which may alter the function of multiple organs, and suggest that supplementation of methionine metabolites may be beneficial for sTBI patients.

MATERIALS AND METHODS

Recruitment of Study Subjects

The University of Texas Health Science Center at Houston Committee for the Protection of Human Subjects approved the human subject protocol in accordance with the Declaration of Helsinki. In total, 60 subjects were recruited and provided written informed consented for participation in this study. All subjects were between 14-57 years of age and provided consent or proxy consent for their participation in this study. None of the subjects had drug dependency, or had active infections. Twenty sTBI (GCS < 8), 20 mTBI (GCS > 12), and 20 HV were recruited for this study. mTBI had no abnormalities on head computed tomography (CT) scans, but experienced one or more of the following: loss of consciousness, post-traumatic amnesia, altered mental status, neurologic deficits, or seizure. Demographic and clinical information on the study subjects is provided in **Table 1**. The predominant causes of all injuries were motor vehicle accidents and falls.

Sample Collection and Analysis

Seventeen of the 20 HV did not eat after midnight, the night before sample collection (at least 12 h before sample collection). The mTBI subjects had a last recorded meal that was 7.5 ± 4.4 h prior to sample collection. sTBI samples were collected 15.07 ± 5.0 h after the time of their injury. Blood samples were obtained within the first 24 h after injury and were coded to protect anonymity. Samples were collected in potassium EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA), placed on ice, and processed within an hour of draw. Plasma was isolated by centrifugation at 4°C as described by

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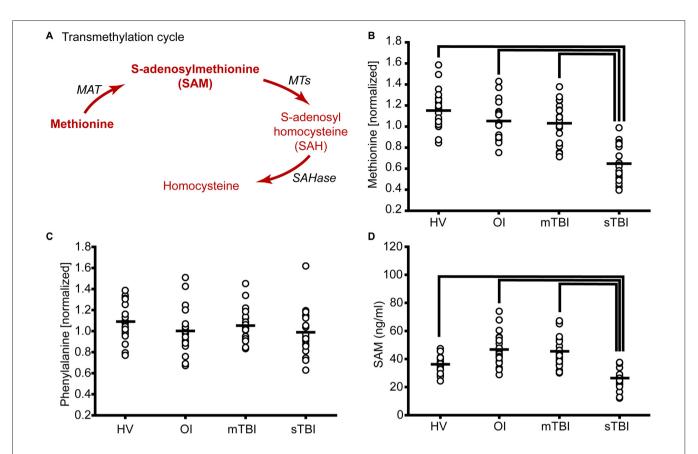


FIGURE 1 | Severe traumatic brain injury (sTBI) reduces plasma methionine and metabolism through the transmethylation pathway. (A) Schematic showing the metabolism of methionine via the transmethylation pathway. Molecules detected and measured are presented in **bold** text. Key enzymes are indicated by *italic* text. The plasma levels of (B) methionine were significantly reduced as a result of TBI. (C) S-adenosylmethionine (SAM) was significantly reduced in sTBI compared to healthy volunteers (HV) and patients with a mild TBI (mTBI). When the data were segregated by gender, similar changes in both (D) methionine and (E) SAM were observed in males and females. MAT: methionine adenosyltransferase; MTs: methyltransferases; SAHase: S-adenosyl-L-homocysteine hydrolase. Horizontal bar indicates mean. *p < 0.05.

TABLE 1 | Demographic and clinical data for study subjects.

Group	Healthy volunteers	Mild TBI	Severe TBI
Number of subjects	20	20	20
GCS (24 h of injury)	NA	14.85 ± 0.37	3.65 ± 1.2 (intubated)
Injury Severity Score	NA	5.4 ± 2.4	27.5 ± 8.2
Age (years)	25.2 ± 6.7	36.1 ± 13.3	25.8 ± 9.8
Female/Male	4/16	6/14	4/16
Hispanic ethnicity	6	3	4
Race			
White	16	18	16
African American	3	2	3
Asian	1	0	1

TBI, traumatic brain injury; values in mean \pm standard deviation.

the vendor. Aliquots were prepared and frozen at -80° C until needed. Plasma was processed by Metabolon, Inc. (Durham, NC, USA) using a proprietary series of extractions designed to increase the sensitivity of small molecule detection. Samples were placed briefly on a TurboVap® (Caliper Technologies Corp., Hopkinton, MA, USA) to remove any organic solvent. Each

sample was then frozen and dried under vacuum. The samples were analyzed by liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS) depending on the analyte being interrogated. Methionine and its metabolites were identified by comparison to purified standards. A selection of quality control compounds was added to every sample. Relative levels of each metabolite were quantified using Metabolon's proprietary peak integration software. The integrated peak values from all subjects were averaged and used for normalizing the values for each individual subject.

Enzyme-Linked Immunosorbent Assays (ELISAs)

SAM was measured using a competitive ELISA as described by the vendor (BioVendor, Asheville, NC, USA). A standard curve for calculating the abundance of SAM was generated by serial dilution of a purified standard. The range of the standards was based on the vendors' instructions. Standards and plasma samples (50 $\,\mu$ l) were added to a 96-well plate containing immobilized antibodies specific to SAM. Biotinylated SAM was immediately added to the well, after which the plate

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was incubated at 37° C for 1 h. After extensive washing, a streptavidin-horseradish peroxidase conjugate was added and incubated for 30 min. The plate was washed, and developed using tetramethylbenzidine (TMB). The reaction was terminated by the addition of 2N sulfuric acid. The optical density was measured using a microplate reader at 450 nm. Concentration of SAM in the plasma was calculated using a 4-parameter logistic curve.

Statistical Analysis

Data was initially evaluated using a Shapiro-Wilk normality test, followed by a one-way analysis of variance (ANOVA) across the four subject groups. Any data found to not have a normal distribution was analyzed using a Kruskal-Wallis ANOVA on ranks. Groups with differences were identified using a Dunn's pairwise comparison as the *post hoc* test. Differences were considered significant at p < 0.05, with groups with altered levels identified using critical p-values calculated after compensation for multiple comparisons.

RESULTS

Methionine is metabolized by two primary metabolic cycles: the transmethylation pathway to generate SAM and homocysteine (Figure 1A); and the transsulfuration cycle to generate glutathione (Figures 3A, 4A). While not all individual metabolites of these cycles could be detected and quantified, intermediate metabolites for all three cycles were detected. Based on the changes in the levels of these intermediate metabolites, alterations in the flux of these three pathways can be inferred.

The Methionine (Transmethylation) Cycle

The transmethylation cycle involves the metabolism of methionine to generate SAM, S-adenosylhomocysteine (SAH), and homocysteine (Figure 1A). When the relative plasma levels of methionine were measured in patients with either sTBI (GCS < 8) or mTBI (GCS > 12) and compared to HV, a significant change across the groups was detected (F = 44.01, p < 0.001). Post hoc analysis revealed a significant reduction of plasma methionine in both mild and severe TBI patients relative to HV, with greater reductions detected in the sTBI group (Figure 1B). In contrast, the relative levels of phenylalanine, a second essential amino acid, were found to be unchanged across all three groups (F = 1.82, p = 0.17). Methionine is converted to SAM by L-methionine S-adenosyltransferase (MAT). Although SAM was not detected by LC/GC-MS, we used a competitive ELISA to measure the levels of this important methyl donor. Figure 1C shows concentration of SAM in individual samples. When analyzed by a one-way ANOVA on Ranks, a significant decrease (H = 29.44, p < 0.001) in plasma SAM levels was detected in the sTBI patients compared to the other groups. As it has been reported that there are gender differences in amino acid metabolism (Lamont et al., 2003), we next parceled the methionine and SAM results to examine sex-related differences. As TBI occurs predominately in males (4:1 male to female ratio), our recruited study subjects followed this distribution resulting in 4-6 females in each of our study groups (Table 1). However, even with this small number of females, we observed that the changes in methionine appear to be independent of gender with both males and females showing significant decreases ($F=17.58,\,p<0.001$) in plasma methionine in the sTBI group (**Figure 1D**). However, due to the reduction in number of subjects in each group, the difference between the mTBI group and HV was no longer statistically significant. Plasma SAM ($F=9.70,\,p<0.001$) was also found to change similarly in males and females (**Figure 1E**), although more error-prone in the females due to the small number of study subjects. No significant differences between males and females within any group were detected.

A number of methyltransferases use SAM as a methyl donor for DNA, protein and lipid methylation and convert SAM to SAH (Cantoni, 1975; Grillo and Colombatto, 2008). SAH is then converted by SAH hydrolase to homocysteine, which can be used as a substrate to regenerate methionine (Figure 2A). In this pathway, choline is metabolized to betaine, which then acts as the methyl donor for the methylation of homocysteine by betaine homocysteine methyltransferase (BHMT). During this reaction, homocysteine is converted to methionine and dimethylglycine. Figure 2B shows the relative levels of choline in the plasma samples of individual subjects from all three groups. Plasma choline levels in the sTBI group were found to be significantly reduced as compared to the other groups (H = 18.64; p < 0.001). Betaine (trimethylglycine) is synthesized from choline via a multistep enzymatic pathway. The plasma levels of betaine were found to be significantly reduced (F = 8.42; p < 0.001) in the sTBI patients relative to the HV and mTBI group (Figure 2C). When the plasma levels of dimethylglycine were interrogated, a significant decrease (H = 8.76 p = 0.013) in its plasma levels were detected in the samples from sTBI patients compared to HV, but not the mTBI group (Figure 2D). Taken together, the decreased levels of these metabolites indicated decreased flux through the transmethylation pathway.

The Transsulfuration Pathway

Transsulfuration of homocysteine is essentially an irreversible reaction that generates cystathionine (via cystathionine β-synthase), which is further metabolized by cystathionine gamma-ligase to produce cysteine and alpha-ketobutyrate. Alpha-ketobutyrate is then reduced by α-hydroxybutyrate dehydrogenase to generate 2-hydroxybutyrate (**Figure 3A**). Although the relative levels of both α-ketobutyrate (**Figure 3B**; H = 22.05, p < 0.001) and 2-hydroxybutyrate (**Figure 3C**; H = 28.65, p < 0.001) were found to be significantly increased in the plasma of sTBI patients, cysteine levels showed a significant reduction (**Figure 3D**; F = 20.23, p < 0.001). Interestingly, the levels of cysteine in the plasma of mTBI was found to be significantly increased relative to HV.

Glutamate cysteine ligase (GCL) ligates cysteine with glutamate to generate gamma-glutamylcysteine that is then combined with glycine by glutathione synthase (GS) to generate glutathione (**Figure 4A**). Although the relative plasma levels of glutamate did not significantly change across groups (**Figure 4B**; H = 2.21, p = 0.331), the plasma levels of glycine were found to

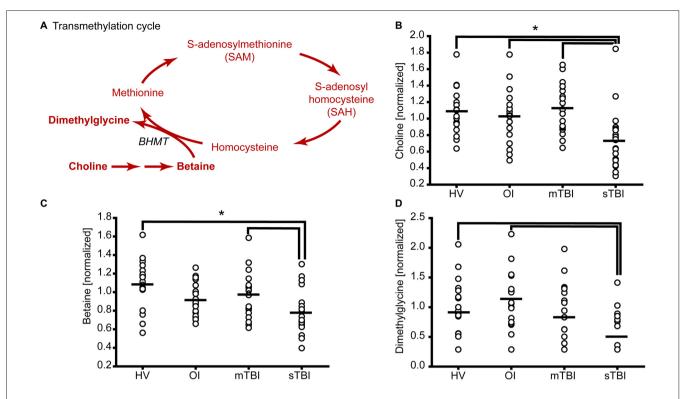


FIGURE 2 | sTBI reduces the levels of methyl donors for methionine remethylation. (A) Schematic showing the methylation of homocysteine to generate methionine. Molecules detected and measured are presented in **bold** text. Key enzymes are indicated by *italic* text. The plasma levels of the methyl donors (B) choline and (C) betaine used in methionine remethylation were significantly reduced in sTBI. (D) The side-product of methionine regeneration, dimethylglycine, is significantly reduced as a result of sTBI. BHMT: betaine-homocysteine S-methyltransferase. Horizontal bar indicates mean. *p < 0.05.

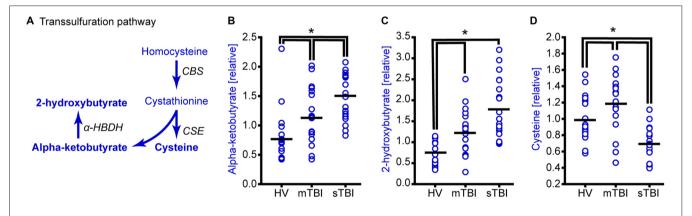


FIGURE 3 | TBI alters methionine metabolism through the transsulfuration pathway. (A) Drawing of the transsulfuration pathway in which homocysteine is converted to cysteine and alpha-ketobutyrate. Alpha-ketobutyrate is reduced to generate 2-hydroxybutyrate. Molecules detected and measured in the current study are presented in **bold** text. Key enzymes are indicated by *italic* text. Although the plasma levels of both (B) alpha-ketobutyrate and (C) 2-hydroxybutyrate were significantly elevated in the plasma of sTBI patients compared to other groups, the plasma levels of (D) cysteine were significantly decreased in sTBI patients. In contrast, the levels of cysteine were significantly increased in the mTBI group. HV: healthy volunteers; mTBI: mild TBI patients; CBS: cystathionine-β-synthase; CSE: cystathionine γ -lyase; α -HBDH: alpha-hydroxybutyrate dehydrogenase. Horizontal bar indicates mean. *p < 0.05.

be significantly reduced in both the mild and severe TBI patients (**Figure 4C**; F = 27.61, p < 0.001).

The Gamma-Glutamyl Cycle

A decrease in cysteine and glycine would be expected to result in a decrease in the levels of glutathione. Unfortunately, plasma

glutathione could not be detected by our mass spectrometry analysis. In addition to its role in the reduction of oxidative stress, glutathione is used to facilitate the transport of amino acids into cells via the gamma-glutamyl cycle (Orlowski and Meister, 1970). This occurs via the conversion of amino acids to gamma-glutamyl amino acids by gamma-glutamyl transpeptidase (GGT).

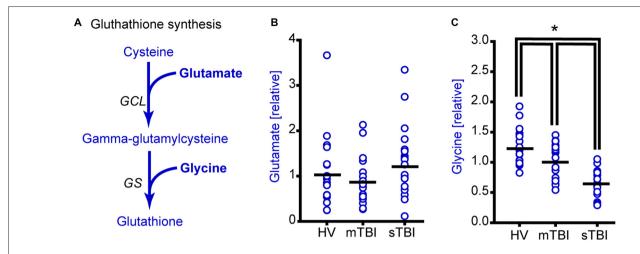


FIGURE 4 | Plasma glycine, but not glutamate levels are reduced as a result of sTBI. (A) Simplified drawing showing the production of glutathione from cysteine, glycine and glutamate. Glutamate cysteine ligase (GCL) catalyzes the reaction between glutamate and cysteine to produce gamma-glutamylcysteine. Glutathione synthase (GS) converts gamma-glutamylcysteine and glycine to generate glutathione. Molecules detected and measured in the current study are presented in **bold** text. Key enzymes are indicated by *italic* text. (B) Glutamate levels did not change across any of the subject groups. Horizontal bar indicates mean. (C) Plasma glycine levels in individual subjects from HV mTBI, and sTBI are shown. The levels of glycine are significantly reduced in the plasma of sTBI patients compared to all other groups. *p < 0.05.

The release of amino acids from gamma-glutamyl amino acids by gamma-glutamyl cyclotransferase (GGCT) generates 5-oxoproline as an intermediate (**Figure 5A**). Thus, a decrease in glutathione availability would be anticipated to result in a decrease in gamma-glutamyl amino acids and 5-oxoproline. The relative levels of gamma-glutamylvaline (**Figure 5B**; F = 13.54, p < 0.001), gamma-glutamylleucine (**Figure 5C**; H = 30.48, p < 0.001), gamma-glutamylisoleucine (H = 23.60, p < 0.001), gamma-glutamyltyrosine (H = 14.12, p < 0.001), and gamma-glutamylphenylalanine (F = 4.56, p = 0.015) were found to be significantly decreased in the plasma of sTBI patients. Consistent with the decrease in gamma-glutamyl amino acids, 5-oxoproline was found to be significantly reduced in the plasma of sTBI patients compared to other groups (**Figure 5D**; H = 17.66, p < 0.001).

DISCUSSION

The essential amino acid methionine not only acts as a building block for protein synthesis, but also serves as the substrate for the synthesis of key molecules such as SAM and glutathione. As such, changes in the levels of methionine metabolites could impact a number of biological processes such as epigenetic regulation of gene expression and cytoprotection. Our measurements of methionine and its metabolites in plasma samples from sTBI, mTBI, and HV revealed four key findings: (1) the relative plasma levels of methionine and SAM are significantly reduced in sTBI patients; (2) the levels of cysteine and glycine, the precursors for the synthesis of glutathione, are also reduced in sTBI patients; (3) in contrast to that observed in sTBI patients, the plasma levels of cysteine were

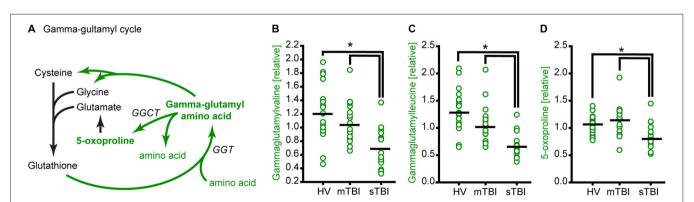


FIGURE 5 | TBI reduces glutathione recycling through the gamma-glutamyl cycle. (A) Simplified schematic showing the enzymes and intermediates for the gamma-glutamyl cycle. Molecules detected and measured in the current study are presented in **bold** text. Key enzymes are indicated by *italic* text. The levels of the gamma-glutamyl amino acids (B) gamma-glutamylvaline and (C) gamma-glutamylleucine, and the intermediate (D) 5-oxoproline are significantly reduced in the plasma of sTBI patients compared to HV, and mTBI groups. GGT: gamma-glutamyl transpeptidase; GGCT: gamma-glutamyl cyclotransferase. Horizontal bar indicates mean. *p < 0.05.

significantly elevated in mTBI; and (4) the relative levels of several gamma-glutamyl amino acids and 5-oxoproline are significantly reduced in the plasma of sTBI patients. These findings are summarized in **Figure 6**. Taken together, our findings suggest that the reduced availability of methionine, SAM and glutathione as a result of sTBI may alter multiple cellular processes including protein synthesis, epigenetic regulation of gene expression, cytoprotection, and the cellular transport of amino acids in multiple organs including the injured brain.

As methionine is an essential amino acid, its decrease in both mild and severe TBI patients could have resulted from a reduction in dietary intake. However, the time from last meal was comparable for the HV and sTBI groups, with all groups having not eaten within 7 h of sample collection. Thus, the changes in methionine levels we observed in TBI patients may not be solely due to lack of food intake. Previous clinical studies have shown that sTBI causes a nitrogen imbalance in which nitrogen excretion exceeds nitrogen uptake (Clifton et al., 1985), suggesting increased protein break-down. Attempts to restore nitrogen balance using enteral feeding found that replacement of 17 g N/day yielded a nitrogen balance of −9.2 g/day, whereas replacement of 29 g N/day restored nitrogen balance to -5 g/day (Clifton et al., 1985). Given that the human body requires up to 1.5 g protein/kg/day in order to maintain lean body mass and normal protein synthesis (Bistrian and Babineau, 1998), higher protein loads are likely required to preserve muscle mass. However, although enteral hyperalimentation can partially restore metabolic expenditure

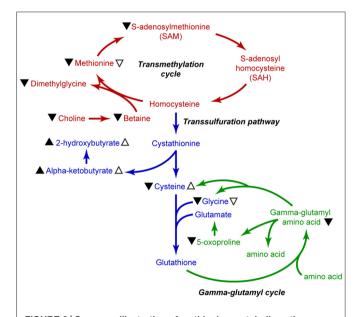


FIGURE 6 | Summary illustration of methionine metabolic pathways. Metabolites of the transmethylation pathway are indicated in red, the transsulfuration pathway is indicated in blue, and the gamma-glutamyl cycle is shown in green. Black arrowhead (Δ) indicates changes in metabolite levels in the plasma of sTBI patients. Open arrows (Δ) indicate significant changes measured in the mTBI patients as compared to HV. Up arrowheads indicate

increased levels, whereas down arrowheads indicate decreased levels.

in sTBI patients, feeding rarely achieves nitrogen balance resulting in weight loss of up to 15% by the second week after injury (Clifton et al., 1985). As the principal source of excreted nitrogen is urea, and urea is generated from the deamination of amino acids, this suggests that circulating amino acids levels should also be reduced in TBI patients. Consistent with this, it has been reported that sTBI patients have low levels of serum amino acids, including essential amino acids such as branched-chain amino acids (BCAAs), histidine, and methionine when measured 2 months after injury (Aquilani et al., 2000). In addition to chronic reductions in amino acid levels, the reduced levels of some amino acids have been seen as early as 24 h after TBI (Jeter et al., 2012, 2013). Irrespective of the mechanism by which methionine levels are reduced, decreased methionine availability would be expected to reduce its brain levels. In support of this, it has been demonstrated that the administration of intravenous amino acids results in an increase in their levels in the brain (Lajtha and Mela, 1961).

Only a few clinical studies have examined the consequences of amino acid supplementation after TBI. Ott et al. (1988) compared the effects of two different intravenous amino acid formulations on amino acid levels and nitrogen balance in sTBI patients. Their results indicated that positive nitrogen balance could be achieved with early administration of some combinations of amino acids, although neither the specific amino acids responsible for this improvement, nor their effect on outcome was assessed (Ott et al., 1988). BCAAs play important roles in regulating protein synthesis, gluconeogenesis, and energy metabolism as well as functioning as a major source of nitrogen for producing glutamine and nitric oxide (Fernstrom, 2005; Jeter et al., 2013). Experimental studies have shown that dietary supplementation of BCAAs improves cognitive function and can reduce injury-associated sleep disturbances (Lim et al., 2013; Elkind et al., 2015). Aquilani et al. (2000) tested if supplementation of BCAAs for 15 days during rehabilitation (average time from injury: 2 months) improved outcome, and found that patients receiving BCAAs had improved cognitive function as indicated by a reduction in their Disability Rating Scale (DRS) scores. Nägeli et al. (2014) tested if inclusion of an alanine-glutamine dipeptide as a part of normal feeding (initiated 3 days after injury) could be used to increase plasma and brain glutamine levels without corresponding increases in cerebral glutamate. Although alanine and glutamine levels in the brain were found to be increased in the absence of a net increase in cerebral glutamate, the benefit of this treatment on outcome was not evaluated (Nägeli et al., 2014). At present, it has not been determined if supplementation of methionine would improve outcome in sTBI patients. However, in an animal model of TBI, administration of methionine was found to increase neuronal apoptosis via yet unidentified mechanisms (Akkaya et al., 2014). As we observed that methionine and a number of its metabolites were reduced as a result of TBI, supplementation of these or other intermediates may be beneficial and may reduce complications associated with methionine administration.

SAM is a key methyl group donor for methylation of intracellular molecules including DNA, proteins and phospholipids (Grillo and Colombatto, 2008). A methyl group is transferred to these substrates by different methyl transferases. Methylation of these molecules regulates a number of important biological processes including epigenetic gene regulation (via DNA and/or histone methylation), axonal transport (via microtubule methylation) and plasma membrane integrity and signal (via phospholipid methylation; Cantoni, 1975; Hirata and Axelrod, 1980; Bhaumik et al., 2007; Bird, 2007). Second only to ATP, SAM is one of the most commonly used molecules in living organisms. In experimental models of TBI, changes in substrate methylation have been observed for histones and cellular DNA, and have been suggested to contribute to ongoing pathologies including reactive gliosis and impaired axonal regeneration, as well as contribute to the development of chronic neurodegeneration (Gao et al., 2006; Zhang et al., 2007; Portela and Esteller, 2010; Lardenoije et al., 2015). Several studies have evaluated the consequences of SAM supplementation to treat diseases such as depression, Alzheimer's disease, tauopathy, liver disease, and various cancers (Purohit et al., 2007; Lu et al., 2009; Papakostas, 2009; Lee et al., 2012; Montgomery et al., 2014). For example, Papakostas (2009) tested if SAM could be used as an adjunct therapy for depressed patients who do not respond to serotonin reuptake inhibitors (SSRIs). By comparison to patients maintained only on SSRIs, those whose received SAM supplementation had higher Hamilton Depression Rating Scale (HAM-D) response and remission rates (Papakostas, 2009), indicating a reduction in depression severity and an increase in wellness, respectively. As our measurements indicate a significant reduction in the relative levels of SAM in the plasma of sTBI patients, SAM supplementation may be useful to treat some of the behavioral problems seen in these patients.

As the brain has a high metabolic activity and high lipid content, it is particularly vulnerable to oxidative damage. Glutathione is the major antioxidant molecule, and a decrease in its levels can exacerbate brain damage. Although intracellular glutathione levels are relatively high, circulating levels of glutathione are low, due to its extremely short half-life (ranging from seconds to minutes; Wendel and Cikryt, 1980). This likely contributed to our inability to detect glutathione in our plasma samples. However, our findings that glutathione precursors cysteine and glycine were significantly reduced, as well as corresponding reductions in gamma-glutamyl amino acids, suggest a decrease in its levels. It is therefore anticipated that supplementation of precursors of glutathione may offer protection. Consistent with this, systemic administration of N-acetylcysteine, in combination with minocycline, improves cognitive function in brain injured animals (Haber et al., 2013).

Amino acids are thought to be transported into the cell via their conversion to gamma-glutamyl amino acids, a process dependent on glutathione (Orlowski and Meister, 1970). Gamma-glutamyl amino acids are then transported into the cytosol where they are processed by gamma-glutamylcyclotransferase to yield free amino acids and

5-oxoproline. As we observed decreased levels of several gamma-glutamyl amino acids, as well as 5-oxoproline, these findings suggest that intracellular transport of amino acids via the gamma-glutamyl cycle is decreased. These decreases, in conjunction with (or resulting from) decreased amino acid levels, could further reduce the intracellular availability of methionine and other amino acids

In addition to changes in methionine metabolism in sTBI patients, we found a modest, but significant, decrease in plasma methionine levels in mTBI as compared to HV (Figure 1). This suggests that a decrease in methionine following TBI may be related to injury severity. In addition to methionine, we also observed TBI severity related changes in the levels of alpha-ketobutyrate, 2-hydroxybutyrate (Figure 3) and glycine (Figure 4). Interestingly, cysteine levels were found to increase in the plasma of mTBI (Figure 3), whereas its levels decreased in sTBI patients as compared to HV. The reason for this divergence is not clear at present.

A number of weakness need to be considered when interpreting our results. As methionine and its metabolites were measured in the plasma, the source of these molecules cannot be ascertained. Additional weakness of the present study include that the activity and levels of the enzymes for methionine metabolism were not measured. Alterations in their activity, in conjunction with decreased methionine levels, could have given rise to the changes we observed. If supplementation of protein/methionine restores the levels of SAM and other metabolites, this would suggest that the metabolic enzymes of methionine metabolism are not altered. However, this is yet to be examined. Another weakness of our study is that the consequence of SAM reduction on the methylation of cellular proteins, DNA and phospholipids has not been determined. A study by Yi et al. (2000) has reported that an increase in plasma SAM levels is associated with hypomethylation of DNA in lymphocytes. This suggests that the reduced plasma SAM levels we measured in sTBI patients may result in the hypermethylation of DNA and subsequent suppression of gene expression. Finally, although we examined the influence of gender on methionine and SAM levels, the number of females in our study population was small. Thus, a larger group of females would need to be assessed to truly evaluate if there are gender differences in methionine metabolism after TBI.

In summary, our results show that sTBI patients have low levels of circulating methionine, and that methionine metabolites generated via the transmethylation and transsulfuration cycle are significantly reduced. In addition, flux through the gammaglutamyl cycle is decreased. As methionine and its metabolites are critical for a number of cellular functions and cytoprotection, decreases in their plasma levels may contribute to brain injury pathology. Alternatively, these biochemical changes may serve a protective function in the acute stage of injury, and their persistence may be detrimental. The systemic changes in methionine and metabolite levels we observed may not only influence the function and pathology of the

injured brain, but may alter the function of other organs as well.

AUTHOR CONTRIBUTIONS

PKD, GWH, CBJ, HAC, NK and ANM all contributed to either the conception, design, acquisition, analysis, or interpretation of data for the manuscript. Further, PKD, GWH, CBJ, HAC, NK and ANM all contributed to either drafting the manuscript or revising it for intellectual content. PKD, GWH, CBJ, HAC, NK and ANM have all approved the version of the manuscript to be published and agree to be accountable for all aspects of the work in ensuring that questions of the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Making Waves in the Brain: What Are Oscillations, and Why Modulating Them Makes Sense for Brain Injury

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Traumatic brain injury (TBI) can result in persistent cognitive, behavioral and emotional deficits. However, the vast majority of patients are not chronically hospitalized; rather they have to manage their disabilities once they are discharged to home. Promoting recovery to pre-injury level is important from a patient care as well as a societal perspective. Electrical neuromodulation is one approach that has shown promise in alleviating symptoms associated with neurological disorders such as in Parkinson's disease (PD) and epilepsy. Consistent with this perspective, both animal and clinical studies have revealed that TBI alters physiological oscillatory rhythms. More recently several studies demonstrated that low frequency stimulation improves cognitive outcome in models of TBI. Specifically, stimulation of the septohippocampal circuit in the theta frequency entrained oscillations and improved spatial learning following TBI. In order to evaluate the potential of electrical deep brain stimulation for clinical translation we review the basic neurophysiology of oscillations, their role in cognition and how they are changed post-TBI. Furthermore, we highlight several factors for future pre-clinical and clinical studies to consider, with the hope that it will promote a hypothesis driven approach to subsequent experimental designs and ultimately successful translation to improve outcome in patients with TBI.

Keywords: traumatic brain injury, electrical neuromodulation, deep brain stimulation, oscillations, hippocampus, theta

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INTRODUCTION

There are an estimated 3.8 million new traumatic brain injury (TBI) cases annually, and well over 5.3 million patients report chronic TBI-related deficits (Langlois et al., 2006; DeKosky et al., 2010). Ultimately, an estimated \$221 billion (combined acute and chronic care) is spent to treat TBI annually (Coronado et al., 2012). Critically, however, only 7% (\$14.6 billion) of the estimated \$221 billion is spent on direct medical costs (Coronado et al., 2012) and therefore, the vast majority of the financial burden is related to the long-term care of patients with chronic disabilities. In addition to the financial cost, there is a significant and well-documented emotional toll of caring for chronic TBI patients both on caregivers and society at large (Roozenbeek et al., 2013). Therefore, there is a critical need to develop innovative strategies to specifically address and improve the quality of life for patients with chronic disability following TBI. In the following review, we propose that oscillations observed in the electroencephalogram (EEG) play a key role in cognitive function

and that a TBI-induced change in oscillations can result in impaired behavioral function. Finally, we discuss the potential for electrical neurostimulation to improve chronic behavioral outcome in TBI patients.

A REVIEW OF THE IMMEDIATE EFFECTS OF TBI

The application of mechanical force on the brain initiates a complex series of interacting (sometimes non-monotonic) biochemical cascades, which, along with the initial impact, characterize TBI pathophysiology. Disruption of the cell membrane can lead to an ionic disturbance of Na⁺, K⁺, Ca²⁺, Mg²⁺, and Zn²⁺ (Vink et al., 1988; Katayama et al., 1990; Soares et al., 1992; Nilsson et al., 1993; Smith et al., 1993). The high concentration of K⁺ and Ca²⁺ in the extracellular space triggers release of neurotransmitters (e.g., glutamate), which can further exacerbate the ionic disturbance creating a vicious cycle (Faden et al., 1989; Katayama et al., 1990; Nilsson et al., 1990; Lyeth et al., 1993; Reeves et al., 1997; Shin and Dixon, 2015). This wave of depolarization can lead to excitotoxic cell death beyond what is observed in the injury core and surrounding penumbra (Sullivan et al., 1976; Dixon et al., 1987; Lowenstein et al., 1992; Hicks et al., 1993; Yamaguchi et al., 1996; Leonard et al., 1997; Yakovlev et al., 1997; Floyd et al., 2002; Witgen et al., 2005; Fedor et al., 2010). Moreover, there is considerable evidence that the pathophysiological release of neurotransmitters can alter the function of glutamatergic (Faden et al., 1989; Miller et al., 1990; Smith et al., 1993; Schwarzbach et al., 2006), cholinergic (Robinson et al., 1990; Yamamoto et al., 1993; Jiang et al., 1994; Lyeth et al., 1994; Delahunty et al., 1995; Shin and Dixon, 2015), GABAergic (Reeves et al., 1997; Witgen et al., 2005; Bonislawski et al., 2007; Gupta et al., 2012), and dopaminergic receptor systems (Donnemiller et al., 2000; Massucci et al., 2004; Shin et al., 2011), resulting in potential long-term cellular and circuit dysfunction independent of cell death.

One specific change related to excessive activation of the nervous system following TBI is the accumulation of intracellular calcium and the subsequent activation of calcium dependent catalytic enzymes such as calpain (Kampfl et al., 1997; Khorchid and Ikura, 2002). While hyperactivation of calpains is commonly associated with apoptosis (Patel et al., 1996), calpains also advance cytoskeletal and plasma membrane breakdown as well as disruption of Na⁺ channel function (Hicks et al., 1995; Folkerts et al., 1998; Saatman et al., 1998; Johnson et al., 2013). Changes in the cytoskeleton and membrane can trigger further ionic imbalance and specifically lead to high intraaxonal Ca²⁺ levels, which further challenge the already damaged axons resulting from the primary injury (Graham et al., 2000; Kita et al., 2000; Baker et al., 2002; Johnson et al., 2013; Li et al., 2014). Not surprisingly, for a prolonged period after the initial injury, neurons in the corrupted neural network can have impaired neurophysiological responses (Reeves et al., 1997; Golarai et al., 2001; Santhakumar et al., 2001; Kao et al., 2004; Goforth et al., 2011) including long-term potentiation (LTP; Miyazaki et al., 1992; Reeves et al., 1995; D'Ambrosio et al., 1998; Sick et al., 1998; Sanders et al., 2000; Schwarzbach et al., 2006; Li et al., 2014). In addition, TBI results in deficits impacting certain forms of behavioral plasticity (Ip et al., 2002; Griesbach et al., 2004) and formation of long term memories (Rimel et al., 1981; Leininger et al., 1990; Fedor et al., 2010; Gurkoff et al., 2013; Zhang et al., 2015). Disruption to cognition and plasticity following TBI is of particular relevance to our research interests and will be the focus of this review. Specific emphasis will be placed on how oscillatory activity contributes to information processing and how modifying injury- perturbed EEG could be relevant to reversing deficits in the clinical population. To this end we will first elaborate on what local field oscillations are and how they are generated in the brain.

DEFINING AN OSCILLATION

There is both growing evidence and excitement that and specifically invasive neuromodulation, neurostimulation, can be used to improve function in patients with neurological disorders (Lozano and Lipsman, 2013; Suthana and Fried, 2014; Tekriwal and Baltuch, 2015). In the case of TBI it is clear that functional consequences can be severe and persist for many years after the insult (Jennett et al., 1981; Whiteneck et al., 2004; DeKosky et al., 2010; Ponsford et al., 2014). And at least some of these cognitive and behavioral deficits could be mitigated with neurostimulation (Buzsáki and Watson, 2012; Lozano and Lipsman, 2013; Shin et al., 2014). Although the precise mechanism is still being delineated, it is hypothesized that driving specific neural circuits can entrain physiological circuit activity ultimately improving behavioral outcomes. Stemming from this, based on our recent findings we hypothesize that TBI-induced alterations in neural connectivity result in altered oscillations, as observed in the EEG. Further, we hypothesize that stimulating the injured nervous system to restore or substitute these oscillations will improve outcome. However, essential to the implementation and assessment of any intervention is the knowledge of the underlying mechanisms involved. Therefore, the next sections will summarize the basic neurophysiology associated with brain oscillations observed in the EEG and how these oscillations contribute to neural function. Critically this overview will introduce concepts from the perspective of developing research strategies to determine whether electrical neurostimulation can be used to improve cognitive outcome in TBI patients.

EEG is the measurement of change in the extracellular field potential recorded from the scalp that is generated by the sum of ionic movements across synapses, dendrites, soma, axons and the electroconductive cerebral spinal fluid. Similar activity measured from intracranial electrodes is commonly referred to as intracranial EEG (iEEG) or electrocorticography (ECoG). For simplicity, we will refer to all recordings, scalp and intracranial, as EEG for the remainder of this manuscript. The average of ionic movements within the immediate surrounding volume of an implanted electrode is referred to as the *local* field potential (LFP). There are many cellular actions that sum together to

contribute to the total change in the ionic balance measured by a depth electrode, such as synaptic activity, Ca²⁺ fluctuations, intrinsic currents and resonances, spike after-hyperpolarization, gap junctions and glial interaction (Berridge and Rapp, 1979; Buzsáki et al., 2012). The magnitude of the electric field detected in the EEG is related to the alignment of the electrode relative to the processes of cells in any given region (Buzsáki et al., 1986; Montgomery et al., 2009). Specifically, an electrode placed parallel to the dipole created by ionic movements will result in the highest amplitude recording (Kringelbach et al., 2007).

A synchronized and reoccurring change in ionic movements results in an oscillation that can be observed in the EEG. Oscillations may arise due to a variety of mechanisms, such alternating excitation-inhibition (or excitation-excitation or inhibition-inhibition) of neurons, pacemaker cells, resonance or subthreshold membrane oscillation (James et al., 1977; Buzsáki et al., 1983; McCormick and Bal, 1997; Marshall et al., 2002; Klausberger et al., 2003; Wang, 2010). There are multiple discrete oscillatory bands ranging from 0.05 to 500 Hz that have been operationally defined based on functional states of the brain (Klausberger et al., 2003; Penttonen and Buzsáki, 2003; Buzsáki and Watson, 2012). While the general structure of many oscillations is similar (e.g., alternating excitation-inhibition, pacemaker cell), granularly each rhythm is quite distinct from one other. How, when, and where an oscillation is generated defines its operation and contribution to information processing, and in the case of a brain injury, the pathophysiology of a disorder. Therefore, in order to understand how TBI might affect the generation or maintenance of oscillations, and how to develop and assess potential strategies to restore oscillations, it is critical to consider how mechanistically an oscillation is generated.

UNDERSTANDING HOW OSCILLATIONS ARE GENERATED

One of the earliest and most studied examples of oscillations observed in the EEG is from studies of sleep progression. For example, a defining characteristic of early non-REM (NREM) sleep is the presence of spindle waves, which are 1-3 s bursts of activity in the 7-14 Hz range every 3-10 s (Brown et al., 2012). To describe spindle generation it is important to consider both which brain regions as well as which specific cellular mechanisms are responsible for generating rhythmicity. Spindles arise due to the thalamic reticular nucleus (TRN) hyperpolarizing thalamocortical neurons with a rhythmic burst of inhibitory synaptic potentials (IPSPs; Avanzini et al., 1989; Bal et al., 1995a,b). This hyperpolarization leads to the activation of low-threshold T-type Ca2+ channels (I_T), which even at low, negative membrane potentials can generate an action potential. Subsequently thalamocortical neurons generate a burst of excitatory synaptic potentials (EPSPs) that activate the TRN as well as corticothalamic neurons giving rise to a spindle (Crunelli et al., 1989; Bal et al., 1995a,b). Convergence of excitatory input onto TRN activates low-threshold Ca²⁺ channels, which send prolonged IPSPs back to thalamocortical neurons starting the oscillatory cycle anew (Steriade and Deschenes, 1984; Avanzini et al., 1989; McCormick and Bal, 1997). Thus, the time to go through one full cycle prescribes the observed frequency of a spindle (Bal et al., 1995a).

Thalamocortical bursting activity gives rise to another dominant NREM sleep oscillation in the delta frequency band (0.5-4 Hz; McCormick and Bal, 1997; Brown et al., 2012). Unlike the spindle waves, delta oscillations are generated in a single cell by the interplay between ionic currents (Steriade et al., 1993b). Low-threshold Ca²⁺ bursting in thalamocortical neurons is followed by a hyperpolarizing overshoot. This de-inactivates I_T and opens the hyperpolarization-activated cation channel causing an h-current (Ih). Ih slowly depolarizes the cell towards the threshold for a Ca²⁺ spike by activating I_T. Depolarization past -65 mV and subsequently -35 mV inactivates I_T and deactivates Ih, respectively, and leads to an action potential (Crunelli et al., 1989; McCormick and Bal, 1997). Repolarization overshoots start the cycle again. However, it should be noted that other mechanisms have been proposed to account for the thalamocortical delta oscillation (Ball et al., 1977; Steriade et al., 1993a).

Specific to our understanding of oscillations during sleep it is easy to imagine how the precise activity of a series of receptor systems and the related interaction of ionic currents would be sensitive to the large ionic imbalance that follows TBI (Vink et al., 1988; Katayama et al., 1990; Soares et al., 1992; Nilsson et al., 1993; Smith et al., 1993). Consistent with this assertion, TBI is associated with sleep disturbances (Mathias and Alvaro, 2012) and specifically a decrease in delta power during NREM sleep for at least 12 weeks post injury (Parsons et al., 1997). Therefore, when considering how TBI alters oscillations and the potential for neurostimulation one has to determine not only which circuits and specific mechanisms are affected, but also when one needs to stimulate.

Our primary interest related to TBI and EEG is how injury may alter hippocampal oscillations and cognitive function. This interest is driven by a rich history in TBI-induced spatial learning deficits, deficits that we now know are concurrent with altered hippocampal oscillations (Fedor et al., 2010; Lee et al., 2013, 2015). Unlike the previously described oscillations, hippocampal theta (3-12 Hz), and specifically in CA1 is generated and maintained by the interaction of multiple rhythm generators as well as intrinsic membrane properties of hippocampal neurons that contribute to the detected rhythmic slow wave (Green and Arduini, 1954; Vanderwolf, 1969; Buzsáki et al., 1986; Kirk, 1998; Kocsis et al., 1999; Mormann et al., 2008; Montgomery et al., 2009; Colgin, 2013; Watrous et al., 2013). In the hippocampal CA1 subfield there are two well characterized dipoles of theta: the distal dendrites and soma (Figure 1). The first dipole, measured strongest near the hippocampal fissure, is attributed to layer 3 entorhinal cortex (EC) and CA3 subfield rhythmic excitation of distal dendrites of CA1 (Bland, 1986; Alonso and García-Austt, 1987; Konopacki et al., 1987; Kamondi et al., 1998; Kocsis et al., 1999). This dendritic depolarization co-occurs

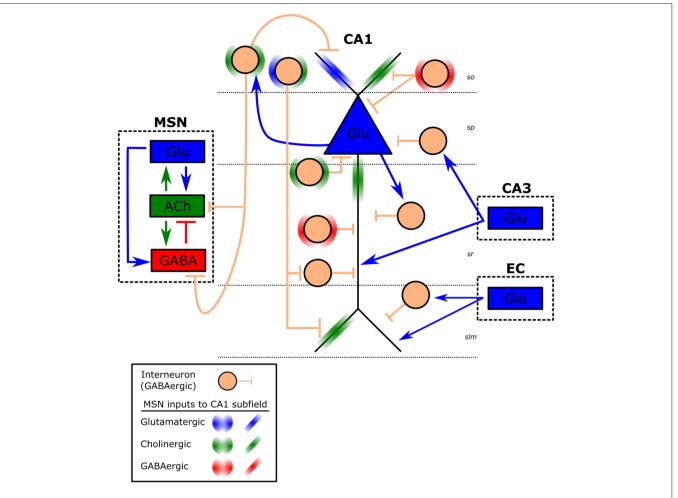


FIGURE 1 | Schematic of CA1 theta generators. Illustrated is a CA1 pyramidal cell (blue triangle) and hippocampal GABAergic interneurons (peach circle) within each CA1 layer. Approximate CA1 layers are indicated by dashed horizontal lines (so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; slm, stratum lacunosum-moleculare). Interneurons within each layer represent a subclass of interneurons (e.g., O-LM, PV basket, axo-axonic) for that layer, which receive different inputs and have distinct projections (e.g., back projecting). Dashed boxes represent CA1 inputs from medial septal nucleus (MSN), entorhinal cortex (EC) and the CA3 subfield. Arrows represent excitatory (blue-Glu: glutamate, green: ACh- acetylcholine), whereas short vertical lines are inhibitory (red and peach: GABA) connections. Projections from the MSN are left out for clarity and are represented by halos (circular on interneurons, linear on CA1 pyramidal cell).

with somatic hyperpolarization, which reflects inputs from the medial septum nucleus (MSN; Green and Arduini, 1954; Petsche et al., 1962; James et al., 1977; Bland, 1986; Vertes et al., 2004). The MSN consists of three types of neurons: GABAergic, cholinergic and glutamatergic. In fact, afferents from each of these neuronal subtypes play a role in the generation of the second dipole. Specifically, the interplay of phasic GABAergic inhibition, tonic cholinergic and glutamatergic excitation of hippocampal interneurons results in CA1 theta (Cole and Nicoll, 1984; Smythe et al., 1992; Tóth et al., 1997; Apartis et al., 1998; Wang, 2002; Hajszan et al., 2004; Colom et al., 2005; Vandecasteele et al., 2014; Fuhrmann et al., 2015). The septal GABAergic cells act as pacemakers of theta generation in CA1 pyramidal cells through disinhibiting hippocampal interneurons (Freund and Antal, 1988; Ylinen et al., 1995; Wang, 2002). In addition, MSN cholinergic and glutamatergic neurons directly modulate excitability in CA1 pyramidal cells, which

in turn excite back projecting hippocamposeptal interneurons completing the septohippocampal loop (Figure 1; Gaykema et al., 1991; Tóth and Freund, 1992; Tóth et al., 1993; Manseau et al., 2008; Mattis et al., 2014; Sun et al., 2014). This interplay between septohippocampal interneurons has the added effect of disinhibiting and inhibiting the soma of CA1 pyramidal neurons at the theta frequency, which can be measured at or dorsal to the pyramidal layer. Interestingly, hippocampal interneurons are vulnerable to cell death after TBI (Tóth et al., 1997b; Almeida-Suhett et al., 2015; Huusko et al., 2015). In addition there is evidence that injury can alter function in these neurons (O'Dell et al., 2000; Ross and Soltesz, 2000; Mtchedlishvili et al., 2010; Gupta et al., 2012; Almeida-Suhett et al., 2015; Drexel et al., 2015). Any change in interneuronal number or function could contribute to changes seen in the theta band post injury. While it is well accepted that TBI can result in cell death and dysfunction in interneurons in general,

in order to get a better understanding of the hippocampal pathophysiology it will be important for future studies to examine which specific classes of interneurons (**Figure 1**, e.g., O-LM, PV basket, axo-axonic) that contribute to CA1 theta generation are also affected by TBI (Klausberger et al., 2003).

These theta rhythm generators also work in concert with the intrinsic properties of hippocampal neurons. Specifically, there are intrinsic resonant and subthreshold membrane oscillating events which contribute to the hippocampal oscillations. For example, CA1 pyramidal cells have resonance (preferred frequency for maximal response) at theta frequency due to the interplay between voltage gated ionic currents. Depolarizations activate I_M (K⁺ current), which has the effect of hyperpolarizing the cell. Hyperpolarization activates I_h (mix Na⁺/K⁺ current), which brings the potential closer to spike threshold. With the addition of a persistent Na+ current (I_{NAP}) these currents oppose each other resulting in a membrane resonance in the theta frequency (Pike et al., 2000; Hu et al., 2002). This CA1 rhythmicity is further amplified with subthreshold membrane oscillations via persistent Na+ and Ca2+ currents (Leung and Yim, 1991; García-Muñoz et al., 1993; Fransén et al., 2004). In a similar vein, both EC cells projecting to the hippocampus (Alonso and Llinas, 1989; Alonso and Klink, 1993; Klink and Alonso, 1993; Dickson et al., 2000; Quilichini et al., 2010) and hippocampal inhibitory interneurons (Maccaferri and McBain, 1996; Chapman and Lacaille, 1999; Pike et al., 2000) have a natural resonance and subthreshold membrane oscillation in the theta frequency range due to a mix of voltage-sensitive Na⁺ and K⁺ currents. Furthermore, the MSN displays intrinsic bursting in the theta range (Vinogradova et al., 1980; Zhadina and Vinogradova, 1983). Thus, the magnitude of synaptically driven theta from the generators in the EC and septum is boosted by multiple intrinsic resonances from cells within the hippocampus as well as extrinsic to the hippocampal formation (Goutagny et al., 2009).

In summary, there are bands of oscillations starting as low as <0.1 Hz and ranging to as high as 600 Hz. Over the years we have operationally defined discrete ranges of oscillations (e.g., theta and gamma) based on specific cellular mechanisms as they relate to observed behavioral relationships. Research into individual oscillatory bands has revealed that mechanisms for the generation and maintenance of oscillations are complex and varied, with interactions of synaptic and intrinsic generators summing together to provide a single detected change in the LFP. However, it remains an open question to what extent TBI alters any, or perhaps all, of the specific mechanisms involved in the generation of individual oscillatory bands and ultimately the neural network underlying cognition.

HOW OSCILLATIONS INTERACT AND CONTRIBUTE TO INFORMATION PROCESSING

While each electrode yields a single LFP measure, that LFP is made up of several components. In fact, the combination

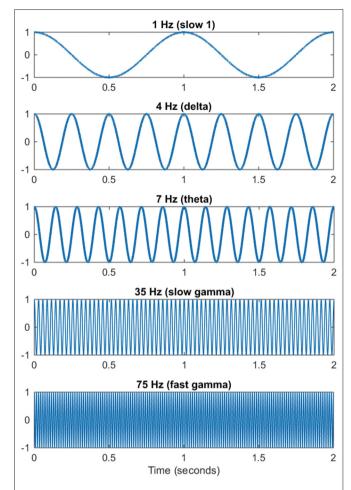


FIGURE 2 | Oscillations of varying frequency. MATLAB generated sine curves to represent neuronal oscillations that make up the unfiltered electroencephalogram (EEG). Each panel represents a distinct frequency and in parentheses the corresponding oscillatory band name most often associated with it.

of synaptic and intrinsic membrane events frequently leads to power in multiple oscillatory bands. Specifically, when one decomposes an individual LFP one can see that each oscillation occurs simultaneously (Figure 2). Figure 2 illustrates sinusoidal waves whose frequency corresponds to the slow, delta, theta and gamma oscillatory bands. A more exhaustive list of oscillatory frequencies was described by Penttonen and Buzsáki (2003). In general slow wave oscillations, and relevant to the current discussion those in the theta frequency range, are thought to synchronize distal regions of the brain promoting plasticity, while faster gamma oscillations are hypothesized to link and/or activate local neuronal ensembles (Bragin et al., 1995; Penttonen and Buzsáki, 2003; Buzsáki and Draguhn, 2004). There are several published reviews relating to a broader analysis of EEG and their role in plasticity and learning (Başar et al., 2001; Buzsáki, 2005; Lakatos et al., 2008; Knyazev, 2012; De Gennaro and Ferrara, 2003). However, based on the current level of understanding of these oscillations as they pertain to TBI is limited and therefore an in depth description of these

findings is beyond the scope of this review. But, if we want to understand the extent of the effect of TBI on oscillations, it is important to not only consider one specific frequency band at a single electrode, for example hippocampal theta, but instead consider how multiple frequency bands are related at a single recording site (i.e., cross frequency coupling), and also how similar frequency bands are related between distal electrodes (i.e., phase coherence). Thus, in order to better understand the effects of brain injury on EEG it will be necessary to sample from multiple regions within a circuit as well as to investigate a range of frequency interactions in addition to a power analysis.

Interactions that take place across different frequencies at a single recording site are referred to as cross frequency coupling. The interplay between two frequencies could take place across several different domains (Figure 3). For example, crossfrequency power-power coupling (amplitude-amplitude) occurs when the power of the low frequency oscillation dictates the power of the high frequency oscillation; cross-frequency phasephase coupling (n:m phase locking) refers to a fixed number of high frequency oscillations nested in each slower cycle; crossfrequency phase-frequency and phase-power coupling indicates that the frequency and power of the faster wave is modulated by the phase of the slower oscillation, respectively (Jensen and Colgin, 2007; Belluscio et al., 2012). For a more thorough review on the significance of each of these interactions as they relate to cognition see (Axmacher et al., 2006; Lisman and Buzsáki, 2008; Colgin, 2013; Lisman and Jensen, 2013).

Phase coherence is the relationship of two oscillations of the same frequency across different electrodes. For example, there can be phase-phase coupling of oscillations such that the phases of each ongoing oscillation are in sync (i.e., the peak of one oscillation always occurs in the same phase of a second oscillation measured at a second site). Likewise, two similar frequencies can correlate in their power, independent of the phase. Specifically, as the power of an oscillation increases at one recording site, a similar increase in power is observed at a second electrode. These types of specific interactions suggest that oscillations are not simply a local phenomenon but instead have a role in network activity.

In addition to phase coherence, oscillations can organize the firing of individual neurons by summing together subthreshold excitatory inputs or organizing the firing procession of assemblies of neurons (O'Keefe and Recce, 1993; Skaggs et al., 1996; Tukker et al., 2007). While there are different implications for the specific type of interaction observed, in general interaction between rhythms supports neural communication, plasticity, formation of functional ensembles and consolidation of long-term memories (Buzsáki and Draguhn, 2004; Fell and Axmacher, 2011; Belluscio et al., 2012). The common variable is that oscillatory patterns contribute to higher order information processing including the formation of neuronal ensembles.

Ensemble formation, or the linking of a group of cells, is at the heart of information processing (Fries, 2005; Buzsáki, 2010). Oscillations are capable of promoting ensembles partly through temporally precise segregation and boosting of

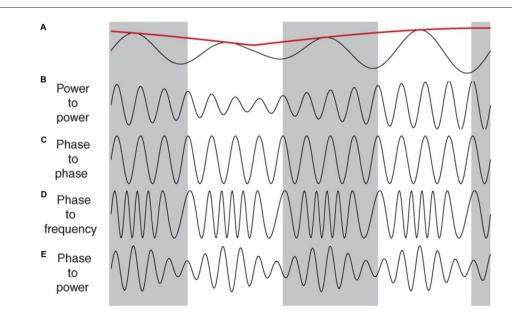


FIGURE 3 | Illustrations of cross-frequency coupling. (A) A slow oscillation in the theta range, along with its power indicated by the red line. (B–E) Illustrate the different types of interplay that faster oscillations (e.g., gamma) can have with the slower trace in (A). (B) Power–power: the power (amplitude) of the faster wave correlates with the power of the slower wave. (C) Phase–phase: a fixed number of faster cycles within each phase of the slower oscillation. In this case there are four cycles within each phase. (D) Phase–frequency: the number of cycles in the faster wave correlates with specific phase of the slower wave. (E) Phase–power: the power of the faster wave correlates with the specific phase of the slower wave. Reproduced with permission from Elsevier (Jensen and Colgin, 2007).

communication between some groups of neurons. Theta-gamma phase modulation (cross frequency phase-amplitude coupling) can explain how one region of the hippocampus, say CA1, can be involved in multiple networks virtually simultaneously. For instance, CA3-CA1 shows the greatest coherence and phase locking of single unit firing in the slow gamma band (gammaS: 25-50 Hz), which peaks in the early descending phase of the CA1 theta. On the other hand, medial EC (MEC)-CA1 has high coherence at mid gamma frequency (gammaM 50-90 Hz) which is strongest at the peak of theta (Colgin et al., 2009; Belluscio et al., 2012; Schomburg et al., 2014). Thus, CA1 inputs are segregated across the phase of theta cycle and therefore individual pyramidal neurons have the potential to temporally align with multiple ensembles within a phase of theta (for detailed discussion, see Buzsáki and Schomburg, 2015). Alternating back and forth between functional networks could be important, as an example, for shifting between encoding new and retrieval of previous information (Montgomery and Buzsáki, 2007; Colgin et al., 2009), assembling discrete stimuli into a single representation (Gray et al., 1989; Engel et al., 1991), selective attention/gain modulation (Fries et al., 2001) and associative binding (Headley and Paré, 2013). Critically, in many cases theta provides the temporal structure for local gamma, while simultaneously coupling cell assemblies between regions and "chunking" (Buzsáki, 2010) information into discrete processing units (Senior et al., 2008; Buzsáki and Moser, 2013; Colgin, 2013). Given that theta is altered post-TBI (Fedor et al., 2010; Lee et al., 2013, 2015), it will be important for future studies to examine how TBI may affect these tightly coupled interactions and whether changes in these interactions might underlie injury-induced behavioral deficits.

Theta modulation can also strengthen synaptic connections and organize information flow. For instance, hippocampal theta phase locks local cortical gamma activity across multiple regions and the firing of individual cortical neurons (Sirota et al., 2003; Hyman et al., 2005; Jones and Wilson, 2005; Siapas et al., 2005; Fujisawa and Buzsáki, 2011). Convergence of cortical inputs onto the hippocampus coincides with a time (i.e., theta phase) that is optimized to support hippocampal synaptic plasticity (Berry and Thompson, 1978; Huerta and Lisman, 1995; Seager et al., 2002). In turn, local hippocampal plasticity is shaped by a difference in gamma phase-phase synchronization between subfields. During tonic REM sleep CA3-CA1 gamma coherence is decreased, while dentate gyrus-CA3 gamma is increased. This releases CA1 from CA3 recurrent collateral control and allows the dentate gyrus to modify CA3 synaptic activity. However, during brief interspersed periods of phasic REM theta and gamma coherence across all three subfields is increased and so is CA1 firing (Montgomery et al., 2008). Thus, it seems that CA1 is excluded until hippocampal information is transmitted back to the cortex (Buzsáki, 1989; Wilson and McNaughton, 1994; Ji and Wilson, 2007). Shifting between local cell assemblies ensures accurate transmission of information, discrete synaptic modifications free of interference and a receptive receiver to form an ensemble. Unfortunately, it is yet to be determined if these interactions are affected by TBI and if they are correlated with cognitive and behavioral deficits.

While to this point we have focused on theta, ensemble formation can be modulated at other frequencies. For example, during NREM sleep cortical slow oscillations (0.02-0.8 Hz) drive the cortex to alternate between a depolarized and a hyperpolarized (up/down) state (Steriade et al., 1993a; Cowan and Wilson, 1994; Timofeev et al., 2001). This slow oscillation also propagates to the thalamus and the hippocampus. A depolarized cortical state is associated with thalamacortical spindles which can bias high frequency CA1 burst activity, commonly referred to as sharp wave-ripples (Buzsáki et al., 1992; Battaglia et al., 2004; Mölle et al., 2006; Buzsáki and Silva, 2012). These ripples are synchronized to a particular phase of the spindles and drive the activation of specific cortical ensembles (Siapas and Wilson, 1998; Sirota et al., 2003; Isomura et al., 2006; Wierzynski et al., 2009). This hippocampocortical interplay may bind hippocampal output with coactive cortical ensembles. Together, these interactions have the added effect of associating two different networks in the spirit of coordinating information storage and promoting formation of long term memories through reciprocal excitation of ensembles. Ultimately, depending on the effect of TBI these ensembles could be as important of targets as those organized by theta oscillations.

It is important to note that the very functions ascribed to oscillations are perturbed in TBI patients. On a variety of neuropsychological exams patients score worse on tests of attention, concentration, working memory, reaction time, judgement and measures of effort (Rimel et al., 1981; Levin et al., 1988a; McDowell et al., 1997; Bales et al., 2009). These failures of information processing consolidate into deficiencies in verbal and visual memory, episodic memory, multitasking, executive function and cognition (Levin et al., 1988b; Hanks et al., 1999; Millis et al., 2001; Alvarez et al., 2003; Bales et al., 2009; Bootes and Chapparo, 2010; McCauley et al., 2014; Mäki-Marttunen et al., 2015). Deficits in higher order information processing are compounded by sleep-wake disturbances (Kempf et al., 2010; Shay et al., 2014; Skopin et al., 2015). TBI patients report a range of sleep-related disorders including difficulty falling asleep, more nighttime awakenings and daytime naps, increased fatigue, and prolonged sleep (Parcell et al., 2006; Kempf et al., 2010; Mathias and Alvaro, 2012). These sleep deficits are accompanied by altered REM and NREM sleep as detected by nighttime scalp recordings, as well as changes in nocturnal hormone secretion (Parsons et al., 1997; Frieboes et al., 1999). Given the overlap between functions associated with oscillations and observed deficits in TBI patients, additional research is critical to understand whether dysfunction can be ascribed to an alteration in oscillations. Moreover, if there is a relationship between oscillations and outcome in brain injured patients treatments aimed at modulating EEG are seemingly an appropriate starting point.

ALTERED EEG AFTER TBI

There are data that clearly indicate that TBI alters oscillations both in pre-clinical models as well as in patients (for detailed

table on altered EEG, see Thatcher et al., 1989; Wallace et al., 2001; Rapp et al., 2015). In rodent models, there is a pronounced decrease in alpha, beta, delta and theta power following mechanical injury (Dixon et al., 1987; Ishige et al., 1987; McIntosh et al., 1987; Paterno et al., 2016). While most of these reductions return to baseline levels within minutes to hours after the injury (Sullivan et al., 1976; Dixon et al., 1988; McIntosh et al., 1989), some, like diminished theta, can persists for as long as 8-10 weeks after the insult (Fedor et al., 2010). A prolonged decrease in theta power is accompanied by other neurophysiological irregularities, even in brain regions spared from significant cell death. One such area, the CA1 subfield, exhibits altered excitation and inhibition, reduced LTP and pathological spine anatomy days to weeks after injury (Reeves et al., 1997; Sick et al., 1998; Sanders et al., 2000; Schwarzbach et al., 2006).

Analysis of human TBI patients resembles the reported prolonged recovery of EEG in experimental models of TBI. While modifications in brain activity can be seen as early as 24 h even after a subconcussive head trauma (Johnson et al., 2014), altered EEG following TBI can last for years after injury (Thatcher et al., 1989; Alvarez et al., 2008; Kempf et al., 2010; Slobounov et al., 2012). The changes in the EEG are not confined to a single oscillatory band, as they have been reported for the alpha, beta, delta, theta and gamma range (Alvarez et al., 2008; Tomkins et al., 2011; Rapp et al., 2015). Alterations of EEG activity are not state dependent, as changes are observed when a patient is at rest (Virji-Babul et al., 2014; Borich et al., 2015), actively moving (Slobounov et al., 2012) and during sleep (Parsons et al., 1997; Frieboes et al., 1999). In fact, abnormalities in scalp EEG are so consistent in patients they have been used to differentiate between injured and non-injured subjects, classify the severity of the injury, and some suggest, predict long term outcome after TBI (Thatcher et al., 1989, 1991, 2001; Alvarez et al., 2003; Arciniegas, 2011). For example, one of the criteria used to diagnose mild TBI many months after injury is an increase in coherence and a decrease in phase offset between frontal and temporal lobes, along with a decrease in power between frontal and posterior cortical regions (Thatcher et al., 1989). Furthermore, reversal of pathological EEG power ratio with administration of a neurotrophic peptide correlated with improvement in attention and working memory (Alvarez et al., 2008). The persistence of an abnormal EEG after a head injury suggests a potential link to prolonged psychological symptoms.

As is clear from the previous sections, it is not rigorous enough to determine that EEG is altered following injury. Unfortunately for the patient, it is also unlikely that there is a single mechanism that can easily explain why the EEG has changed. Therefore, it is critical to identify a starting point for research. While many neural systems and processes may be affected by a head injury, of particular interest (in our laboratory) is hippocampal dysfunction and the generation/maintenance of theta. TBI alters hippocampal neurotransmitter systems involved in the generation of theta, including acetylcholine, glutamate and GABA (Saija et al., 1988; Faden et al., 1989; Katayama et al., 1990; Robinson et al., 1990; Marshall et al., 2002). Rapid and prolonged increases in neurotransmitter levels act on local

receptors causing long-lasting adaptation (Miller et al., 1990; Delahunty, 1992; Jiang et al., 1994; Delahunty et al., 1995; Schwarzbach et al., 2006). Thus, even after the injury-induced alteration of extracellular concentration of neurotransmitters returns to basal levels, modified receptors may have an ectopic response to subsequent activation, potentially affecting the timing and strength of receptor coupled processes essential to rhythm generation (Lyeth et al., 1992; Fineman et al., 1993; Kato et al., 2007; Marcoux et al., 2008). Another consequence of an intense glutamate discharge is excitotoxicity (Choi, 1988). This cell loss is readily observed in CA3 and dentate gyrus (Hicks et al., 1993; Floyd et al., 2002; Witgen et al., 2005), both of which are contributing nodes to CA1 theta (Bland, 1986; Kocsis et al., 1999; Marshall et al., 2002). Within the dentate gyrus, GABAergic interneurons in the hilus seem to be especially vulnerable to excitotoxicity, due to an increased excitatory drive onto their glutamatergic receptors (Tóth et al., 1997b; Hunt et al., 2011). Consequently, GABAergic cell death profoundly alters the excitability of not only the dentate gyrus, but the hippocampus as a whole leading to deficits in LTP and theta generation (Reeves et al., 1995, 1997; van den Pol et al., 1996; Witgen et al., 2005; Mtchedlishvili et al., 2010; Dinocourt et al., 2011). This hippocampal pathological process results in delayed degeneration in the MSN, a critical pacemaker for theta generation (Leonard et al., 1997). More specifically, cholinergic neurons within the septum show a marked susceptibility to cell death days to weeks following mild/moderate fluid percussion (Leonard et al., 1994; Schmidt and Grady, 1995) and a controlled cortical impact injury (Dixon et al., 1997). Such neuronal atrophy leads to enlarged ventricles and a proliferation of astrocytes, detected up to a year after TBI (Smith et al., 1997). Furthermore, downstream structures to the MSN, such as the hippocampus, show changes consistent with cholinergic hypofunction. In order to compensate for a decrease in evoked cholinergic neurotransmission (Dixon et al., 1996), there is an increase in the protein responsible for packing acetylcholine into presynaptic vesicles, downregulation of inhibitory autoreceptors and a hypersensitivity hippocampal cholinergic receptors and subsequent response of 2nd messengers (Jiang et al., 1994; Delahunty et al., 1995; Ciallella et al., 1998). MSN function could be further encumbered by post-traumatic epilepsy (Santhakumar et al., 2001; Frey, 2003; Pitkänen and McIntosh, 2006). Chronic seizure activity is related to a decrease (Garrido Sanabria et al., 2006) and altered firing of putative theta generating GABAergic cells in the septum (Colom et al., 2006). These observations have fueled the hypothesis that at least temporal lobe epilepsy in part arises due to an imbalance in septohippocampal theta and that theta stimulation could potentially be used as antiepileptic (Kitchigina et al., 2013; Fisher, 2015).

Degeneration and white matter damage also likely interfere with normal patterns of brain oscillations. Axonal abnormalities may arise from the initial shearing forces from the impact and an ionic imbalance in the extracellular space (Hicks et al., 1995; Graham et al., 2000; Li et al., 2014). Loss of ionic equilibrium leads to axonal increase of Ca²⁺ permeability, calpain activation, mitochondrial dysfunction and eventually breakdown of the cytoskeleton (Maxwell et al., 1997; Johnson et al., 2013). These

changes may culminate in deafferentation/denervation and inappropriate synaptic plasticity (Povlishock and Katz, 2005; Hunt et al., 2011). Many of these axonal changes can be detected weeks after the insult and correlate with behavioral abnormalities (Kempf et al., 2010; Spain et al., 2010). It is not surprising then that compromised axonal structure and function results in irregular oscillatory interactions, even years after the injury. These structural deformities along with neurochemical aberrations contribute significantly to the observed deficits in the propagation of functionally relevant hippocampal theta (Fedor et al., 2010), and subsequently brain function after TBI (Hanks et al., 1999; Millis et al., 2001). These data clearly indicate that multiple TBI-induced mechanisms can play a role in altered brain oscillations and their interactions thus contributing to long-term impaired cognition.

THETA DBS

Pathologies associated with TBI are wide-ranging, occurring at the molecular, physiological and structural level. These alterations in turn may lead to changes in network activity, affecting neural communication and plasticity. Abnormal rhythm generation could potentially hinder and prolong recovery after a TBI insult (Thatcher et al., 1991; Tomkins et al., 2011). Furthermore, once a patient has progressed out of the acute post-injury phase of the disease, neuroprotection is no longer a viable therapeutic option. Therefore, there is an urgency to develop treatment strategies for TBI patients who have chronic disability. DBS represents a potential intervention that can drive neural networks, improve neurophysiology and ultimately behavioral outcome in a subset of brain-injured patients. The advantage of neurostimulation, say over pharmacology, is its ability to target specific regions, inherent higher temporal resolution and ability to generate specific patterns of electrical input, all of which are critical factors in the generation and interaction of oscillations. Furthermore, neurostimulation has shown promise in alleviating symptoms in motor, cognitive, behavioral and psychiatric conditions (Brunoni et al., 2011; Lozano and Lipsman, 2013; Suthana and Fried, 2014). The relative success of DBS in each specific situation is determined by a growing body of parameters, beyond the scope of this article to survey (Kuncel and Grill, 2004; Butson and McIntyre, 2007; Birdno and Grill, 2008). Therefore, given the focus of this review we will highlight the potential use of one stimulation strategy, the use of low frequency stimulation within the theta range.

Exogenous induction of theta in structures like the hippocampus can improve cognitive processes in experimental animals. Hippocampal theta can be achieved either with direct hippocampal stimulation or by targeting afferent structures such as the fornix or MSN. Using rodent models, pre-training stimulation of MSN decreased the time to acquire discriminatory learning (Deupree et al., 1982) while post-training stimulation facilitated memory consolidation (Landfield, 1977; Wetzel et al., 1977). These results mirror the positive correlations observed between endogenous theta and enhanced acquisition and retention (Landfield et al., 1972; Berry and Thompson, 1978; Seager et al., 2002; Mandile et al., 2003;

Mitchell et al., 2008). Importantly, the uniqueness of these findings lies not in the MSN per se, but in the theta oscillation. The critical role of theta oscillations specifically was revealed in studies were theta stimulation of the fornix was able to drive hippocampal theta oscillations following chemical inactivation of the MSN (Green and Arduini, 1954; Petsche et al., 1962; Vertes et al., 2004). Not only did stimulation drive theta, but also rescued the behavioral impairment (McNaughton et al., 2006). Further substantiating the selectivity of theta range, high frequency MSN stimulation does not facilitate mnemonic processes (Landfield, 1977; Wetzel et al., 1977). Therefore hippocampal theta, generated endogenously or extrinsically, plays a critical role in neural computations supporting animal cognition.

The beneficial effects of stimulation in the theta range are not limited to cognitive processes. For example, low frequency stimulation has been shown to be beneficial after an acute spinal cord contusion where an 8 Hz stimulation of the raphe nucleus improved motor coordination and sensory processing, increased white matter integrity and reduced astrocytosis (Hentall and Burns, 2009; Hentall and Gonzalez, 2012). Therefore it is possible that stimulation of theta following TBI might also have indirect effects that could improve the hippocampal milieu postinjury facilitating anatomical as well as physiological recovery. Epilepsy treatment is also closely associated with low frequency stimulation. Effective reduction of kindling from a 60 Hz induced seizure is achieved with 3-5 Hz stimulation (Gaito et al., 1980; Kile et al., 2010; Koubeissi et al., 2013). Building on this framework, Fisher recently proposed a novel hypothesis that MSN stimulation in the theta range may benefit patients with epilepsy (Fisher, 2015). Therefore stimulation following severe TBI might also have the added benefit of reducing or preventing post-traumatic epilepsy.

THETA DBS IN TBI MODELS

Recently, several studies have described the restorative effect of theta stimulation after TBI injury. Lee et al. (2013, 2015) stimulated the MSN at 7.7 Hz and recorded an increase in hippocampal theta power along with better spatial performance in the Barnes maze following a moderate lateral fluid percussion injury (Figure 4). Several stimulation controls bolstered the hypothesis that theta band stimulation was specifically augmenting the septohippocampal system in TBI rats. In particular, successful MSN stimulation in the theta range was intensity specific, there was no effect on overall motor output (i.e., distance traveled) and MSN stimulation at 100 Hz did not rescue the deficit in spatial performance (i.e., spatial search strategy) in the maze (Lee et al., 2015). Moreover, the authors concluded the effect was restorative and not simply enhancing function as similarly stimulated sham animals experienced no improvement in spatial learning. Likewise, the Hentall group observed positive effects on spatial memory in the watermaze and forelimb reaching movements when, following lateral fluid percussion, they stimulated the raphe nucleus, part of the ascending system that generates theta, at 8 Hz (Vertes et al., 2004; Carballosa Gonzalez et al., 2013).

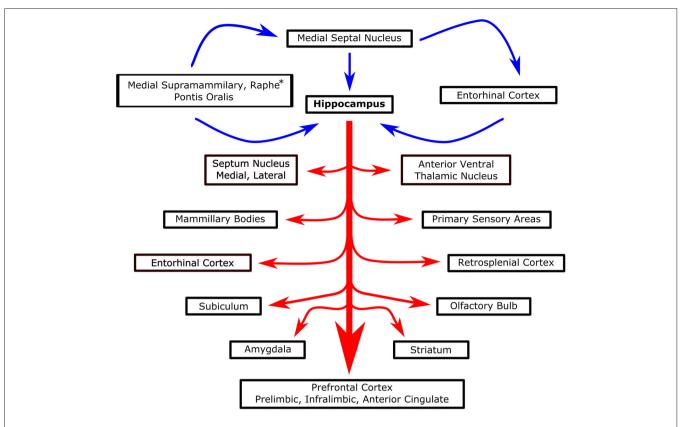


FIGURE 4 | Systems level overview of septohippocampal theta. Blue arrows represent proposed theta generators and essential modulators of hippocampal theta. Red arrows, stemming from the hippocampus, represent various structures that are known to be modulated/interact with hippocampal theta. *Raphe only projects to the hippocampus, not MSN.

In conjunction with behavioral outcomes, stimulation reversed cortical cell loss, white matter degeneration and decreases in cortical and hippocampal levels of cAMP, an intracellular second messenger (Carballosa Gonzalez et al., 2013). These proof of principle studies illustrate the potential of theta stimulation to augment physiological and behavioral outcome following TBI.

Importantly, theta stimulation has not been the only successful stimulation paradigm observed in experimental models of TBI. In a model of mild TBI, theta burst stimulation (TBS) was able to rescue working memory in the T-maze delayed non-match to sample task. Rather than using a continuous single pulse (7.7 or 8 Hz) fixed stimulation (Sweet et al., 2014) used a TBS protocol to stimulate the fornix, specifically with five 50 ms long bursts of high frequency (200 Hz) pulses per second. The hypothetical advantage of TBS as compared to continuous theta stimulation is that the 200 Hz gamma stimulation partially recapitulates endogenous patterned firing at a physiologically relevant theta frequency. In fact TBS has been demonstrated to induce long lasting LTP (Rose and Dunwiddie, 1986; Staubli and Lynch, 1987; Diamond et al., 1988; Kirkwood et al., 1993). Accordingly, Sweet et al. (2014) reported that TBS, but not low (5 Hz) or high (130 Hz) frequency stimulation of the fornix, improved performance of TBI rats in the T-maze. Spatial memory in the water maze was also improved with TBS; however, 5 Hz stimulation was not tested.

There are several key takeaways from these two successful stimulation paradigms. The first is that task might matter. There is evidence that, depending on task, there is a shift in the frequency of the theta oscillation (Kramis et al., 1975; Watrous et al., 2013). Therefore, the specific frequency within the theta range may be critical to improving outcome and the target may be different for different behaviors. Following that reasoning, while 5 Hz stimulation may not have improved T-maze performance, it is possible that 7.7 (or some other frequency) may have. In fact, in a study by McNaughton et al. (2006), it was observed that to optimally restore behavior in the watermaze after chemical inactivation of the MSN, it was best to stimulate the fornix with an endogenous EEG pattern recorded from the supramamillary nucleus as compared to fixed 7.7 Hz or an irregular theta stimulation pattern (with an average frequency of 7.7 Hz). These data indicate that not all "theta" is the same, and that the specific frequency within the theta band may be highly relevant.

The fact that these initial reports of DBS in TBI did not report completely homogenous results is worth noting. The data highlights the need for additional research to investigate the large parametric space available for potential

stimulation parameters. There are many additional variables to be considered: intermittent vs. constant stimulation; endogenous stimulation (where theta is recorded from an uninjured site and played back in the injured hippocampus as in McNaughton et al., 2006) vs. exogenous fixed frequency; theta burst vs. single pulse; variants in voltage, pulse width and square as compared to sinusoidal; which regions, nuclei or subfields to target; at which point during the task/behavior to stimulate or whether to stimulate offline during sleep or to stimulate relative to an endogenous oscillation independent of the behavior. A closed loop system where stimulation was based on the recorded EEG (from a different region) would subsequently be amenable to biofeedback (Wallace et al., 2001; Rosin et al., 2011; de Hemptinne et al., 2015).

Different stimulation parameters will not only influence the efficacy of the treatment, but also most likely the extent of unintended effects. The most commonly reported adverse events are related to the implant rather than stimulation and include inflammation, headache, pain at the implant site, and mild paresthesia surrounding the implant (Kenney et al., 2007; Fisher et al., 2010; Salanova et al., 2015). However, in studies of stimulation for treatment of epilepsy there are reports of cognitive dysfunction, depression and suicide in a small number of patients (Bergey et al., 2015; Salanova et al., 2015). Therefore it will be important to monitor which symptoms TBI patients report receiving low frequency stimulation of the septohippocampal system. While complications with the surgery and device itself are minimal, as argued by Fisher (2015) in his proposal to stimulate the MSN in epileptic patients, there are however potential risks of eliciting seizures or promoting addiction to constant stimulation (for discussion on long-term safety of DBS, see Kenney et al., 2007). These potential risks (e.g., prior epileptic activity, predisposition to addiction, mood/affect disorders) should be taken into consideration when enrolling patients so as to minimize potential harm. The inclusion criterion could be further refined based on the mechanism of action of neurostimulation in the theta range. If DBS is masking an enduring effect or if it is restorative will potentially influence the type of therapy one gets, such as; when should DBS be administered relative to the injury and in response to what type of injury? Will immediate intervention interfere with the healing process or will waiting too long make the system unamenable? How long will the benefits of DBS persist, if stimulation is discontinued? Should the treatment continue indefinitely or should there be a clinical marker/threshold to stop or augment the stimulation? Thus, there is a clear need for considerable pre-clinical animal work and potentially computational modeling to better understand and explore the complex parameter space that is DBS and the mechanisms behind it, if we are to optimize the potential of neurostimulation for clinical translation.

CONCLUSION

After years of research, there are few proven interventions that reduce injury-induced cellular cascades and ultimately, cell death and dysfunction following TBI. While the latest census estimates over 5.3 million patients live with chronic disability, it is clear that that number has grown and continues to grow. Therefore, there is a clear need for pre-clinical research expressly focused on the injured nervous system in the chronic stages of the disease. Oscillations are known to play a key role in physiological circuit function, whether it is the progression of oscillations through the sleep cycle or theta oscillations in the hippocampus. Initial evidence suggests that injury-induced disruption of these oscillations has a profound impact on neural connectivity and behavior. In fact, changes in EEG can be used as a biomarker to confirm mild and moderate TBI. Additionally, limited studies of DBS in brain injured rats demonstrate that the injured brain can be modulated by entraining or replacing oscillations, with improved outcomes. Future preclinical studies are needed to explore a very large parametric space that spans not only multiple stimulation targets and paradigms but also different injury mechanisms as well as a range of cognitive behavioral tasks and dependent measures, extending beyond spatial navigation. The potential for DBS is clear. We believe that further research into electrical neuromodulation of the injured brain will result in an exciting avenue to promote behavioral, cognitive and neurophysiological recovery following TBI.

AUTHOR CONTRIBUTIONS

AP, KS, and GGG contributed to the conception, writing and editing of the manuscript. AI, DJL contributed significantly to the conception and editing of the manuscript.

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Hippocampal Neurophysiologic Changes after Mild Traumatic Brain Injury and Potential Neuromodulation Treatment Approaches

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Traumatic brain injury (TBI) is the leading cause of death and disability in individuals below age 45, and five million Americans live with chronic disability as a result. Mild TBI (mTBI), defined as TBI in the absence of major imaging or histopathological defects, is responsible for a majority of cases. Despite the lack of overt morphological defects, victims of mTBI frequently suffer lasting cognitive deficits, memory difficulties, and behavioral disturbances. There is increasing evidence that cognitive and memory dysfunction is related to subtle physiological changes that occur in the hippocampus, and these impact both the phenotype of deficits observed and subsequent recovery. Therapeutic modulation of physiological activity by means of medications commonly used for other indications or brain stimulation may represent novel treatment approaches. This review summarizes the present body of knowledge regarding neurophysiologic changes that occur in the hippocampus after mTBI, as well as potential targets for therapeutic modulation of neurologic activity.

Keywords: hippocampus, mild traumatic brain injury, neurophysiology, neuromodulation, cognitive dysfunction, drug repositioning

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INTRODUCTION

Every year, one out of every 200 people worldwide will suffer a traumatic brain injury (TBI), including 1.7 million people in the United States alone, with an annual associated cost of approximately \$76.5 billion (Faul et al., 2010). It is estimated that 5.3 million Americans live with long-term disability as a result of their injury (Centers for Disease Control and Prevention [CDC], 2013). Young men and the elderly are particularly vulnerable because of an increased incidence of trauma and falls, respectively (Susman et al., 2002). TBI is also prevalent in the military, with approximately 20% of all returning deployed American troops suffering some form of brain injury from a blast exposure (Terrio et al., 2009), the vast majority of these being mild injuries.

Mild TBI (mTBI), sometimes termed concussion, accounts for 75% of all head injuries (Faul et al., 2010). Symptoms include headache, sleep impairment, memory deficit, difficulty concentrating (Levin et al., 1987), and an increased incidence of affective disorders such as depression and anxiety (Park et al., 2008). These clinical problems are persistent and severe in 10% of cases, lasting for months to years, sometimes indefinitely (Greig et al., 2014). Due to the striking prevalence and disruptive clinical manifestations of mTBI, research has focused on elucidating the pathophysiological mechanisms underlying primary and secondary

brain injury in order to develop therapies that may alter or prevent these processes. More recently, changes in neural circuitry underlying many of the clinical manifestations of mTBI, especially memory and affective disorders, has been studied in an attempt to understand mechanisms of disruption and how modulation of these networks may be used to therapeutic advantage. In order to determine how novel neuromodulation treatments might be able to improve TBI-associated deficits, it is important to establish how individual neurons and subpopulations of neurons contribute to network activity and how these networks respond to injury. This review will discuss neurophysiological changes in the hippocampus associated with mTBI, and ways in which therapeutic neuromodulation might be used to reverse these deficits.

BIOCHEMICAL AND GENETIC CHANGES IN THE HIPPOCAMPUS FOLLOWING TBI

Neurological injury that occurs after a traumatic insult can result from two different mechanisms: primary and secondary injury. Primary injury refers to the mechanical forces of shearing and compression at the time of impact, while secondary injury involves the subsequent cascade of pathological events that occurs minutes to days after the insult (Greve and Zink, 2009). Such secondary processes include hypoxia/ischemia, edema, raised intracranial pressure, excitotoxity through glutamate release, calcium dysregulation, cytoskeletal proteolysis, free radical oxidative damage, altered synaptic physiology, cytokine release causing inflammation, and ultimately neuronal cell death (Choi et al., 1987; Raghupathi, 2004; Maas et al., 2008; Barkhoudarian et al., 2011; Greig et al., 2014; Merlo et al., 2014). These secondary injury mechanisms are especially important for therapeutic consideration as they can theoretically be altered or inhibited with the aim of preventing injury or protecting brain tissue that has the potential to recover.

There are several commonalities between the biochemical cascades involved in secondary mTBI injury mechanisms and those found in degenerative central nervous system (CNS) diseases such as Parkinson's disease (PD; Eakin and Miller, 2012; van Bregt et al., 2012), Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral sclerosis (Daneshvar et al., 2011). As a result, pathways that have been extensively characterized in these diseases can be extrapolated and applied to analogous pathways involved in TBI in order to better understand the mechanisms of injury and potential novel therapeutic approaches. This has important implications for diagnosis: a complex mathematical model of semantic predications was recently used to search almost 100,000 citations of neural injury networks and produced a list of 17 potential biomarkers that may indicate mTBI, with glutamate, glucose, and lactate being the most common (Cairelli et al., 2015). In combination with other compounds, such as the apolipoprotein E-4 allele which adversely influences recovery following brain injury (Lawrence et al., 2015), these biomarkers may aid in the early diagnosis of mTBI, allowing the prompt administration of neuroprotective drugs or neuromodulatory therapies.

Glutamate is particularly important, as this excitatory neurotransmitter is known to play a strong role in secondary injury mechanisms throughout the brain. Microdialysis studies, in both rodents (Folkersma et al., 2011) and humans (Chamoun et al., 2010), have shown that glutamate concentrations increase in the acute phase following injury, whereas magnetic resonance spectroscopy (MRS) studies have shown a persistent decrease in glutamate concentrations following injury. The reason for this discrepancy is that microdialysis measures extracellular glutamate, while MRS measures both intra and extracellular glutamate (Guerriero et al., 2015).

Specifically in the hippocampus, glutamate decreases following moderate to severe temporal cortex injury, as measured by *in vivo* proton MRS in a direct cortical injury model in rodents (Harris et al., 2012). Although the hippocampus was considered perilesional tissue in this experiment, the temporal neocortex, i.e., the lesional tissue, also showed a decrease in glutamate in the acute phase. Human MRS studies also in the acute phase following concussion, however, showed no changes in hippocampal glutamate concentrations, despite finding decreased glutamate levels in the motor cortex (Henry et al., 2010). A reason for this discrepancy may be that the human study was in patients with mTBI, whereas the rodent study looked at moderate to severe TBI.

Another important compound altered in the hippocampus after TBI is the neurotransmitter gamma-aminobutyric acid (GABA). The expression of various GABA receptor subunits is changed, causing reductions of inhibitory postsynaptic currents and increases in excitatory post-synaptic currents after TBI in rodents (Hunt et al., 2011; Almeida-Suhett et al., 2015; Drexel et al., 2015). As a result, deficits occur in long-term potentiation, which can translate into cognitive impairment. Similarly, up and down-regulation of other proteins can lead to impairments in hippocampal synaptic transmission, including those involved in the extracellular signal-regulated kinase (ERK) pathways (Atkins et al., 2009), and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (Carlson et al., 2016).

The regulation of gene transcription affects not only the creation of proteins involved in neurotransmission after injury, but also the synthesis of neurons themselves. Because TBI is associated with hippocampal neuronal damage and death, it is important to develop therapeutic strategies to either protect immature neurons or enhance neurogenesis in the hippocampus after injury. Methods shown to drive neurogenesis post injury include forced overexpression of insulin-like growth factor 1 (IGF-1; Carlson et al., 2014), and the transplantation of synthetic human progenitor cells into the hippocampus after TBI (Blaya et al., 2015). Interestingly, TBI severity has been found to correlate with the degree of post-traumatic neurogenesis in rat hippocampus. Whereas mTBI had no effect on neurogenesis, moderate TBI promoted neural stem cell proliferation without increasing neurogenesis, and severe TBI increased neurogenesis (Wang et al., 2015a). A summary of the biochemical and genetic changes following TBI in rodent hippocampus can be found in Table 1.

TABLE 1 | Biochemical changes in rodent hippocampus following TBI, with effects categorized by subsection of the hippocampal complex.

Structure	Biochemical changes after TBI		
CA1	Decreased surface expression of α 1, β 2/3, and γ 2 subunits of the GABA _A receptor caused reductions in the frequency amplitude of spontaneous and miniature GABA _A -receptor mediated inhibitory postsynaptic currents after TBI in rats. This led deficits in long-term potentiation of synaptic transmission (Almeida-Suhett et al., 2015).		
CA1, CA3	Expression of GABA _A and GABA _B receptor subunit mRNAs α 4 (GCL, CA3, CA1), α 5 (CA1) and γ 2 (GCL, CA3, CA1) was up regulated after TBI in rats, with many of the changes being reversible (Drexel et al., 2015).		
CA1, dentate	Mechanical stimulation using a stretchable microelectrode array disrupted bicuculline (GABA _A antagonist) induced long-lasting network synchronization 24 h after TBI, despite the continued ability of injured neurons to fire (Kang et al., 2015).		
CA4	Action potential and excitatory post-synaptic current frequencies were increased in hilar GABA neurons after TBI in mice, with further increase observed after photostimulation of dentate granule cell or CA3 pyramidal cell layers (Hunt et al., 2011).		
Dentate	TBI severity affected hippocampal neurogenesis in rats: mild TBI did not affect neurogenesis; moderate TBI promoted neural stem cell proliferation without increasing neurogenesis; severe TBI increased neurogenesis (Wang et al., 2015a).		
	Survivin (apoptosis protein inhibitor) down-regulation inhibited adult hippocampal neurogenesis, promoted apoptotic cell death, and worsened memory capacity on water maze testing, after TBI in mice (Zhang et al., 2015).		
Entire hippocampal complex	Several microRNAs are significantly altered in hippocampal mitochondria and cytoplasm, including elevated levels of miR-155 and miR-223 (play a role in inflammation), after TBI in rats (Wang et al., 2015b).		
	Synthetic, human multineurotrophin neural progenitor cells (MNTS1-NPCs) conferred significant preservation of pericontusional host tissues and enhanced hippocampal neurogenesis after TBI in rats (Blaya et al., 2015).		
	Inhibition of population spikes was reduced in the Schaffer collateral pathway (CA3 to CA1) 2 days after TBI in rats, while increases in inhibition in the dentate gyrus (corresponding to increased GABA levels) was seen at both 2 and 15 days after injury (Reeves et al., 1997).		
	Decreased field excitatory post-synaptic potentials were recorded in hippocampal subfield CA1 in response to electrical stimulation of the Schaffer collaterals, following TBI in vitro, indicating that expression of long-term potentiation and synaptic plasticity was inhibited following TBI (Sick et al., 1998).		
	Gene transcriptome study identified upregulation of 193 transcripts and downregulation of 21 transcripts in the hippocampus, affecting mostly the transcription of non-neuronal genes, 24 h after mild TBI in mice (Samal et al., 2015).		
	Hippocampus nuclear factor of activated T cells (NFAT) c3 levels (expressed in astrocytes) were decreased both in the cytoplasmic and nuclear fractions, while NFATc4 levels (expressed in neurons) were increased in the cytoplasmic fraction but decreased in the nuclear fraction, after TBI in rats (Yan et al., 2014).		
	Insulin-like growth factor 1 (IGF-1) promotes neurogenesis after TBI in mice, in that overexpression of IGF-1 resulted in a marked increase in immature neuron density in the subgranular zone at 10 days after injury (Carlson et al., 2014).		
	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex was reduced in rat hippocampus following TBI, and resulted in a significant reduction in synaptic vesicle number (Carlson et al., 2016).		
	TBI in rats results in chronic signaling deficits through the extracellular signal-regulated kinase (ERK)—cAMP response element-binding protein (CREB) pathway in the hippocampus (Atkins et al., 2009).		
	Up-regulation of mRNA and protein for Nav1.6 (a voltage-gated sodium channels alpha subunit) occurred in rat hippocampal neurons after TBI (Mao et al., 2010).		
	Nuclear factor erythoid related factor 2 (NRF2, transcription factor) mRNA increased significantly post-TBI at 48 and 72 h and 1 week in the hippocampus with a coincident increase in glial fibrillary acidic protein mRNA, overlapping with heme-oxygenase-1, nicotinamide adenine dinucleotide phosphate-quinone-oxidoreductase 1, glutathione reductase, and catalase mRNA (Miller et al., 2014).		

CA, cornu ammonis; GABA, gamma-aminobutyric acid; GCL, granule cell layer; RNA, ribonucleic acid.

HIPPOCAMPUS BIOCHEMICAL PATHWAYS AND NEUROPROTECTIVE AGENTS IN mTBI

Since mechanisms of secondary injury after mTBI are similar to other diseases, it is likely that drugs that are already in use for other indications may have efficacy for mTBI as well. One example of such a mechanism is the glucagon-like peptide-1 (GLP-1) signaling pathway. GLP-1 was initially described based on its role in the pancreatic regulation of insulin, but receptors are also found in the CNS and changes in this pathway have been implicated in a number of neurodegenerative diseases (Li et al., 2009, 2010, 2012; Martin et al., 2009; Salcedo et al.,

2012). Through coupling to a cAMP second messenger pathway, activation of GLP-1 receptors elicits neurotrophic actions such as neuritic growth while protecting neurons against various insults (Perry et al., 2002). As a result, there have been a number of investigations into modulation of this pathway to treat neurological disease.

Exendin-4 is a synthetic GLP-1 receptor agonist currently approved as a drug for the treatment of hyperglycemia in type-2 diabetes that crosses the blood brain barrier (Kastin and Akerstrom, 2003). When administered after mTBI, this neuroprotective agent has been shown to prevent changes in rodent hippocampal gene expression following mTBI. Many of these genes protected by Exendin-4 are also associated with

memory loss in AD (which has been associated with TBI), and administration ameliorated memory deficits after injury (Tweedie et al., 2013). Additional rodent studies using markers of cell death have shown that Exedin-4 improved hydrogen peroxide mediated oxidative stress and glutamate toxicity in neuronal cultures *in vitro*. *In vivo*, using markers of cognitive function, the drug ameliorated mTBI-induced deficits in novel object recognition and maze testing, both when administered prior to and after the insult (Eakin et al., 2013; Rachmany et al., 2013). Therefore, data is emerging to support a shift in indication for the use of Exedin-4 from an antihyperglycemic medication to a potential agent to minimize secondary injury after mTBI.

Another agent with potential efficacy for secondary injury after mTBI is N-Acetyl-cystein (NAC), which is currently used as a mucolytic agent and an antidote to acetaminophen overdose. NAC has been shown to have antioxidant and neurovascularprotective effects after mTBI (Ellis et al., 1991; Hicdonmez et al., 2006; Chen et al., 2008) and has been demonstrated to reverse behavioral deficits in mTBI rodents when administered 30-60 min after injury (Eakin et al., 2014). Specifically in the hippocampus, NAC was found to decrease levels of cytosolicfree Ca2+ and reactive oxygen species, reduce apoptosis, and lower caspase-3 and -9 neuronal activities, following TBI in rats (Naziroğlu et al., 2014). In humans, NAC has been shown in a randomized double-blind trial to improve auditory, vestibular, and cognitive functional sequelae after blast-induced mTBI in military personnel (Hoffer et al., 2013). Although the mechanism of action in the trauma setting has yet to be elucidated, the 40 year safety profile and ease of administration of the medication makes it an attractive option for widespread use.

Numerous other compounds have been found to exert effects in the hippocampus following injury. As summarized in **Table 2**, different substances affect different subsections of the hippocampal complex. Although all these investigations were performed in rodents, several of these biochemical compounds may prove to have beneficial effects in humans suffering from cognitive and memory impairments following TBI.

HIPPOCAMPAL CIRCUITRY AND NEUROPHYSIOLOGIC CHANGES AFTER mTBI

In addition to biochemical changes after injury, changes in electrical neural activity also play a role in the sequelae of TBI. The high prevalence of memory deficits after injury point to a relative vulnerability of the hippocampus and mesial temporal structures as compared to the rest of the brain (Hamm et al., 1993; Finset et al., 1999; Comper et al., 2005), and electrophysiologic studies have shown that changes occur in hippocampal circuit excitability after TBI (Witgen et al., 2005; Ortiz and Gutiérrez, 2015). In addition, moderate and severe TBI is associated with neuronal cell loss in several hippocampal regions (Lowenstein et al., 1992) as well as alterations in cellular homeostasis (D'Ambrosio et al., 1999) and dysregulated synaptic transmission (Toth et al., 1997; D'Ambrosio et al., 1998) in this

region. On the other hand, mTBI has been associated with more subtle changes in firing patterns that are present even in the absence of morphological changes.

Studies comparing control rats to those who had undergone lateral fluid percussion induced mTBI found changes in working memory in the absence of differences in histology or neuronal loss (Eakin and Miller, 2012). Single unit recordings from cornu ammonis 1 (CA1) and CA3 subfields during a working memory task showed no differences in firing rates or spike characteristics between the two groups, but rats exposed to mTBI were found to have significantly fewer cells with spatiotemporal activity, and this correlated with task performance. Moreover, in a similar experiment looking at the burst characteristics of hippocampal cells during an object exploration task, memory deficits were found to be associated with decreased burst activity of certain subsets of neurons within the pyramidal cell layer (Munyon et al., 2014). Therefore, functional impairment may stem from an alteration in the activity of certain subpopulations of cells within the hippocampus.

These changes in neuronal firing patterns within the hippocampus can persist after TBI and correlate with changes in cognitive function (Witgen et al., 2005). In the analysis of local field potentials after injury, hippocampal broadband power decreased (Paterno et al., 2015), and specifically disruption of theta rhythm (4-7 Hz) has been implicated in the pathophysiology of memory loss (Winson, 1978; Fedor et al., 2010). It has been theorized that using electrical stimulation to artificially counteract and reverse these disrupted firing patterns within the hippocampus and other structures may regulate cellular and network processes and restore functional memory circuits (Diamond et al., 1988; Nakao et al., 2003; Shirvalkar et al., 2010; Tabansky et al., 2014; Hanell et al., 2015). Accordingly, theta burst stimulation (200 Hz in 50 ms trains, five trains per second; 60 mA biphasic pulses) delivered to the fornix of rats with mTBI improved deficits in learning and memory, whereas non-oscillatory low or high frequency stimulation did not (Sweet et al., 2014). In a related manner, theta band stimulation of the medial septal nucleus of mTBI rats resulted in a transient increase in hippocampal theta activity and improved spatial working memory (Lee et al., 2013), whereas 100 Hz gamma stimulation did not (Lee et al., 2015).

In humans, theta burst stimulation of the fornix has been studied in patients undergoing stereo-electroencephalography evaluation for drug-resistant epilepsy, and was shown to produce evoked potentials in the ipsilateral hippocampus. This resulted in significant and reversible improvements in immediate and delayed performance on a visual-spatial memory task (Miller et al., 2015). Temporally patterned stimulation paradigms may therefore present novel neuromodulatory strategies in the treatment of post-traumatic memory loss and cognitive deficits. Potential targets include the hippocampus, its associated white matter tracts, as well as the medial prefrontal cortex. The dysfunction of the latter is thought to play a role in the failure of emotion regulation after mTBI through top down amygdalar regulation (van der Horn et al., 2016), a mechanism that may result in persistent post-concussive symptoms.

TABLE 2 | Biochemical compounds investigated in rodent hippocampus following TBI, with effects categorized by subsection of the hippocampal complex.

Structure	Compound	Effects
CA1	WIN55, 212-2	WIN55,212-2 (synthetic cannabinoid) restored CA1 interneuron GABAergic signaling after TBI in mice (Johnson et al., 2014).
CA2, CA3	NT4/5	Recombinant Neurotrophin-4/5 (Trk-B ligand) increased survival of CA2/3 pyramidal neurons after TBI in rats, but did not improve functional outcome (Royo et al., 2007).
CA1, CA3, Dentate gyrus	MK-801	MK-801 (competitive NMDA receptor antagonist) ameliorated hippocampal neuronal loss after TBI in rats, and improved anxiety and hippocampus dependent memory (Sönmez et al., 2015).
Dentate gyrus	Rapamycin	Rapamycin (mTOR inhibitor) reduced dentate granule cell area, neurogenesis, and mossy fiber sprouting; increased recurrent excitation of dentate granule cells; and diminished seizure prevalence after TBI in rats (Butler et al., 2015).
	Ara-C	Ara-C (arabinofuranosyl cytidine, antimitotic agent) reduced progenitor cell proliferation and neurogenesis in the dentate gyrus, and completely abolished innate cognitive recovery on water maze performance, after TBI in rats (Sun et al., 2015).
	Clioquinol	Clioquinol (zinc chelator) reduced progenitor cell proliferation and neurogenesis after TBI in rats (Choi et al., 2014).
Entire hippocampal complex	DHF	DHF (7,8-dihydroxyflavone, brain derived neurotrophic factor imitator) increased the number of adult- born immature neurons in the hippocampus, and promoted their dendrite arborization in the injured brain following TBI in mice (Zhao et al., 2015).
	NAC + selenium	N-acetylcysteine (NAC) and selenium (antioxidants) decreased levels of cytosolic-free Ca ²⁺ , apoptosis, cytosolic reactive oxygen species levels, and caspase-3 and -9 activities in hippocampal neurons, after TBI in rats (Naziroğlu et al., 2014).
	SB-3CT	SB-3CT (matrix metallopeptidase nine inhibitor) preserved hippocampal neurons and prevented declines in motor function and memory, following TBI in rats Jia et al., 2014).
	Oxaloacetate + pyruvate	Oxaloacetate and pyruvate (blood glutamate scavengers) increased hippocampus neuronal survival and neurologic severity scores, after TBI in rats (Zlotnik et al., 2012).
	Thymoquinone	Thymoquinone (phytochemical compound) increased hippocampus neuronal densities and malondialdehyde levels, after TBI in rats (Gülsen et al., 2015).
	Indomethacin	Indomethacin (anti-inflammatory) suppressed Nogo-A (membrane protein important in axonal remodeling) expression, leading to decreased levels of IL-1β, therefore lessening neuronal damage after TBI in rats (Chao et al., 2012).

CA, comu ammonis; GABA, gamma-aminobutyric acid; Trk, tyrosine kinase; NMDA, N-methyl-D-aspartate; mTOR, mammalian Target Of Rapamycin; IL-1β, Interleukin-1 beta.

IMAGING IN mTBI

Recent advances in imaging have allowed for in-depth structural analysis of brain regions both acutely after concussive injury and in the chronic phase. This is important because the ability to identify abnormalities that may previously have been missed is the first step in developing modalities to treat these phenomena, or in the least decide which patients require treatment. Although no single imaging modality has yet proven to be sufficient for all patients with TBI due to the heterogeneity of the condition (Amyot et al., 2015), each method offers its own advantages.

Conventional magnetic resonance imaging (MRI) and computed tomography (CT) are typically negative in mTBI patients with persistent symptoms of post-concussive syndrome. However, compared to controls, mTBI patients were found to have lower cortical thickness of the right temporal lobe and left insula, and increasing number of mTBIs was associated with lower cortical thickness in more brain areas, including bilateral insula, right middle temporal gyrus, and right entorhinal area (List et al., 2015). These findings are worrisome because if further deterioration of these structures occur as part of the aging process, subtle cognitive symptoms may progress to

clinical dementia earlier than in age-matched controls. Diffusion tensor imaging is another technique that has recently emerged as a powerful adjunct to conventional MRI. It enables the measurement of fractional anisotropy, a value that indicates the degree of restriction experienced by water molecules diffusing along white matter fibers. Such a tool offers a noninvasive glimpse into the neurophysiology of these tracts and may allow detection of demyelination and axonal damage that would otherwise be missed on a conventional MRI. Indeed, fractional anisotropy was significantly reduced in the right superior longitudinal fasciculus of patients with mTBI and normal MRI as compared with controls, showing that diffusion tensor imaging is more sensitive than standard imaging in this population (Adam et al., 2015). Another study showed similar but subtler findings, as only those with mTBI who had abnormalities on conventional MRI had white matter changes on diffusion tensor imaging (Panenka et al., 2015).

Although increasing abnormality on post-injury CT and MRI correlate with lower neuropsychological performance (Bigler et al., 2015), diffusion tensor imaging has also shown promise in predicting neuropsychological outcomes following mTBI in patients with normal CT and MRI.

Immediately following injury and 6 months post-trauma, patients with mTBI showed significant differences in fractional anisotropy and radial diffusivity as compared to controls, specifically in the corona radiata, anterior limb of internal capsule, cingulum, superior longitudinal fasciculus, optic radiation, and genu of corpus callosum, changes which demonstrated associations with neuropsychological outcomes across both time points (Veeramuthu et al., 2015). Another study has shown significantly reduced fractional anisotropy in the internal capsule of both TBI and depression patients (Maller et al., 2010), further underscoring the potential role for diffusion tensor imaging as a noninvasive biomarker useful in the prognostication of post-concussive symptoms.

MRS is another noninvasive technique that measures metabolic changes within brain tissue. In combination with vascular imaging techniques such as transcranial Doppler ultrasound, these modalities have begun to shed light on metabolic processes and the control and regulation of cerebral blood flow following mTBI (Len and Neary, 2011). In the hippocampus, N-acetyl aspartate to choline and N-acetyl aspartate to creatine ratios were decreased in patients with mTBI in comparison to control subjects (Hetherington et al., 2014). Similar changes were found following mTBI in the thalamus, with a reduction of N-acetyl aspartate to creatine and choline to creatine ratios, findings that are classically associated with ischemia (Sours et al., 2015). Of interest, these results correlated with self-reported sensory symptoms, leading the authors to speculate that novel interventions could target these changes in the hopes of treating patients with persistent post-concussive symptoms, as well as use the absence of such imaging features as an indicator to help predict safe return to work.

Magnetoencephalography is an imaging modality largely restricted to the study of epilepsy, but may prove useful as a sensitive detector of local field potentials in brain areas affected by mTBI. A recent innovative study looked at the tracking of eye movements as a marker for sustained attention over time and internal anticipatory control, measurements that are impaired in chronic mTBI while the patients were undergoing magnetoencephalography (Diwakar et al., 2015). Compared to controls, mTBI patients demonstrated impaired eye tracking that was concurrent with abnormal beta activity, which was suppressed in the right parietal cortex and enhanced in the left caudate and frontal-temporal areas. The clinical significance of these findings is yet to be seen, but this is a strong example of novel imaging methods being used in new and original ways to better understand the neurophysiology of injured neural circuits, methods that may alter the manner in which mTBI is diagnosed and treated in the future.

FUTURE DIRECTIONS

Direct modulation of neural activity via deep brain stimulation (DBS) currently does not play a direct role in the setting mTBI, but it does carry promise in the treatment of

post-concussive affective disorders, trauma-induced tremor, and neurodegenerative disorders. DBS is an effective treatment for certain patients with severe major depression (Lozano et al., 2012) and obsessive-compulsive disorder (Kisely et al., 2014), but has yet to be investigated in the treatment of these disorders following brain injury. Likewise, PD often responds well to DBS of the subthalamic nucleus or globus pallidus internus (Okun, 2012), but data is lacking in the setting of trauma-induced Parkinson's. However, tremor following severe brain injury has been shown in small studies to respond to both thalamic and pallidal DBS (Issar et al., 2013; Carvalho et al., 2014).

If neurostimulation augments improvements in cognitive deficits after brain injury, memory disorders may present other potential applications for DBS (Shin et al., 2014). Forniceal DBS in a mouse model of Rett syndrome restored *in vivo* hippocampal long-term potentiation and neurogenesis, and rescued contextual fear memory and spatial learning (Hao et al., 2015). In humans, the fornix and nucleus basalis of Meynert are being investigated as possible DBS targets for improvement of memory in the treatment of AD (Sankar et al., 2014; Suthana and Fried, 2014). Although these strategies seem promising, they will likely be tested for efficacy and safety in the setting of severe TBI, prior to being applied in patients with mTBI.

Less invasive neuromodulation options for the treatment of mTBI include transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (TDCS). TMS uses a surface coil to create a low frequency electric current that is delivered to the surface of the brain, while TDCS uses electrodes placed directly on the scalp. There is evidence in severe TBI that TMS may aid in motor recovery, aphasia, visuospatial neglect (Castel-Lacanal et al., 2014), and cognitive function (Polanowska and Seniów, 2010), but high frequencies are contraindicated due to the risk of seizures, especially in the setting of trauma as the injured brain is already predisposed to epileptiform activity. TDCS uses lower currents and therefore has a lower risk of seizures, but subsequently may also have less clinical effect, as current studies show mixed results in the treatment of post-concussive symptoms (Kang et al., 2012; Leśniak et al., 2014; Ulam et al., 2015). TMS is currently the most extensively studied brain stimulation modality in TBI, but most interventions have been non-targeted and focused on the chronic phase of recovery after the injury (Li et al., 2015). As can be expected, better clinical outcomes occur when these neuromodulatory approaches are coupled with cognitive therapy and neurorehabilitation (Chantsoulis et al., 2015; Page et al., 2015).

Finally, nanotechnology and brain-computer interfaces may present new opportunities. By using a computer to bridge the gap between the brain and peripheral nerves in the setting of an injured spinal cord, brain-computer interfaces are "a form of extended embodiment that become integrated into the individual's conception of himself as an autonomous agent" (Glannon, 2014). Such systems are still in the experimental stage, but offer hope to those patients with severe motor deficits. Similarly, carbon nanotubes are being used to synthesize nanoelectrode arrays that permit real time monitoring and modulation of electrochemical events at the neural and synaptic

level (Andrews, 2007), and may offer a glimpse into the future of the field of neuromodulation. As with DBS, however, these modalities will likely be reserved for patients with severe TBI until efficacy, safety, and economic feasibility can be established.

CONCLUSION

Despite the high incidence and prevalence of mTBI, only recently have the pathophysiologic mechanisms begun to emerge. Understanding the biochemical signaling pathways involved in the propagation of secondary injury mechanisms has led to the discovery of novel uses for medications

AUTHOR CONTRIBUTIONS

condition.

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currently used for other indications. Biochemical markers

and advances in imaging are allowing for earlier diagnosis

of concussions, and developments in the area of DBS for the

treatment of memory and affective disorders are creating

impact the lives of patients and families suffering from this

new avenues for the management of post-concussive syndrome. Ultimately, these advances in neuromodulation strive to improve outcomes in mTBI and substantially

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Facilitating Mitochondrial Calcium Uptake Improves Activation-Induced Cerebral Blood Flow and Behavior after mTBI

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Mild to moderate traumatic brain injury (mTBI) leads to secondary neuronal loss via excitotoxic mechanisms, including mitochondrial Ca²⁺ overload. However, in the surviving cellular population, mitochondrial Ca²⁺ influx, and oxidative metabolism are diminished leading to suboptimal neuronal circuit activity and poor prognosis. Hence we tested the impact of boosting neuronal electrical activity and oxidative metabolism by facilitating mitochondrial Ca2+ uptake in a rat model of mTBI. In developing rats (P25-P26) sustaining an mTBI, we demonstrate post-traumatic changes in cerebral blood flow (CBF) in the sensorimotor cortex in response to whisker stimulation compared to sham using functional Laser Doppler Imaging (fLDI) at adulthood (P67-P73). Compared to sham, whisker stimulation-evoked positive CBF responses decreased while negative CBF responses increased in the mTBI animals. The spatiotemporal CBF changes representing underlying neuronal activity suggested profound changes to neurovascular activity after mTBI. Behavioral assessment of the same cohort of animals prior to fLDI showed that mTBI resulted in persistent contralateral sensorimotor behavioral deficit along with ipsilateral neuronal loss compared to sham. Treating mTBI rats with Kaempferol, a dietary flavonol compound that enhanced mitochondrial Ca²⁺ uptake, eliminated the inter-hemispheric asymmetry in the whisker stimulation-induced positive CBF responses and the ipsilateral negative CBF responses otherwise observed in the untreated and vehicle-treated mTBI animals in adulthood. Kaempferol also improved somatosensory behavioral measures compared to untreated and vehicle treated mTBI animals without augmenting post-injury neuronal loss. The results indicate that reduced mitochondrial Ca²⁺ uptake in the surviving populations affect post-traumatic neural activation leading to persistent behavioral deficits. Improvement in sensorimotor behavior and spatiotemporal neurovascular activity following kaempferol treatment suggests that facilitation of mitochondrial Ca²⁺ uptake in the early window after injury may sustain optimal neural activity and metabolism and contribute to improved function of the surviving cellular populations after mTBI.

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INTRODUCTION

Preclinical systems level studies of TBI during development can be approached using neuroimaging combined with behavioral assessment from the same subjects as currently done in younger TBI patients. As the number of children and young adults seeking care for TBI continues to increase, developmental aspects of mild to moderate traumatic brain injury (mTBI) which lead to disruption of neuronal circuits and formation of maladaptive circuits with aberrant behavioral responses (Pitkanen et al., 2009; McNamara et al., 2010; Li et al., 2015) need to be characterized. As brain activity related to behavioral tasks such as cognition, sleep, sensory processing, and motor responses result from spatially segregated neuronal assemblies (Harris, 2005), spatiotemporal mapping of stimulus-induced hemodynamic representatives of neural activity are significant for systems-level evaluation of neural plasticity (Logothetis et al., 2001). Hence functional Magnetic Resonance Imaging (fMRI) methods that enable spatiotemporal mapping of brain activity through changes in cerebral blood oxygenation, water diffusion or cerebral blood flow (CBF) are increasingly used to assess TBI patients (Kim et al., 2010; Slobounov et al., 2010; Johnson et al., 2012). Functional Laser Doppler Imaging (fLDI) is a preclinical neuroimaging method enabling high spatial resolution (100 µm) mapping of stimulus inducedneural activity represented by changes in microvascular CBF (Kannurpatti and Biswal, 2004, 2006). Accurate spatiotemporal mapping of neural events is limited by the sensitivity to microvascular signals compared to the contribution from large vessels. This ability to selectively discriminate signal change from arterioles and capillary compartments (closest in range to neural activity) is defined as functional resolution (Truong and Song, 2009). As shown by our previous studies, fLDI provides excellent functional resolution (Kannurpatti and Biswal, 2011) and complements preclinical fMRI for brain spatiotemporal mapping (Sanganahalli et al., 2013a). In this study, we used fLDI in a similar configuration that provided high microvascular specificity to detect activity close to neural ensembles (Kannurpatti and Biswal, 2011). Neural activity change in response to whisker stimulation between sham and mTBI animals was evaluated by assessing the spatiotemporal change in microvascular CBF in the somatosensory barrel field (S1BF), a region well characterized by electrophysiological and optical mapping studies of neuroplasticity (Welker, 1976; Diamond et al., 1993; Dowling et al., 1996; Kannurpatti and Biswal, 2011).

Secondary neuronal damage due to excitotoxic mechanisms and mitochondrial Ca2+ overload by increased Ca2+ influx is inevitable after TBI (Yokobori et al., 2014). However, the developing brain is relatively resistant to excitotoxic damage including relatively lesser excitotoxic Ca²⁺ dependent mitochondrial oxidative stress when compared to the adult (Kannurpatti et al., 2004). Hence the surviving neurons and their relatively Ca2+ resistant mitochondria may influence TBI prognosis during the developmental years. Human mitochondrial energy metabolism and Ca²⁺ uptake capacities diminish after TBI (Verweij et al., 2000), making the potentially surviving mitochondrial populations a critical therapeutic target (Giorgi et al., 2012). Slowing of mitochondrial Ca²⁺ uptake reduces neuronal activity in vivo (Mathiesen et al., 2011; Fluegge et al., 2012; Sanganahalli et al., 2013a,b) along with decreased oxidative energy metabolism (Kann et al., 2003; Mathiesen et al., 2011; Sanganahalli et al., 2013a,b). Hence we tested the impact of facilitating Ca2+ influx, known to boost neuronal electrical activity, and oxidative metabolism (Sanganahalli et al., 2013a), on long-term outcome after mTBI in developing rats. Kaempferol, a dietary flavonol compound and a known enhancer of the mitochondrial Ca²⁺ uniporter channel (mCU) was used to facilitate mitochondrial Ca²⁺ influx after mTBI. Kaempferol treatment improved post-injury behavioral outcomes and reduced alterations in neural circuit activity without augmenting mTBI-induced neuronal loss. The results indicate that facilitation of mitochondrial Ca²⁺ influx, known to boost ongoing neuronal electrical activity, and metabolism, may be a primary mechanism sustaining normal neural circuit functions in the surviving neuronal population leading to improved outcomes after mTBI.

METHODS

Animals

Male Sprague-Dawley rats (23-24 days old; weighing 60-80 g) were procured from Charles River Laboratories, Wilmington, MA, USA and used in this study. Rats were housed in pairs under controlled conditions. All procedures were carried out in accordance with the institutional guidelines and approved by the Institutional Animal Care and Use Committee of Rutgers-New Jersey Medical School and ARRIVE guidelines.

Lateral Fluid-Percussion Injury

Lateral fluid percussion injury (FPI) is a mixed injury model (both focal and diffuse) leading to axonal, somal, and microvascular swelling. This leads to tissue distortion and axonal shearing both proximal and distal to the injury location (Lindgren and Rinder, 1965; Dixon et al., 1987; McIntosh et al., 1989). In comparison to a more focal cortical contusion injury (CCI), lateral FPI induces significant cell death in distant regions even as far as the corresponding contralateral cortex (Peterson et al., 2015), and neurophysiological changes in deeper regions such as the hippocampus (Gupta et al., 2012; Li et al., 2015; Pang et al., 2015). In the current mTBI model, lateral FPI was performed in rats when they reached an age of 25-26 days according to the procedures established previously in our laboratory (Gupta et al., 2012; Li et al., 2015). The injury age (P25-P26) in the current rat model corresponded to the developmental time range of approximately a year in humans (Sengupta, 2013) and can be classified as a developmental mTBI model.

In brief, rats were anesthetized with ketamine (80 mg/kg i.p)xylazine (10 mg/kg i.p) and positioned on a stereotaxic frame. Ketamine provided rapid surgical plane anesthesia followed by quick recovery for the survival surgery procedure. A 3 mm craniotomy was performed on the left side of the skull −5 mm posterior to the bregma and 3 mm lateral to the sagittal suture keeping the dura intact. A Luer-Lock syringe hub was glued surrounding the exposed dura using a cyanoacrylate adhesive. After 24 h, injury was induced by attaching the Luer-Lock hub of an isoflurane-anesthetized rat to the FPI device (Virginia Commonwealth University, VA, USA). A pendulum drop delivered a brief 20 ms impact on the intact dura. The impact pressure was measured by an extra-cranial transducer and controlled between 2.0 and 2.2 atm. For sham group (n=5), the rats were anesthetized and attached to the FPI device without the pendulum drop. Righting reflex recovery time was recorded after injury in all animals and was in the range of 5.352 ± 2.97 min. Sham and injured animals were monitored within their cage environment on a daily basis.

Drug Treatments

A subset of mTBI rats (n = 5) were administered 3 doses of kaemferol (1 mg/Kg i.p) diluted in saline containing 10% dimethyl sulfoxide (DMSO). The first dose was delivered 1 h following the FPI and the other doses on consecutive days. Another subset of mTBI-rats (mTBI + vehicle, n = 4) was administered 3 doses of vehicle (saline with 10% DMSO). The kaempferol dose level used (1 mg/Kg i.p) was significantly lower than the dose level (20 mg/Kg i.p) shown to have significant antioxidative effects in vivo (Lagoa et al., 2009). Intraperitoneal administration was adopted due to several benefits such as gradual absorption and broader time-peaks of plasma concentrations (Barve et al., 2009). Oral administration was not preferred due to concerns of intestinal microflora-related metabolism of the compound (Vissiennon et al., 2012). As the average half life of intravenous delivered kaempferol was 6 h and average lifetime 24 h (Barve et al., 2009), we designed the dosing regimen in 24 h intervals. 1 mg/Kg intravenous infusion of kaempferol can produce an estimated peak plasma concentration of \sim 250 μ M/L, which is known to boost neural activity and oxidative metabolism in vivo (Sanganahalli et al., 2013a,b). Hence the intraperitoneal route of 1 mg/Kg kaempferol used would lead to plasma concentrations comparable to that achieved by human dietary intake of flavonol rich foods (DuPont et al., 2004).

Behavioral Testing

Following mTBI, all animals exhibited neurological deficits as assessed by a forelimb flexion test (data not shown). In order to access whisking-related sensorimotor impairment, a specific behavioral test namely the whisker stimulation-induced motor response (WSIMR) was performed at 1, 3, 7, 14, and 21 days following mTBI. We developed the WSIMR as a modification of the whisker-stimulated forelimb placement test (Schallert et al., 2000), since the forelimb placement responses in younger rats (23-30 days range) were not fully mature. During the WSIMR test, the animal was placed in a test cage and the whiskers on one side were gently stroked multiple times (10 stroke maximum) at a frequency ~5 Hz in a caudo-rostral direction with an applicator stick. The animal did not have any initial visual cue to the approaching stroke from the experimenter. Ten trials with an inter-trial gap of 30 s were performed and repeated 5 times on each side of the animal with a repetition gap of 2 min. Whisker deflections during the behavioral experiments were performed by a single experimenter to maintain consistency of the force, frequency, and angle of stimulation. Motor responses such as avoidance of stick and the proficiency of reaction to each stroke was noted and scored on a scale of 0-3: Active avoidance by running away = 3, quick head movement away from the stroking stick = 2, slower head movement away from the stroking stick = 1 and no reaction to stroking = 0. A cumulative score was obtained after averaging all measurements from each side of whisker stroking.

Functional Laser Doppler Imaging (fLDI)

We performed initial pilot experiments in a different set of animals (not included in this study) adopting the mTBI experimental approach to enable fLDI measurements. Removal of the luer-lock cap from the skull was piloted in three animals post-injury which led to increased thickness of the skull around and adjoining the capped area. During the preparation of the thinned skull, the texture of the skull area was very flakey with presence of extra intracranial blood vessels which resulted in excessive bleeding and comparably different quality of ipsilateral-contralateral window preparations. Hence cap removal immediately after injury was discontinued. In the next pilot, we retained the cap after injury using normal cyanoacrylate adhesive strength. However, cap removal in the adult animal prior to fLDI measurements became difficult. Hence a gradual reduction of the cyanoacrylate adhesive strength was tested and an appropriate concentration enabling easy cap removal prior to the thinned skull window preparation was used in all animals included within the study.

Rats after 42-48 days following mTBI (P67-P73) and agematched shams were anesthetized with urethane 1.2 g/kg i.p and placed on a warm pad for fLDI measurements. Animal core temperature was monitored and maintained at 37 \pm 0.5°C with a rectal probe and homeothermic blankets (Baxter K-MOD100, Gaymar Industries, USA). After endotracheal intubation and mechanical ventilation, end-tidal CO₂ (ETCO₂) and arterial oxygen saturation (SPO2) were continuously monitored throughout the experimental duration (8100 Poet-Plus Vital Signs Monitor, Criticare, WI, USA). ETCO2 levels were maintained between 32 and 35 mmHg through appropriate ventilator adjustments. Animals received a bolus of gallamine triethiodide (250 mg/kg, i.p) after the start of ventilation to achieve muscle relaxation. To enable fLDI measurements animals were positioned on a stereotaxic frame with the scalp retracted from the frontoparietal cortex by a dorsal midline incision to expose the cranium. The Luer-Lock syringe hub implanted for the induction of mTBI was gently dislodged and removed from the skull using a pair of scissors. Inflammation was evaluated visually by ensuring that the fur growth was normal with no scalp wounds around the cap. After the midline scalp incision and cap removal for fLDI measurement, the exposed skull area beneath the cap was examined to ensure that the prior craniotomy area was dry and closed by skull and tissue growth. On inspection in almost all the rats imaged, the skull beneath the cap region had completely grown and closed at 42-48 days post-injury. There was no inflammation in any of the cap-retained animals during observations prior to the fLDI measurements. Furthermore, no inflammation was observed in two animals from which the cap fell off during the duration

of housing for the experiments. The temporalis muscle was disconnected from the cranium and a window of skull area measuring $5 \times 5 \,\mathrm{mm}$ centered at $-2 \,\mathrm{mm}$ and $+5 \,\mathrm{mm}$ lateral to the bregma enclosing the somatosensory barrel field (S1_{BF}) on either hemisphere was thinned to translucency using an aircooled dental drill. Functional imaging using fLDI was carried out using a Moor LDI scanner (Moor Instruments, Sussex, UK) as described previously (Kannurpatti and Biswal, 2011). Briefly, a beam of laser from a low power (2 mW \pm 20%, 632.8 nm) He-Ne source scanned the thinned skull window in a raster pattern and the scattered Doppler shifted beam was collected and focused onto a photo-detector. The laser Doppler flux signal which was proportional to the tissue perfusion/CBF at each measurement point was calculated and stored in the resulting image pixels. Distance between the scanner head and the thinned skull was 23 cm leading to an in-plane resolution of about 120 imes120 μ m² with fLDI images of 42 \times 54 pixel matrix obtained covering a field of view of $5 \times 6.5 \,\mathrm{mm}^2$. Functional images were obtained from the somatosensory field of view on both hemispheres at a rate of 16 sec/image and a dead time of 4s between each image. The current configuration was optimized for maximum microvascular specificity to detect activity close to neural ensembles (Kannurpatti and Biswal, 2011). Urethane was used due to its minimal cardiovascular effects, respiratory, spinal reflexes and confounds on neurovascular coupling (Masamoto and Kanno, 2012). Urethane also provided stable anesthesia over longer periods (several hours) (Sanganahalli et al., 2013a) and was best suited for the imaging procedure using fLDI.

Whisker Stimulation during fLDI Acquisition

Whiskers from B-E rows and the stradlers α , β , γ , and δ were glued together using a masking tape of rectangular area of $0.5 \times 0.6 \,\mathrm{cm}^2$ to include up to 5 arcs. Whisker deflections were carried out in the caudo-rostral direction using air puffs of 15 ms duration from a pressurized air source delivering a pressure of 60 psi through a solenoid (James Long Company, Caroga Lake, NY) controlled by a A310 pulse generator (WPI instruments, Sarasota, FL). Air puffs were delivered through a short plastic tubing of length 50 cm connected to a standard 1 ml pipette tip with sufficient velocity within the duration of 15 ms that deflected the whiskers by $\sim 40^{\circ}$ in the rostro-caudal direction as characterized previously in our laboratory (Kannurpatti and Biswal, 2011). Deflected whiskers returned back to their original position before the delivery of the next air puff for the stimulus frequency of 5 Hz used in the current experiments. Whisker stimulation was performed on both sides of the animal in a sequential manner with contralateral whisker stimulation matched by ipsilateral fLDI measurement.

Analysis and Statistical Parametric Mapping of fLDI Image Data Sets

The Moore LDI scanner acquisition program processed the blood flow related signal derived from the Doppler spectrum via an analog to digital (A/D) converter. The light intensity and the blood flux signals from the A/D converter was

compensated for noise effects and color-coded in arbitrary flux units (relative perfusion units; rpu) before image reconstruction. Further data processing and statistical analysis was performed on the reconstructed image data using custom made programs developed in our laboratory (MATLAB, Natik, MA). Each experimental run contained 3 periods of whisker stimulation interspersed with 4 periods of rest representing an "ON/OFF" cycle along a block of 25 images. Experimental runs were repeated 5 times on each cortical field of view and averaged. An idealized box-car function representing the "ON/OFF" cycle of whisker stimulation was used as the reference waveform. The stimulus reference function was statistically cross correlated with the fLDI flux signal on a pixel-wise basis to determine activation. Cross correlation estimates the degree to which any two time-dependent signals are correlated. The distribution of the cross correlation coefficient throughout the image when no stimulus was presented (null distribution) was approximately a normal distribution and used to determine the threshold cross correlation coefficient value. A threshold of 1.96 times the standard deviation for the null distribution corresponding to an error probability of P < 0.05 was used for determining the activation threshold that corresponded to a correlation coefficient value of 0.22. Activated pixels in the functional maps were further controlled for false positives through a family-wise error control using a cluster size threshold of 0.04 mm². Functional images were color coded according to the correlation coefficient values and overlaid on the gray scale baseline CBF images obtained by averaging all resting period images. The functional images (color overlay) and the baseline CBF images have identical spatial resolution.

Histology

After the terminal fLDI experiments, rats were perfused with 4% paraformaldehyde in PBS, pH 7.4. Brains were removed and postfixed for 24 h in the same paraformaldehyde solution. Following post-fixation, brains were cryoprotected in 30% sucrose in PBS for 24-48 h. Coronal sections (40 µm-thickness) were cut at approximately three horizontal planes with respect to the bregma (-0.1, -2.0, and -4.0 mm) using a Leica cryostat and mounted on gelatin-coated glass slides for histological analyses. The sections were stained with cresyl violet to mark the neurons. Brain tissue sections from both the injured-ipsilateral hemisphere and the uninjured-contralateral hemisphere were used for histological evaluations. To assess neuronal cell survival, six random counting frames/animal (three in each hemisphere), were considered in three distinct regions depending upon their lateral distance and depth from the injury site. (1) layer-IV of the S1_{BF} region (-0.1 mm to bregma), (2) Layer-II and III of the S1_{BF} region (-2 mm to bregma), and (3) hippocampal hilus (-4 mm to bregma). Neuronal cell bodies were counted from each frame obtained at a magnification of 40x using an oil immersion objective on an inverted optical microscope.

Statistics

Data are presented as mean \pm SEM for the behavioral results and mean \pm SD for all others. WSIMR test on different time points were analyzed using ANOVA with *post-hoc*

pair-wise comparisons to assess differences in performances between ipsilateral and contralateral-side. For fLDI and histology measurements, average from each group was calculated and the significant differences between the sham and treatment groups were analyzed using ANOVA followed by post-hoc HSD test or a paired t-test. A P < 0.05 was considered to be statistically significant.

RESULTS

Using the lateral fluid percussion rat model of TBI (Gupta et al., 2012), mTBI was performed on the hippocampal plane on the left hemisphere at -5 mm to the bregma (**Figures 1A,B**). This model, producing both focal and diffuse injuries, is a good representative of mild to moderate injuries in humans and well characterized in its functional impact on hippocampal (Gupta et al., 2012) and somatosensory neural circuits in rats (Lifshitz and Lisembee,

Sensorimotor behavior was assessed using the WSIMR test in order to correlate the S1_{BF} circuit activity mapped using fLDI. Scores were assigned depending upon the vigor of face withdrawal when the whiskers on each side were stroked. In sham animals, a progressive improvement was observed in the WSIMR scores as rats reached a young-adult age of 1.5 months (Figure 2A). In mTBI animals, there was a significant ipsilateral-contralateral asymmetry in addition to a time-dependent improvement in WSIMR scores (Figure 2B). Ipsilateral-contralateral asymmetry in behavioral performance was significant at all time points up to 21 days post-injury

(Figure 2B) without any change in contralateral performance when compared to sham (Figures 2A,B; ANOVA). As WSIMR testes higher order cortical functional integration requiring the engagement of sensorimotor circuits to elicit behavioral responses, the progressive decrease in the ipsilateral-contralateral asymmetry during the 21 day period after injury indicates that recovery in higher order brain functions may continue through the late stage (>21 days post-injury). In two additional groups of mTBI animals, one was treated with the mitochondrial Ca²⁺ uniporter mCU channel enhancer, kaempferol, during the early window (0-3 days post injury) while the other received vehicle treatment. Kaempferol (1 mg/Kg, i.p) or vehicle (saline with 10% DMSO) treatments were performed at 1 h, 24 h, and 48 h after injury. Behavioral assessment revealed that kaempferol treatment significantly improved WSIMR scores after and abolished the ipsilateral-contralateral asymmetry (Figure 2D; ANOVA) compared to vehicle treated rats which showed persistent statistically significant ipsilateralcontralateral asymmetry (Figure 2C; ANOVA, P < 0.01; Post-hoc Tukey's HSD test). The brief 3 day post-injury kaempferol treatment was able to completely normalize the ipsilateral-contralateral asymmetry around 14 days post-injury and thereafter (Figure 2D).

After completion of behavioral monitoring, each animal underwent functional imaging of CBF using fLDI 42-48 days post-injury (P67-P73). fLDI spatiotemporal mapping was performed during the adult stage as the immature somatosensory cortical barrel representation continues to develop exhibiting dynamic changes in metabolism, electrical activity, neural cell

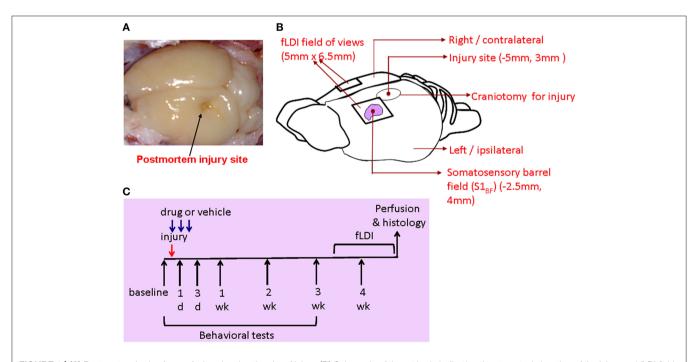


FIGURE 1 | (A) Postmortem brain after perfusion showing the site of injury. (B) Schematic of the rat brain indicating the stereotaxic location of the injury and fLDI fields of view on either hemisphere covering the somatosensory whisker barrel representation. (C) Description of the integrated experimental design to assess mTBI prognosis after mitochondrial treatment through behavioral, fLDI, and histological markers.

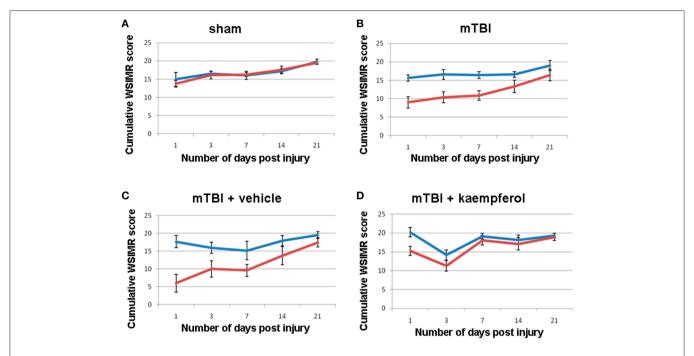


FIGURE 2 | Whisker stimulation-induced motor reactivity (WSIMR) behavioral responses in sham and mTBI animals. (A) Sham group (n = 5), (B) mTBI without any treatment (n = 15), (C) mTBI animals treated with vehicle (n = 4), and (D) mTBI animals treated with kaempferol (n = 5). Blue curves represent WSIMR responses due to ipsilateral whisker stimulation and red curves represent WSIMR responses due to contralateral whisker stimulation. ANOVA; (P < 0.01; Post-hoc pairwise comparison) indicated significant ipsilateral-contralateral asymmetry at all behavioral time points in the mTBI and mTBI + vehicle groups. No significant difference was observed in the ipsilateral-contralateral asymmetry for all time points in the sham whereas the 1 and 3-day time points showed significant asymmetry (P < 0.01) in mTBI + kaempferol group. Data points represent mean \pm SEM.

types, and transmitter systems which are complete and stable after 2 months of age. As detailed in the methods section, FPI models produce neuronal damage spread across a wider distance in the brain with cell death occurring even in the contralateral cortex (Pang et al., 2015; Peterson et al., 2015). The somatosensory barrel field (S1BF) chosen for functional imaging of brain circuit activity to correlate with whisker deflection-induced sensorimotor behavior in the intact animal model was distal to the site of impact. fLDI imaging field of view was placed on the S1_{BF} region centered -2.5 mm caudal, 5 mm lateral to the Bregma and was \sim 3 mm away from the site of fluid percussion impact (Figure 1). fLDI flux value in a pixel represents the product of the number of red blood cells and their velocity. Hence larger vessels with relatively higher CBF show larger fLDI flux values (>500 rpu) relative to smaller vessels such as arterioles and venuoles (<500 rpu), which can be distinguished in the baseline CBF images (Figures 3A,D). CBF change induced by underlying neural activity and oxygen metabolism forms the basis of spatiotemporal functional mapping of brain activity (Logothetis et al., 2001). Brain activation in response to a stimulus is determined by statistical parametric mapping of the hemodynamic response on a pixel-by-pixel basis. We performed statistical parametric mapping (Methods Section Analysis and Statistical Parametric Mapping of fLDI Image Data Sets) to detect whisker stimulation-induced CBF changes

within the somatosensory cortex. Increases in CBF response yielded positive correlation coefficients whereas CBF decreases yielded negative correlation coefficients. Pixels with correlation coefficients \geq 0.22 (positive CBF activation) or \leq -0.22 (negative CBF activation) corresponding to a significance threshold of P < 0.05 were considered activated, representing the stimulusinduced cortical activation (color overlay in Figures 3B,E). Spatiotemporal mapping of hemodynamics in response to evoked stimuli can be positive or negative in direction. However, the negative hemodynamic responses cannot always be attributed to deactivation as CBF decreases can occur during various situations such as neuronal deactivation (Shmuel et al., 2002), blood diversion to an adjacent active cortical region (Harel et al., 2002; Kannurpatti and Biswal, 2004), or neurovascular uncoupling due to pathology (Schridde et al., 2008). Hence the positive and negative spatiotemporal CBF changes can be more appropriately defined as "activation" with respect to the resting state. Positive CBF responses contralateral to the injury, was similar to that observed in sham animals, but qualitatively appeared to be less focal than sham (Figures 3A-C). Small clusters of negative CBF changes in response to evoked stimulus accompanied the positive CBF responses (Figure 3B). The sparse negative CBF responses, usually in small clusters, originated from higher baseline flux pixels (over large vessels >500 rpu), and consistent with our previous fLDI mapping studies on normal rats. These small clusters of negative CBF change can

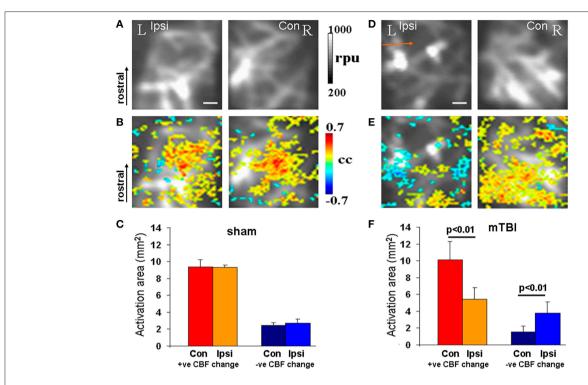


FIGURE 3 | fLDI mapping of somatosensory (S1_{BF}) activity. (A,D) Baseline CBF in sham and mTBI animals respectively showing ipsilateral decrease in the S1_{BF} region in mTBI (arrow) when compared to sham. (B,E) Whisker deflection-induced functional activation indicating S1_{BF} CBF changes (color map) overlaid on the baseline CBF images. A functional asymmetry in mTBI can be observed, not present in the sham. (C,F) Activation area of positive and negative CBF responses to whicker stimulation at the group level represented as Mean \pm SD. Sham group; n = 3 and mTBI group; n = 6. Significance tested using one-way ANOVA, *Post-hoc* Tukey's HSD test. Scale bar = 1 mm, R, right; L, left; lpsi, ipsilateral; Con, contralateral; cc, correlation coefficient; rpu, relative perfusion units.

be attributed to a passive upstream decrease in CBF in certain large vessels diverting blood to adjacent cortical areas (Harel et al., 2002; Kannurpatti and Biswal, 2004). Activation area was estimated as the product of pixel area and the number of activated pixels in response to whisker stimulation. In the mTBI animals, fLDI activation was asymmetric where the injured ipsilateral hemisphere's positively activated area decreased by about 50% (Figures 3D-F). A prominent negative CBF activation with large clusters of activated pixels predominantly over microvasculature with low baseline fLDI flux values (<500 rpu) was observed in the mTBI animals (Figure 3E). These larger clusters of negative CBF responses from the microvascular pixels arise due to vasoconstriction originating from suppressed neuronal activity (Devor et al., 2007) and are distinct from the small negative CBF clusters observed mostly over large vessels in sham animals. The large cluster negative CBF responses from the microvascular pixels indicated substantially reduced excitability within the S1_{BF} region ipsilateral to the injury in the mTBI animals. Such an alteration of stimulus-induced ipsilateral CBF response in mTBI animals may have multiple origins including impaired excitatory neuronal electrical activity, enhanced neuronal inhibition (Johnson et al., 2014), impaired neurovascular coupling and/or impaired blood perfusion in the injured and adjoining regions (Hayward et al., 2010). Overall, the results highlight mTBI induced neurodegeneration with sustained behavioral deficit and altered stimulus-induced spatiotemporal CBF responses indicative of increased neuronal inhibition and/or neurovascular uncoupling.

In separate mTBI animal groups the impact of mitochondrial Ca²⁺ uptake facilitation was assessed using fLDI after kaempferol or vehicle treatments. fLDI responses in vehicle treated mTBI animals (**Figures 4A–C**) was similar to that observed in the untreated mTBI animals where the stimulus-induced positive CBF response was significantly asymmetric (**Figures 4A–C**). Stimulus-induced positive CBF response not only improved in the kaempferol-treated animal group but the asymmetry in the ipsilateral-contralateral neurovascular activity was also significantly reduced (**Figures 4D–F**) compared to vehicle-treated mTBI animals (**Figures 4A–C**). No prominent microvascular-negative CBF responses were observed in the ipsilateral hemisphere after kaempferol treatment.

As brain perfusion is diminished chronically in TBI patients matching spatially with regions of neural functional deficits (Kim et al., 2010), we assessed the impact of mTBI on the baseline CBF in the developmental mTBI model used in this study. Baseline CBF was averaged over all pixels within the image field of view during the resting condition (in the absence of whisker stimulation). Baseline CBF significantly decreased across the ipsilateral cortex compared to contralateral (Figures 5A,B) accompanied by notable vascular rearrangements

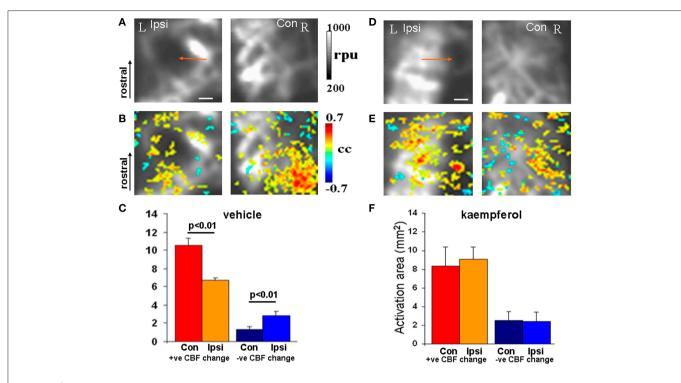


FIGURE 4 | fLDI somatosensory ($S1_{BF}$) activation in mTBI animals after vehicle or kaempferol treatment. (A,D) Baseline fLDI flux in the vehicle and kaempferol-treated animals respectively, indicating baseline CBF decrease in the $S1_{BF}$ region on the injured (ipsilateral) side (arrows). (B,E) Whisker deflection-induced functional activation indicating CBF changes overlaid on the baseline image. A reduced functional asymmetry is evident after kaempferol treatment. (C,F) Activation area of positive and negative CBF responses at the group level represented as Mean \pm SD. Vehicle group; n=3, kaempferol group; n=6. Significance tested using one-way ANOVA, *Post-hoc* Tukey's HSD test. Scale bar = 1 mm, R, right; L, left; Ipsi, ipsilateral; Con, contralateral; cc, correlation coefficient; rpu, relative perfusion units.

within the ipsilateral cortical hemisphere (**Figure S1**). These results are consistent with earlier MRI findings of reduced CBF accompanied by increased vascular density in the peri-lesional cortex months after the injury (Hayward et al., 2010). Kaempferol treatment, however, did not lead to any improvement in the baseline CBF decreases observed after mTBI (**Figure 5D**).

To assess neural survival after injury, coronal sections of fixed brain tissue were stained with cresyl violet to evaluate neuronal density in the distal regions such as layer IV of S1BF and hippocampal hilus and $S1_{BF}$ layers II-III stereotaxically closer to the injury impact site. Figures 6A-C show representative cresyl violet stained section of a typical mTBI brain across both hemispheres from the distal and proximal regions relative to the site of impact Average neuronal counts obtained from multiple animals indicated a significant neuronal loss within the ipsilateral hippocampal hilus (Figure 6F), typical to this injury model (Gupta et al., 2012; Li et al., 2015). Cortical layers of the S1_{BF} region distal and proximal to the injury site also indicated significant neuronal loss in the ipsilateral hemisphere compared to contralateral (Figures 6D,E). No significant difference in neuronal cell density was observed between the ipsilateral and contralateral hemispheres of sham animals. However, neuronal density in the ipsilateral cortex in all injured groups significantly reduced compared to shams (Figure 6; paired t-test). Kaempferol and vehicle treatments did not induce further changes in neuronal density in injured cortex either proximal or distal to the injury site (**Figure 6**; ANOVA).

DISCUSSION

Using a systems approach, the current study assessed mTBI outcomes in developing rats with clinically relevant neuroimaging markers. As mitochondrial Ca²⁺ uptake capacity is known to decrease after TBI along with impaired oxidative metabolism, neural loss, and neurovascular changes (Verweij et al., 2000; Pandya et al., 2007; Hayward et al., 2010; Lifshitz and Lisembee, 2012), we tested if mitochondrial Ca²⁺ influx facilitation, known to boost neuronal electrical activity and metabolism (Sanganahalli et al., 2013a), will impact mTBI prognosis.

Kaempferol, a dietary flavonoid compound and a specific enhancer of the mitochondrial Ca²⁺ uniporter channel (mCU) (Montero et al., 2004), capable of crossing the blood brain barrier and diffusing freely into cells and organelles (Liu et al., 2006), was administered in a dose relevant to dietary intake. Dietary therapy is recognized to be advantageous to patients recovering from mild TBI without necessitating hospitalization and intravenous access for drug administration and considered important in preclinical treatment studies of TBI (Elkind et al., 2015). Based on previous dose-dependent studies determining that

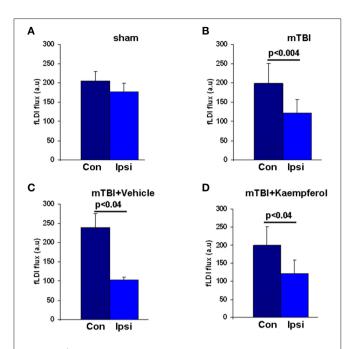


FIGURE 5 | Baseline fLDI flux representing resting CBF in all animals within the Ipsi-ipsilateral and Con-contralateral hemispheres relative to the site of injury. (A) Sham, (B) mTBI, (C) mTBI animals treated with vehicle, and (D) mTBI animals treated with kaempferol. Data represent group level Mean \pm SD, sham group; n=3 and mTBI group; n=6 vehicle group; n=3, kaempferol group; n=6. Significantly different, one-way ANOVA, Post-hoc Tukey's HSD test. mTBI (Ipsi) vs. (Con); P<0.004 and F=11.88. mTBI + vehicle (Ipsi) vs. (Con); P<0.04 and F=11.84. mTBI + kaempferol (Ipsi) vs. (Con); P<0.04 and F=6.57. No significant differences were observed in the baseline CBF levels within the same hemispheres when compared between the mTBI + vehicle and mTBI + kaempferol groups.

1--3 mg/Kg kaempferol delivered intravenous could enhance the baseline spontaneous/evoked neural activity and CBF responses (Sanganahalli et al., 2013a,b), a similar dose range was used with daily intraperitoneal injections of 1 mg/Kg. The current treatment can be expected to produce plasma concentrations significantly lower than 250 μ M/L and comparable to ranges of kaempferol concentrations produced by human dietary intake of flavonol rich foods (DuPont et al., 2004). All animals receiving kaempferol survived with no adverse effects during the treatment which was 1000 times lower than the suggested safety limits of 1000 mg/Kg (Shih et al., 2013).

States of impaired mitochondrial Ca²⁺ uptake capacity has the potential to disrupt normal neural signaling and shown to diminish both spontaneous and activation-induced CBF and fMRI-BOLD functional responses in the working brain *in vivo* (Kannurpatti and Biswal, 2008; Sanganahalli et al., 2013a). Early post-injury brain functions are suboptimal and may coincide with the window of neuronal death/survival events (Lifshitz and Lisembee, 2012). Eelectroencephalography (EEG) and fMRI studies of humans in the early stages after mTBI indicate diminished neural circuit activity (Gosselin et al., 2011). The early-stage evolution of brain events may critically depend on mitochondrial functions within the surviving neuronal populations (Vagnozzi et al., 2007; Calderon-Cortes et al.,

2008; Lifshitz and Lisembee, 2012; Sauerbeck et al., 2012; Bartnik-Olson et al., 2014). The current in vivo results reflect robust changes in cortical excitation where mTBI-induced brain functional activation showed decreased stimulus-evoked positive CBF responses and increased stimulus-evoked negative CBF responses (Figures 3D-F). Stimulus-evoked negative CBF responses were prominent on the injured hemisphere indicating a post-traumatic alteration in the underlying neural circuit activity or their neurovascular coupling, which may not be a desirable outcome in TBI patients. The prominent negative CBF responses in mTBI animals (large clusters of activated area) could be driven by suppressed neuronal activity within the somatosensory cortex (Shmuel et al., 2002) or neurovascular uncoupling due to pathology (Schridde et al., 2008). This is distinct from the smaller negative CBF response clusters observed in sham animals which likely reflects a passive reduction in CBF in upstream vessels of larger caliber (Kannurpatti and Biswal,

Diminished oxidative metabolism measured by oxygen utilization (indicative of mitochondrial dysfunction) has been observed in pediatric TBI patients (Ragan et al., 2013). As demonstrated by our earlier studies, diminished oxidative metabolic state achieved by inhibiting mitochondrial Ca²⁺ uptake after treatment with the mCU inhibitor Ru360 attenuated stimulus-evoked somatosensory neuronal activity and CBF responses (Kannurpatti and Biswal, 2008; Sanganahalli et al., 2013a). In contrast to Ru360, kaempferol treatment was shown to have the opposite neurophysiological effects of enhanced oxidative metabolism and neuronal activity (Sanganahalli et al., 2013a). In the current study where mTBI rats were treated with kaempferol, the brief and early mCU enhancement resulted in sustained beneficial effects eliminating inter-hemispheric asymmetry in the stimulus-evoked CBF response into adulthood. Additionally, the treatment reduced changes in neurovascular activity and activation-induced negative CBF responses across the injured ipsilateral hemisphere (Figures 4D-F). Kaempferol treatment improved somatosensory behavioral performance as early as 7 days after mTBI and the effects were sustained during the entire duration of testing up to 21 days after mTBI (Figure 2D). Hence, mitochondrial Ca²⁺ uptake impairment may be an early event within the mitochondrial populations after developmental mTBI and a potential early treatment target. Since inter-hemispheric asymmetry in somatosensory behavior was eliminated after kaempferol treatment by 7 days after mTBI and was sustained at 21 days post injury, no further behavioral testing was conducted beyond 21 days. Development of inter-hemispheric asymmetry in the spatiotemporal CBF responses measured by fLDI and restoration of inter-hemispheric symmetry by kaempferol treatment observed over 48 days after injury parallels the mTBI-induced behavioral impairment and its mitigation by kaempferol treatment 21 days after injury.

No significant neuronal survival differences were observed between kaempferol and vehicle treatments (**Figure 6**; ANOVA), indicating that mitochondrial Ca²⁺ uptake facilitation effects of the drug did not trigger adverse mitochondrial Ca²⁺ deregulation events including mitochondrial permeability transition. As the

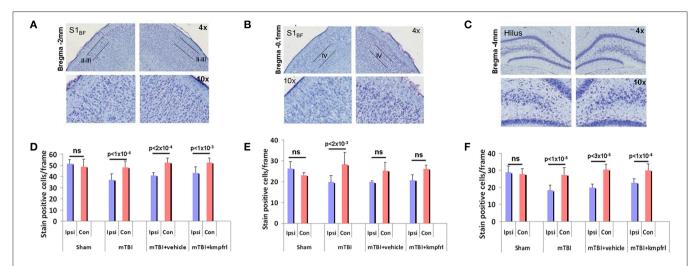


FIGURE 6 | Cresyl violet histological staining of coronal sections of post-mortem brains after terminal fLDI imaging. (A–C) Representative sections over the S1_{BF} and hippocampal hilar regions in different magnifications. (D–F) Number of stain positive neuronal cell bodies estimated per frame from different layers of the S1_{BF} region and hippocampal hilus. Data represent group level Mean \pm SD, sham group; n=3 and mTBI group; n=6 mTBI + vehicle group; n=3, mTBI + kaempferol group; n=6. Paired t-test comparisons between ipsilateral and contralateral neuronal counts indicated significant neuronal loss in the ipsilateral cortex and no significant protective effect of kaempferol treatment. Ipsilateral neuronal counts tested across all groups with a one-way ANOVA followed by a *post-hoc* Tukey's HSD test indicated significant differences between sham and all injured groups whereas no significant difference was observed in the contralateral neuronal counts. Kaempferol treatment after mTBI did not change neuronal survival in the adult. (S1_{BF} layer II-II; ANOVA; F=9.82; Sham vs. mTBI; F<0.01; Sham vs. mTBI + vehicle; F<0.01; Sham vs. mTBI + kaempferol; ns; mTBI vs. mTBI + vehicle; F<0.01; Sham vs. mTBI + kaempferol; F<0.01; Sham vs. mTBI + kaempferol; ns. mTBI + vehicle; ns; mTBI vs. mTBI + kaempferol; F<0.01; Sham vs. mTBI + kaempferol; ns. mTBI + vehicle; ns; mTBI + vehicle; F<0.01; Sham vs. mTBI + kaempferol; F<0.01; Sham vs. mTBI + vehicle vs. mTBI + vehicle; F<0.01; Sham vs. mTBI + vehicle vs. mTBI + vehicle; F<0.01; Sham vs. mTBI + vehicle vs. mTBI + vehicle; F<0.01; Sham vs. mTBI + vehicle vs. mTBI + vehicle; F<0.01; Sham vs. mTBI + vehicle vs. mTBI + vehicle; F<0.01; Sham vs. mTBI + vehicle vs. mTBI + vehicle vs. mTBI + vehicle vs. mTBI + vehicle; F<0.01; Sham vs. mTBI + vehicle vs

developing brain is relatively resistant to excitotoxic Ca²⁺ dependent mitochondrial oxidative stress and mitochondrial Ca²⁺ overload compared to the adult (Kannurpatti et al., 2004; Robertson et al., 2004), mitochondrial Ca²⁺ uptake facilitation treatments may have beneficial effects of improved neuronal activity with minimal impact on detrimental mitochondrial Ca²⁺ dependent events. Hence, mitochondrial Ca²⁺ influx facilitation in the immature brain may prove to be beneficial as it maintained optimal neuronal activity in the surviving neurons with no exacerbation of secondary neuronal death. Mitochondrial Ca²⁺ uptake enhancement also did not improve the baseline CBF decreases observed after mTBI (Figure 5D). This indicated that pharmacological facilitation of mitochondrial Ca²⁺ uptake activity might not have a significant effect on cerebrovascular cellular compartments and their functions. However, possible effects on vascular cell survival and proliferation may need further investigation as increased vascularization along with decreased CBF has been observed as a long term response in the peri-lesional cortex after mTBI (Hayward et al., 2010) and reproduced in the adulthood stage in the current studies (Figure 5 and Figure S1).

In vivo Ca²⁺ fluxes in the working brain in different compartments are difficult to demonstrate directly but its functional consequences can be measured *in vivo*. Impact of enhanced mitochondrial Ca²⁺ influx leads to increased mitochondrial oxidative metabolism and increased neuronal electrical activity which has been demonstrated in the intact animal model by our previous studies (Sanganahalli et al.,

2013a). Hence enhanced mitochondrial Ca²⁺ uptake is the most likely event leading to altered neural functional states affected by kaempferol. Kaempferol as a dietary flavonol possesses antioxidative and anti-inflammatory effects (Middleton et al., 2000; Hamalainen et al., 2007) and may possibly impact the observed changes in brain circuit activity after treatment. Independent in vivo studies suggest that dose levels in the range 20 mg/Kg i.p and above are required for any significant antioxidative effects from kaempferol treatments (Lagoa et al., 2009). Although the 1 mg/Kg i.p dose of kaempferol used in this study may be suboptimal for any antioxidative effects as no neuroprotective impact was apparent in our results, non-specific antioxidative, and anti-inflammatory effects of kaempferol treatment in the developmental mTBI model cannot be excluded and needs further investigation. The current study in vivo tested a single compound targeting mCU activity. In order to quantify the complete physiological impact of mitochondrial Ca²⁺ uptake after developmental mTBI, similar systems level evaluation with several other mCU-targeting compounds may be required. Furthermore, stimulus-induced CBF response alterations after mTBI can have a neuronal and/or neurovascular origin. Hence future single subject electrophysiological and functional imaging correlations may be needed to determine the exact origins of the mTBI-induced functional activity changes within the brain.

In summary, suboptimal mitochondrial Ca²⁺ uptake during the early window after developmental mTBI may lead to impaired ongoing neuronal activity accompanied by neuronal loss and

neurovascular coupling changes with persistent behavioral deficits. Kaempferol treatment known to facilitate oxidative metabolism and the ongoing neuronal activity improved somatosensory behavior and favorably altered the post-injury S1_{BF} neurovascular activity. The results highlight the importance of maintaining mitochondrial Ca²⁺ homeostasis in the surviving mitochondrial population in the early window (0-3 days) after mTBI. Mitochondrial Ca²⁺ influx pathway can be a viable treatment target in patients with mTBI and its physiological effects can be monitored at the systems level with clinically relevant functional imaging markers.

AUTHOR CONTRIBUTIONS

Designed the study: SK. Performed the experiments and analyzed data: MM, SK. Contributed to laboratory resources and expertise

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnsys. 2016.00019

for the animal model of Traumatic Brain Injury: VS. Wrote the

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manuscript: MM, SK. Edited the manuscript: MM, VS, SK.

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Figure S1 | Typical mTBI rats whose postmortem brains were sectioned and stained with cresyl violet 4-5 weeks after injury indicate significant increase in vasculature in the ipsilateral compared to the contralateral cortex proximal to the site of injury.

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Effects of Rapamycin Treatment on **Neurogenesis and Synaptic Reorganization in the Dentate Gyrus** after Controlled Cortical Impact **Injury in Mice**

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Post-traumatic epilepsy (PTE) is one consequence of traumatic brain injury (TBI).

A prominent cell signaling pathway activated in animal models of both TBI and epilepsy is the mammalian target of rapamycin (mTOR). Inhibition of mTOR with rapamycin has shown promise as a potential modulator of epileptogenesis in several animal models of epilepsy, but cellular mechanisms linking mTOR expression and epileptogenesis are unclear. In this study, the role of mTOR in modifying functional hippocampal circuit reorganization after focal TBI induced by controlled cortical impact (CCI) was investigated. Rapamycin (3 or 10 mg/kg), an inhibitor of mTOR signaling, was administered by intraperitoneal injection beginning on the day of injury and continued daily until tissue collection. Relative to controls, rapamycin treatment reduced dentate granule cell area in the hemisphere ipsilateral to the injury two weeks post-injury. Brain injury resulted in a significant increase in doublecortin immunolabeling in the dentate gyrus ipsilateral to the injury, indicating increased neurogenesis shortly after TBI. Rapamycin treatment prevented the increase in doublecortin labeling, with no overall effect on Fluoro-Jade B staining in the ipsilateral hemisphere, suggesting that rapamycin treatment reduced posttraumatic neurogenesis but did not prevent cell loss after injury. At later times post-injury (8-13 weeks), evidence of mossy fiber sprouting and increased recurrent excitation of dentate granule cells was detected, which were attenuated by rapamycin treatment. Rapamycin treatment also diminished seizure prevalence relative to vehicle-treated controls after TBI. Collectively, these results support a role for adult

Keywords: epilepsy, trauma, hippocampus, mTOR, dentate granule cell, mossy fiber sprouting, adult neurogenesis, doublecortin

neurogenesis in PTE development and suggest that suppression of epileptogenesis by

mTOR inhibition includes effects on post-injury neurogenesis.

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INTRODUCTION

Traumatic brain injury (TBI) can result in post-traumatic epilepsy (PTE) in a significant proportion of moderate to severe TBI patients, and PTE accounts for about 20% of symptomatic epilepsies (Caveness et al., 1979; Annegers et al., 1998; Englander et al., 2003). PTE most commonly manifests as neocortical or temporal lobe epilepsy (TLE; Diaz-Arrastia et al., 2000; Hudak et al., 2004). Preventative therapies for PTE have been largely ineffective or have had varying outcomes depending on the type of epilepsy, leaving ~30% of PTE patients intractable to medical therapies (Temkin et al., 1998, 2001; Temkin, 2009). One treatment proposed to prevent PTE in mice is the use of the mammalian target of rapamycin (mTOR) inhibitor rapamycin after injury (Guo et al., 2013). Rapamycin has shown promise in reducing aberrant axonal sprouting and some forms of epileptogenesis, but its effectiveness in preventing seizures in models of acquired epilepsy has been inconsistent (Zeng et al., 2008; Buckmaster and Wen, 2011; Guo et al., 2013; Heng et al., 2013). Studies of rapamycin effects in chemical convulsant models of TLE indicated that rapamycin reduced or eliminated mossy fiber sprouting, but did not prevent spontaneous seizures (Buckmaster and Wen, 2011; Heng et al., 2013). Rapamycin suppressed the development of PTE in mice after controlled cortical impact (CCI) injury, but mossy fiber sprouting recurred after cessation of treatment (Guo et al., 2013). Although mossy fiber sprouting is a hallmark of TLE in animal models and in patients, its causative association with epilepsy development is still controversial and functional outcomes of rapamycin treatment on synaptic reorganization in models of acquired epilepsy are not well described.

Expanding our understanding of how rapamycin treatment exerts its disease-modifying effects in a model of PTE may identify key antiepileptogenic components of mTOR inhibition and guide future treatments and therapeutics for PTE. The mechanism(s) by which mTOR inhibition may alter epileptogenesis, however, are not fully described. Some of the known biochemical and structural cellular effects of increased mTOR signaling include increased protein synthesis, cell growth, and cell proliferation, which may contribute to several outcomes associated with both TBI and TLE, including mossy fiber sprouting, recurrent excitation of dentate granule cells, and enhanced neurogenesis in the dentate gyrus (Buckmaster and Dudek, 1997; Parent and Lowenstein, 1997; Winokur et al., 2004; Parent et al., 2006). Selective genetic upregulation of mTOR activity in newborn granule cells leads to an epilepsy phenotype (Hester and Danzer, 2013), and increased adult neurogenesis has been hypothesized to contribute substantially to epileptogenesis (Parent et al., 2006; Kron et al., 2010). In this study, we investigated cellular, electrophysiological, and disease modifying effects of rapamycin treatment after CCI in mice, a model of PTE (Hunt et al., 2009, 2010, 2011, 2012; Guo et al., 2013). We tested the hypothesis that continual rapamycin treatment after CCI injury reduces post-injury neurogenesis, mossy fiber sprouting, and synaptic reorganization in the dentate gyrus, which may correlate with reduced seizure expression.

MATERIALS AND METHODS

Animals

Six to eight week old (28-32 g) CD-1 mice (n=139; Harlan, Indianapolis, IN, USA) were housed in a 14 h light/10 h dark cycle. Mice were housed for a minimum of 7 days prior to experimentation in the University of Kentucky vivarium and food and water was provided *ad libitum*. All procedures were approved by the University of Kentucky Animal Care and Use Committee and adhered to NIH guidelines for the care and use of animals.

Traumatic Brain Injury

Mice were subjected to a severe unilateral, cortical contusion injury by CCI, as described previously (Hunt et al., 2009, 2010, 2011, 2012). Briefly, mice were anesthetized by 2% isoflurane inhalation and placed in a stereotaxic frame. The skull was exposed by midline incision, and a \sim 5 mm craniotomy was made lateral to the sagittal suture and centered between bregma and lambda. The skull cap was removed, taking care to avoid damage to the exposed underlying dura. The contusion device consisted of a computer-controlled, pneumatically driven impactor fitted with a beveled stainless-steel tip 3 mm in diameter (TBI-0310; Precision Systems and Instrumentation, Fairfax, VA, USA). Brain injury was delivered using this device to compress the cortex to a depth of 1.0 mm at a velocity of 3.5 m/s and 500 ms duration. This brain injury model consistently produced a focal cortical lesion. Although there is no direct damage to the hippocampus from the injury, hippocampal evulsion usually occurs (Hunt et al., 2009, 2012). A qualitative postoperative health assessment was performed daily for 4 days after CCI and periodically thereafter.

Rapamycin Injection

Rapamycin (LC Laboratories, Woburn, MA, USA) was initially dissolved in 100% ethanol (20 mg/ml), stored at −20°C, and diluted in a vehicle solution containing 5% Tween 80, 5% PEG 400, and 4% ethanol (all from Fisher Scientific, Pittsburgh, PA, USA) dissolved in distilled, deionized water immediately before intraperitoneal (i.p.) injection (Guo et al., 2013; Heng et al., 2013). Rapamycin (3 mg/kg or 10 mg/kg) or vehicle was injected i.p. after mice regained consciousness following CCI injury (20-30 min) and the treatment was continued once daily until the day of experimentation. Hippocampal homogenates from mice 24 h after injury indicated an increase in phosphorylated S6 protein (pS6) levels in the ipsilateral hemisphere of CCI-injured mice with vehicle treatment; rapamycin treatment reduced pS6 to sham levels at this post-injury time point (unpublished observation), similar to previous reports (Buckmaster et al., 2009; Zeng et al., 2009; Guo et al., 2013).

Slice Preparation

Slices used for electrophysiological studies were obtained from mice 8–13 weeks post-CCI. Mice were deeply anesthetized by isoflurane inhalation to effect (i.e., lack of tail pinch response) and decapitated while anesthetized. The brain was removed and

placed in ice-cold (2-4°C) oxygenated artificial cerebrospinal fluid (ACSF) containing, in mM: 124 NaCl, 3 KCl, 1.3 CaCl₂, 26 NaHCO₃, 1.3 MgCl₂, 11 glucose and 1.4 NaH₂PO₄ equilibrated with 95% O₂₋5% CO₂ (pH 7.2-7.4). Brains were blocked and glued to a sectioning stage, and 350 µm-thick slices were cut in the coronal or horizontal plane in cold, oxygenated ACSF using a vibrating microtome (Vibratome Series 1000; Technical Products International, St. Louis, MO, USA). The hippocampus was isolated from surrounding tissue, making sure to completely remove the entorhinal cortex. Slices were transferred to a chamber containing oxygenated ACSF at 32-34°C, where they were equilibrated for at least one hour prior to recording. Slices of the septal and temporal hippocampus from the hemispheres ipsilateral and contralateral to CCI injury were used in these experiments and compared to comparable slices from shaminjured mice (i.e., craniotomy, but no impact injury).

Extracellular Field Potential Recordings

Field potential recordings were obtained from the granule cell layer of the dentate gyrus in horizontal slices. Slices were placed into a submersion type recording chamber (RC21-BW, Warner Instruments, Hamden, CT, USA) on an upright, fixed stage microscope (Olympus BX50WI, Center Valley, PA, USA) and continuously perfused with oxygenated, nominally Mg²⁺ free ACSF containing 30 μM bicuculline to block GABA_A receptors, and unmask recurrent excitation (Winokur et al., 2004; Hunt et al., 2009). Extracellular recording electrodes were filled with 1 M NaCl and placed near the apex of the dentate granule cell layer. A concentric bipolar electrode made of platinum-iridium wire (125 µm, FHC Inc., Bowdoinham, ME, USA) was used to apply a single stimulus to the mossy fiber pathway at 0.1 Hz. Stimulus intensity was adjusted to evoke a population response of ~50% maximum amplitude after a single stimulus. Electrical signals were recorded using an Axopatch 200 B amplifier (Axon Instruments, Sunnyvale, CA, USA), low pass filtered at 2-5 kHz, digitized at 20 kHz using a 1322A Digidata (Axon Instruments), and analyzed on a PC computer using pClamp 10.2 (Clampfit, Molecular Devices, Sunnyvale, CA, USA). The number of population spikes following antidromic stimulation of mossy fibers in the hilus was measured as described previously (Hunt et al., 2009).

Whole Cell Recordings

Coronal hippocampal slices containing the dorsal third of the dentate gyrus were transferred to a recording chamber on an upright, fixed-stage microscope equipped with infrared, differential interference contrast optics (i.e., IR-DIC; Olympus BX50WI), where they were perfused with continuously warmed (32–34°C) ACSF. Recordings were performed from dentate granule cells, which were identified using DIC imaging. Recording pipettes were pulled from borosilicate glass (1.65 mm outer diameter, 0.45 mm inner diameter; King Precision Glass, Claremont, CA, USA) with a P-87 puller (Sutter Instrument, Novato, CA, USA). The intracellular solution contained (in mM): 130 K⁺-gluconate, 1 NaCl, 5 EGTA, 10 HEPES, 1 MgCl₂, 1 CaCl₂, 3 KOH, and 2 ATP. Open tip series resistance was 2–5

MOhms. Recordings were obtained using an Axon Multiclamp 700 B amplifier (Molecular Devices), low-pass filtered at 6 kHz, digitized at 20 kHz with a Digidata 1550A (Molecular Devices), and acquired using pClamp 10.5 programs (Clampfit, Molecular Devices). Cells were voltage-clamped at -70 mV for 5–10 min to allow equilibration of pipette and intercellular solutions prior to data collection, after which time whole-cell patch-clamp recordings of spontaneous excitatory postsynaptic currents (sEPSCs) were obtained. All sEPSCs were assessed over a 2–3 min period (>100 events) to assess frequency; amplitude was measured only from unitary events. Although the investigator was not blinded to experimental group for electrophysiological experiments, the investigator was blinded to experimental group for all offline data analyses.

Timm Staining

After the recording experiment concluded, slices were placed in 0.1 M sodium phosphate buffer containing 0.37% sodium sulfide (pH 7.4) for \sim 30 min followed by 4% paraformaldehyde in 0.15M sodium phosphate buffer (pH = 7.4) overnight. Slices were then equilibrated in a 30% sucrose solution in phosphate buffered saline (PBS; 0.1 M) overnight, embedded in Optimal Cutting Temperature (OCT) compound (Fisher Scientific), sectioned at 30 µm on a cryostat, rinsed in PBS, mounted on charged slides (Superfrost Plus; Fisher Scientific), and dried on a slide warmer. Sections were subsequently treated as in previous protocols, using Timm stain to reveal mossy fibers and Nissl counterstain to reveal cell bodies (Tauck and Nadler, 1985; Shibley and Smith, 2002; Winokur et al., 2004; Hunt et al., 2009, 2010, 2011, 2012; Bhaskaran and Smith, 2010). To semi-qualitatively assess mossy fiber sprouting after CCI, sections at equivalent positions relative to bregma ipsilateral and contralateral to injury were examined and assigned Timm scores ranging from 0-3, with a score of 0 corresponding to little to no granular staining, 1 indicating moderate Timm staining through the granule cell layer, but not into the inner molecular layer, 2 indicating continuous staining through the granule cell layer with discontinuous puncta in the inner molecular layer, and 3 indicting a continuous band of staining in the inner molecular layer. The scorer was blinded to treatment. Using a modified scoring scale (Hunt et al., 2009, 2010), regions of the dentate gyrus with Timm scores >1 were considered to exhibit mossy fiber sprouting (Tauck and Nadler, 1985; Patrylo and Dudek, 1998; Shibley and Smith, 2002; Hunt et al., 2009, 2010, 2011, 2012). To obtain the Timm score for each animal, each 350 µm slice used for recording was analyzed using two 30 μ m sections, ~180 μ m apart mounted onto slides. Slices at equivalent hippocampal levels ipsilateral and contralateral to the injury were assessed for each animal. Each blade of the dentate gyrus was assessed independently and the average score of the two blades was given for each slice. The scores from each slice were then averaged per hemisphere per animal to obtain the Timm score for each animal.

Immunohistochemistry

Mice were perfused transcardially with a 0.15M sodium phosphate buffer followed by 4% paraformaldehyde fixative

solution (0.15M sodium phosphate buffer). The brain was removed and placed in fixative overnight and then transferred to a 30% sucrose solution in PBS until the tissue equilibrated. Brains were covered in OCT compound and sectioned serially on a cryostat (-22°C) at 20 μm. Sections (every 6th section in series) were rinsed in Tris-buffered saline (TBS; pH = 7.4) briefly before being mounted onto slides and incubated in a solution containing Triton X-100 (0.3%) and normal goat serum (10%) in TBS for 30 min at room temperature. Sections were then incubated overnight at 4°C with a rabbit primary antibody against doublecortin (DCX; 1:5000; Abcam; Cambridge, MA, USA) in blocking solution (2% normal goat serum; 0.15% Triton X-100; TBS). Sections were rinsed 3 times for 5 min in blocking solution and then incubated for 1 h at room temperature in a goat anti-rabbit secondary antibody (IgG) conjugated to Alexa Flour 488 (IgG; 1:1000; Molecular Probes; Grand Island, NY, USA) in the same blocking solution. Sections were then rinsed 3 times for 5 min with TBS. Slides were covered with Vectashield mounting medium with DAPI (Vector Labs; Burlingame, CA, USA) to image immunofluorescence.

Dentate Granule Cell Layer Area

In order to measure changes in area of the dentate granule cell layer after CCI, adjacent tissue sections to those used for DCX labeling were stained with cresyl violet (i.e., every 6th section in series). Sections were imaged using a SPOT RT camera (Diagnostic Instruments; Sterling Heights, MI, USA) mounted on an upright microscope (BX-40; Olympus) and dentate granule cell area measurements were made using ImageJ software. Images were taken at 4× and 10× magnification to capture the entire dentate gyrus. After scaling the ImageJ software, the cresyl violet-stained dentate granule cell layer was traced freehand and the area measured. The area measurements were assessed in two different ways. The first was to assess overall changes in dentate granule cell layer area across the hippocampus from approximately -1.22 to -3.52 mm from bregma. In this assessment, measurements from each tissue section were made to obtain the overall average of the dentate granule cell layer area for each hemisphere in each animal. Additionally, this data set was assessed as a function of anatomical location along the septo-temporal hippocampal axis relative to the injury epicenter using a mouse brain atlas (Paxinos and Franklin, 1997).

Fluoro-Jade B (FJB) Staining

Protocols used were similar to those reported previously (Hall et al., 2008). In brief, sections (30 μ m) from perfused brains were mounted on slides and treated with a solution of 1% NaOH in 80% ethanol for 5 min followed by 70% ethanol (2 min) and distilled water (2 min). Sections were then incubated in a 0.06% permanganate solution for 10 min on a rotating stage, rinsed in distilled water (3 min) and incubated in a 0.0004% solution of FJB (Histo-Chem Inc., Jefferson, AR, USA; 10 min). They were then rinsed in distilled water and air dried before being placed on a 50°C slide warmer for 30 min. They were then placed in xylene for 20 min and coverslipped in permount.

Cell Counts

Numbers of DCX-immunolabeled dentate granule cells and FJBlabeled neurons were counted between -1.22 to -3.52 mm from bregma in the upper and lower blade of the dentate granule cell layer at 20× and 40× magnification (Olympus, BX40) by an investigator blinded to animal treatment. For figures, representative images were taken at 4× and 10× magnification to display the whole dentate gyrus. DCX cell counts were normalized to dentate granule cell layer area, as above. The area of the dentate gyrus and hilus was obtained to normalize FJB labeling counts and included the dentate granule cell layer and the hilus inside the region outlined by a line around the outer edge of the granule cell layer and connecting the tips of the dentate granule cell layer with the most proximal point of the CA3 pyramidal cell layer. For each animal, cell density was calculated per section and then averaged across the entire hippocampus to obtain an overall measure of cell density. Additionally, cell density was measured as a function of distance from bregma. For these measurements tissue sections were placed in anatomical order using a mouse brain atlas (Paxinos and Franklin, 1997) and cell density was averaged for each animal at each anatomical location.

Seizure Observations

As described previously (Hunt et al., 2009), mice were monitored for behavioral seizures by observation for 6 h per week beginning at 6 weeks post-injury and ending 10 weeks post-injury. Using a modified Racine scale (Racine, 1972) only behavioral seizures at or above a grade 2 (i.e., prolonged freezing and wet dog shakes) and lasting longer than 10 s were counted as behavioral seizures.

RESULTS

We compared cellular and behavioral outcomes (i.e., FJB staining, DGC area, DCX staining, mossy fiber sprouting, field potential responses, sEPSC frequency, and seizures) in the dentate gyrus of mice from sham-injured, CCI-injured with vehicle treatment, and CCI-injured with rapamycin treatment (3 or 10 mg/kg). For cellular outcomes, each hemisphere was assessed independently for all groups. Initial comparisons between hemispheres from sham-injured mice and the hemisphere contralateral to CCI injury in vehicle- and rapamycin-treated mice indicated there were no differences in the cellular outcomes for these groups. Data from sham and contralateral hemispheres were compared using a Oneway ANOVA with Tukey's post hoc analysis, or Kruskal Wallis/Chi-square statistic where necessary, and significance was set at p < 0.05. No significant differences were found for any measure between sham-injured mice and those made in the hemisphere contralateral to CCI injury (p > 0.05). For each analysis of cellular outcomes, these groups were therefore combined as a single control group for clarity of presentation. Table 1 compares results of measurements from both hemispheres after sham surgery with results from the hemisphere contralateral to CCI injury in mice treated with vehicle or rapamycin.

Behavioral Seizure Monitoring

Subsets of mice were monitored for behavioral seizures after severe (1.0 mm depth) unilateral CCI injury from 6-10 weeks post-injury (6 h/week) to qualitatively assess spontaneous seizure development. Consistent with previous reports, four of 10 mice (40%) that received CCI injury and vehicle treatment displayed spontaneous seizures during this period post-injury (Hunt et al., 2009, 2010; Guo et al., 2013). All were S2 seizures with tail stiffness and freezing for more than 30 s. One of twelve mice (8%) in the low-dose (3 mg/kg) rapamycin-treated group and one of 11 mice (9%) in the high-dose (10 mg/kg) rapamycintreated group were observed to have spontaneous seizures (all category S2). Although numerically lower in the rapamycin treatment groups, and a trend toward reduced seizures was apparent, the prevalence of observed seizures in CCI injured mice with no treatment and CCI injured mice with rapamycin treatment was not significantly different using a Chi-square test of probability (3 mg/kg, p = 0.078; 10 mg/kg, p = 0.097). These data indicate that mTOR activity may influence seizure development after CCI in some cases, but mTOR inhibition is not sufficient to prevent epileptogenesis after CCI injury in all mice.

Fluoro-Jade B (FJB) Labeling

Regional cell loss (i.e., dentate gyrus, hilus, and CA3 pyramids) in the hippocampus is a common feature after TBI (Lowenstein et al., 1992; Hicks et al., 1993; Smith et al., 1995). The role of mTOR in neuronal death and survival after TBI has been controversial (Guo et al., 2013; Tanaka et al., 2013). This is likely due to the complex role mTOR plays in the balance of autophagy and apoptosis after injury. Based on previous reports, FJB staining in the ipsilateral hemisphere peaks following CCI injury in the first 3 days after injury (Anderson et al., 2005). We therefore measured FJB staining at 72 h post-CCI injury to evaluate this peak FJB staining. Representative images of FJB stained sections from CCI-injured mice treated with vehicle, low-dose rapamycin (3 mg/kg), and high-dose rapamycin (10 mg/kg) in hemispheres ipsilateral to the injury

are shown in **Figure 1A**. The granule cell layer and hilus of mice with CCI injury + vehicle (309.1 \pm 37.8 FJB-positive cells/mm²; n=7 mice), CCI + low-dose rapamycin (256.6 \pm 27.3 FJB-positive cells/mm²; n=6 mice), and CCI + high-dose rapamycin (238.6 \pm 27.0 FJB-positive cells/mm²; n=7 mice) treatment displayed significantly more FJB-labeled cells ipsilateral to injury than sham-operated, vehicle-treated controls (7.38 \pm 0.73 FJB-positive cells/mm²; n=20; one-way ANOVA; ($F_{(3,37)}=66.58$, Tukey's; p<0.0001; **Figure 1B**). There was no difference in the density of FJB-labeled cells between the ipsilateral hemispheres of vehicle- or rapamycin-treated mice after CCI injury (p>0.05).

Previously, rapamycin treatment was reported to reduce FJB staining in one region of the dentate gyrus (Guo et al., 2013), so we investigated FJB staining throughout the septotemporal dentate gyrus and hilus at 180 μm intervals. We found only two intervals in which FJB staining in CCI-injured mice treated with high dose rapamycin was reduced in the ipsilateral hemisphere relative to CCI + vehicle (Figure 1C). These locations were between -2.62 to -3.18 mm from bregma, which is $\sim\!1$ mm posterior to injury epicenter (Figure 1C). There was no significant reduction in FJB staining at any other location along the hippocampal axis after CCI injury in rapamycin-treated mice and FJB labeling was significantly increased relative to controls at all septo-temporal locations.

Dentate Granule Cell Layer Area

Both cell death and cell proliferation are common features in the dentate granule cell layer following injuries such as TBI and seizures (Parent et al., 1997; Rola et al., 2006; Carlson et al., 2014). After TBI in rodents, a reduction in dentate granule cell count or area has been observed early (48 h) after injury (Smith et al., 1995). This reduction persists for up to 7 days (Wiltgen et al., 2009) and is alleviated by 2 weeks post-injury (Grady et al., 2003), suggesting cell proliferation compensates for early cell loss after injury. To test the effect of rapamycin on dentate granule cell layer thickness at a time point corresponding to its restoration after CCI injury, dentate granule cell layer area

TABLE 1 | Measures from sham-operated mice (both hemispheres) and the hemisphere contralateral to injury after CCI in mice treated with vehicle or rapamycin.

Group	FJB + cell density (FJB-positive cells/mm²)	DGC area (mm²)	DCX + cell density (DCX-positive cells/mm ²)	Timm score	Recurrent excitation (% slices with secondary depolarization)	sEPSC frequency (Hz)
Sham	N/A	0.130 ± 0.012	774.50 ± 96.37	0.285 ± 0.02	0% (0/5 slices)	N/A
(contralateral)		(n = 7)	(n = 6)			
Sham	N/A	0.130 ± 0.012	637.42 ± 44.42	0.248 ± 0.047	20% (1/5 slices)	N/A
(ipsilateral)		(n = 7)	(n = 6)			
CCI + vehicle	7.07 ± 1.20	0.133 ± 0.013	957.00 ± 133.99	0.260 ± 0.034	25% (2/8 slices)	0.83 ± 0.11
(contralateral)	(n = 7)	(n = 8)	(n = 6)			(n = 14)
CCI + Rapa	8.33 ± 1.30	0.130 ± 0.013	742.77 ± 142.46	0.333 ± 0.108	20% (2/10 slices)	0.5 ± 0.10
(3 mg/kg; contralateral)	(n = 7)	(n = 6)	(n = 5)			(n = 9)
CCI + Rapa	7.19 ± 1.32	0.126 ± 0.014	760.86 ± 131.20	0.437 ± 0.091	25% (2/8 slices)	0.81 ± 0.26
(10 mg/kg; contralateral)	(n = 6)	(n = 7)	(n = 6)			(n = 12)

One-way ANOVA with Tukey's post hoc analysis, or Kruskal Wallis/Chi-square statistic where appropriate, were used to obtain the p-value; p > 0.05 for all analyses.

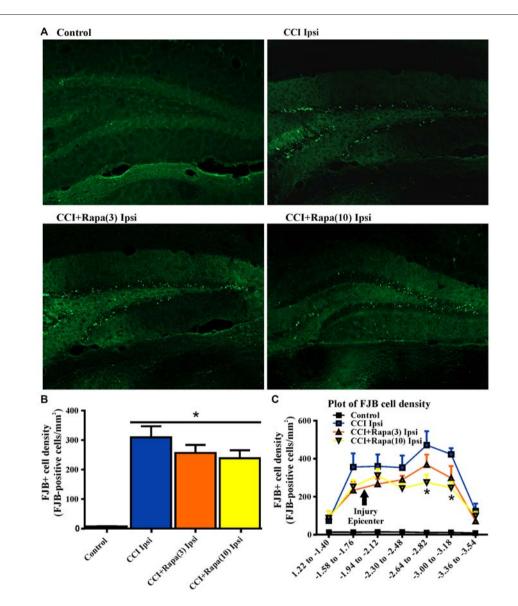


FIGURE 1 | Fluoro-Jade B labeling in dentate gyrus 3 days after control treatment, CCI or CCI with daily rapamycin administration. (A) Representative images of Fluoro-Jade B (FJB) labeling from four different groups: control, ipsilateral to CCI injury + vehicle (CCI lpsi), ipsilateral to CCI injury + rapamycin at 3 mg/kg (CCI + Rapa(3) lpsi), and ipsilateral to CCI injury + rapamycin at 10 mg/kg (CCI + Rapa(10) lpsi). (B) Mean FJB labeling in control, CCI lpsi, CCI + Rapa(3) lpsi, and CCI + Rapa(10) lpsi groups. FJB-positive cell density (cells/mm²) was averaged across coronal slices from -1.22 to -3.52 mm from bregma. All CCI ipsilateral hemispheres exhibited increased FJB-positive cell density relative to controls. No significant difference was observed after CCI injury between vehicle- and rapamycin-treated mice. (C) FJB-positive cells/mm² as a function of distance from bregma along the septo-temporal axis of hippocampus. The CCI + Rapa(10) group exhibited reduced FJB-positive cell density only in a limited region of posterior hippocampus relative to CCI + vehicle treatment. Error bars indicate SEM; *p < 0.05.

was measured 14 days after injury in Nissl stained sections from control $(0.125 \pm 0.006 \text{ mm}^2; n = 35; \text{Figure 2B})$, CCI with vehicle $(0.110 \pm 0.009 \text{ mm}^2; n = 8; \text{Figure 2B})$, CCI with low-dose rapamycin treatment $(0.094 \pm 0.010 \text{ mm}^2; n = 6; \text{Figure 2B})$, and CCI with high-dose rapamycin treatment $(0.093 \pm 0.005 \text{ mm}^2; n = 7; \text{Figure 2B})$. Representative images of Nissl stained sections are shown in **Figure 2A**. Rapamycin treatment resulted in a significant reduction of DGC layer area ipsilateral to the injury relative to control (One-Way ANOVA; $F_{(3,41)} = 6.476$, Tukey's;

p = 0.0003; **Figure 2B**). There was a trend toward decreased granule cell layer area after CCI, but a significant change was not detected (p = 0.0846).

Doublecortin (DCX) Immunolabeling

Shortly after CCI injury there is an initial decrease in DCX expression ipsilateral to injury, but from days 7–14 after injury an increase in DCX expression ipsilateral to the injury has been reported (Dash et al., 2001; Rola et al., 2006; Barha

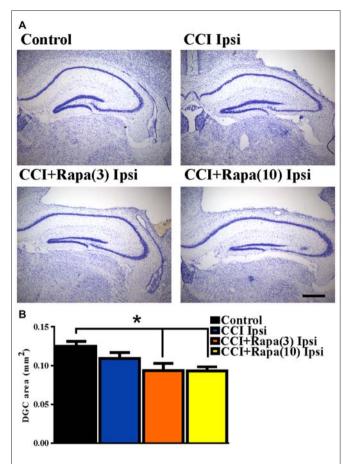


FIGURE 2 | Dentate granule cell area 14 days after injury in mice from control, CCI-injured, and CCI-injured with rapamycin treatment. (A) Representative images of Nissl stained sections ipsilateral to injury from the four different treatment groups: sham-operated control, CCI injury + vehicle (CCI lpsi), CCI injury + 3 mg/kg rapamycin (CCI + Rapa(3) lpsi), and CCI injury + 10 mg/kg rapamycin (CCI + Rapa(10) lpsi). Hippocampal sections were from similar anterior-posterior coordinates. (B) Mean dentate granule cell area from Nissl stained sections in control, CCI lpsi, CCI + Rapa(3) lpsi, and CCI + Rapa(10) lpsi groups. Scale bar indicates 0.5 mm. Error bars indicate SEM; *p < 0.05.

et al., 2011). This proliferation of newborn dentate granule cells after injury or status epilepticus has been proposed to contribute substantially to epileptogenesis (Parent et al., 2006; Hester and Danzer, 2013; Lasarge et al., 2015). Therefore, DCX expression was examined 14 days after injury in control mice (n = 29), vehicle-treated CCI-injured mice (n = 6), CCIinjured mice with low-dose rapamycin treatment (n = 5), and CCI-injured mice with high-dose rapamycin treatment (n = 6). Figure 3A shows representative images of DCX staining from the dentate gyrus ipsilateral to the injury (or sham surgery) in these groups. In the ipsilateral hemisphere, a significant increase in DCX expression was observed in vehicle-treated CCI-injured mice relative to controls (Control: 730.04 ± 51.71 DCX-positive cells/mm²; CCI: 1154.15 \pm 114 DCX-positive cells/mm²; One Way ANOVA; $F_{(3,39)} = 4.838$, Tukey's; p = 0.0059; **Figure 3B**). The relative increase in DCX expression after CCI was observed up to ~1.5 mm temporal to the injury epicenter (**Figure 3C**). Rapamycin treatment after CCI injury significantly reduced DCX expression to levels similar to control (rapamycin 3 mg/kg: 653.94 ± 85.99 DCX-positive cells/mm², p = 0.6098 vs. control; rapamycin 10 mg/kg: 728.51 ± 117.2 DCX-positive cells/mm², p = 0.8848 vs. control; **Figure 3B**). These results are consistent with an inhibitory effect of rapamycin treatment on post-injury neurogenesis.

Timm Staining

Several weeks after CCI, there is an increase in Timm staining in the inner molecular layer of the dentate gyrus ipsilateral to the injury relative to the contralateral hemisphere or in sham-treated mice (Hunt et al., 2009, 2010, 2011, 2012; Guo et al., 2013). Rapamycin treatment for 4 weeks post-injury reduced Timm staining five weeks after CCI (Guo et al., 2013). However, as with studies done in the pilocarpine-induced status epilepticus model of TLE (Buckmaster et al., 2009), mossy fiber sprouting recurred after cessation of treatment. To assess the effects of continuous rapamycin treatment on mossy fiber sprouting after injury, Timm staining was examined in control mice and in CCI-injured mice treated daily for 8-13 weeks with rapamycin or vehicle. Slices used for extracellular field potential recordings, as well as mice perfused for histology, were used for Timm staining measurements. There was no significant difference between the contralateral hemispheres of any of the groups (all exhibited Timm scores < 1; **Table 1**). Figure 4A shows representative images of Timm stained sections ipsilateral to the injury from control, CCI + vehicle, CCI + rapamycin (3 mg/kg), and CCI + rapamycin (10 mg/kg) treated mice. In vehicle-treated mice, Timm scores in hemispheres ipsilateral to CCI injury were increased relative to control hemispheres (control: 0.364 \pm 0.05, n = 31; vehicle + CCI: 2.335 ± 0.300 , n = 12; Kruskal Wallis stat = 41.41, p < 0.0001; Figure 4B). In low-dose rapamycin-treated mice, Timm scores were reduced (1.275 \pm 0.315, n = 12, Figure 4B) ipsilateral to the injury relative to vehicle-treated mice after CCI injury (p = 0.025), but remained greater than in controls (p < 0.0001). Mossy fiber sprouting was not different from controls in the high-dose rapamycin treatment group (0.55 \pm 0.09, n = 11; Kruskal Wallis stat = 16.41; p = 0.145 vs. control; **Figure 4B**). Although the average mossy fiber sprouting score was reduced in mice that received rapamycin treatment, localized areas of mossy fiber sprouting into the inner molecular layer were always observed in the dentate gyrus of mice that expressed spontaneous behavioral seizures. These data indicated that continual rapamycin treatment reduced mossy fiber sprouting after CCI injury and this reduction was maintained during treatment for up to 12 weeks.

Network Excitability in Dentate Gyrus

Increased network excitability (Hunt et al., 2009) and synaptic connectivity between granule cells (Hunt et al., 2010) emerge in the dentate gyrus several weeks after CCI injury. Antidromically-evoked field potentials following electrical stimulation of the hilus were examined in slices perfused with nominally Mg²⁺ free ACSF with bicuculline (30 μ M) 8–13 weeks after injury.

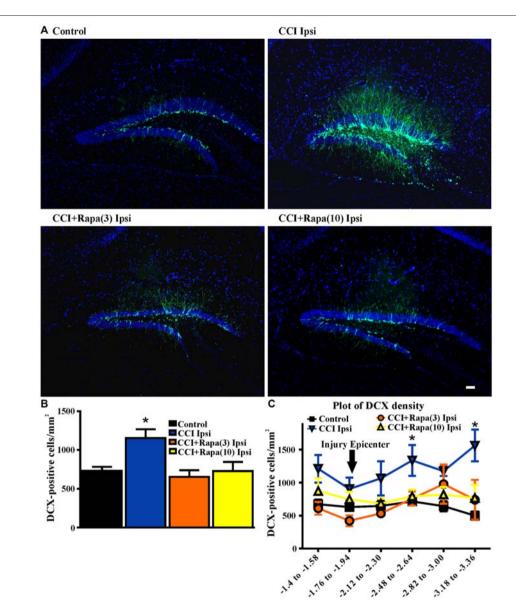


FIGURE 3 | Doublecortin (DCX) immunolabeling in dentate gyrus 14 days after injury in mice from control, CCI-injured, and CCI-injured with rapamycin groups. (A) Representative images of DCX expression from four treatment groups: control, CCI lpsi, CCI + Rapa(3) lpsi, and CCI + Rapa(10) lpsi. (B) Mean DCX expression in control, CCI lpsi, CCI + Rapa(3) lpsi, and CCI + Rapa(10) lpsi groups. Ipsilateral CCI exhibited greater DCX-positive cell density compared to controls. The injury-induced increase in DCX-positive cell density in CCI ipsi mice was not observed in either CCI + Rapa treatment group. (C) DCX-positive cells/mm² as a function of distance from bregma along septo-temporal axis of hippocampus. Scale bar indicates 0.1 mm. Error bars indicate SEM; *p < 0.05.

In these recordings, a single antidromic population spike was elicited after hilar stimulation in most slices (29/36) from five control animals (**Table 1**; **Figure 5**). In contrast, a secondary after discharge was observed in most slices (11/14 slices from 9 mice) from the ipsilateral hemisphere of vehicle-treated, CCI-injured mice (**Figure 5**), similar to previous findings at the same time point post-injury in this model (Hunt et al., 2009; Chi-square statistic = 15.295; p < 0.0001; **Figure 5B**). Low-dose rapamycin treatment reduced, but did not normalize the percentage of slices with secondary depolarization in the ipsilateral hemisphere (5/9)

slices from 8 mice; Chi square statistic = 1.3707; p = 0.241 vs. CCI ipsilateral; **Figure 5B**). In mice treated with high-dose rapamycin, the percentage of slices ipsilateral to the injury that responded with a secondary depolarization (2/10 slices from 10 mice) was significantly lower than for vehicle-treated CCI-injured mice (Chi-square statistic = 8.0607; p = 0.0045; **Figure 5B**), and was similar to controls (p = 0.9687). Notably, 86% of slices from CCI-injured, rapamycin-treated mice that displayed increased network excitability also exhibited localized mossy fiber sprouting near the recording site upon *post*

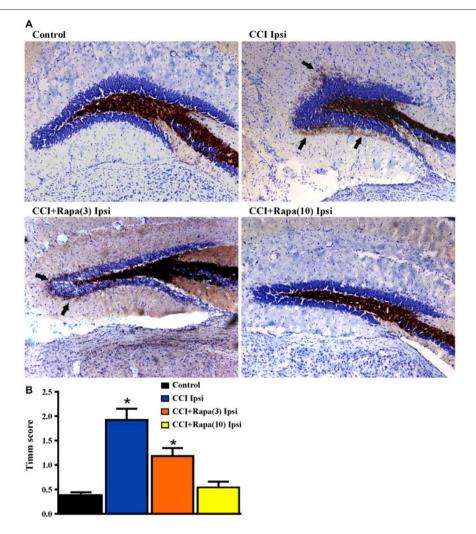


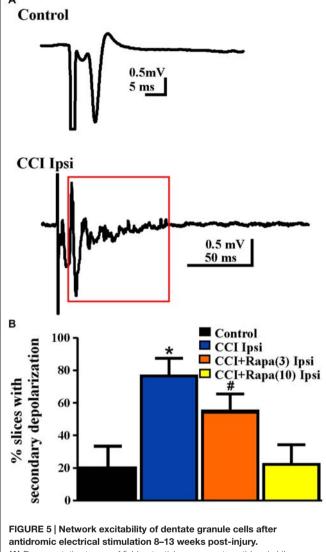
FIGURE 4 | Timm staining in the dentate gyrus 8–13 weeks post-injury from sham-operated control, CCI injured, and CCI-injured with rapamycin treatment groups. (A) Representative images of Timm staining from the four different groups: control, CCI lpsi, CCI + Rapa(3) lpsi, and CCI + Rapa(10) lpsi. (B) Mean Timm scores in control, CCI lpsi, CCI + Rapa(3) lpsi, and CCI + Rapa(10) lpsi groups. The injured hemisphere of both CCI lpsi and CCI + Rapa(3) lpsi groups exhibited higher Timm scores relative to the control group. Mice receiving rapamycin treatment (10 mg/kg) after CCI had Timm scores similar to controls. Error bars indicate SEM; *p < 0.05.

hoc examination. Overall, rapamycin treatment after CCI significantly reduced dentate granule cell network excitability following hilar antidromic stimulation, but excitability was maintained in slices with mossy fiber sprouting.

sEPSCs in DGCs

Spontaneous EPSCs (sEPSCs) were recorded from dentate granule cells in slices from vehicle- and rapamycin-treated mice 8–13 weeks after CCI. Slices were perfused with nominally Mg²⁺ -free ACSF containing 30 μ M bicuculline and cells were voltage-clamped at -70 mV (**Figure 6A**). sEPSC frequency was greater in DGCs ipsilateral to CCI injury relative to controls (control: 0.72 ± 0.08 Hz; n = 29, CCI: 1.51 ± 0.38 Hz, n = 14 cells from 7 mice; $F_{(3,56)} = 5.336$, p = 0.0026; One Way ANOVA, Tukey's; **Figure 6B**). The increase in sEPSC frequency after

CCI injury was reduced in rapamycin-treated mice (CCI + rapamycin 3 mg/kg: 1.16 ± 0.18 Hz; n = 11 cells from 9 mice; p = 0.187 vs. CCI + vehicle; CCI + rapamycin 10 mg/kg: 0.67 ± 0.30 Hz; n = 12 cells from 7 mice; p = 0.0267 vs. CCI + vehicle). Relative to controls, however, sEPSC frequency in dentate granule cells from rapamycin-treated (3 mg/kg) mice remained significantly elevated (p = 0.0154), with no difference between controls and rapamycin treated (10 mg/kg) mice (p = 0.847). No differences in sEPSC amplitude were found between any of the experimental groups (Control: 12.04 \pm 0.49 pA; CCI + vehicle: 11.12 \pm 1.09 pA; CCI + rapamycin 3 mg/kg: 12.06 \pm 0.78 pA; CCI + rapamycin 10 mg/kg: 10.84 \pm 1.54 pA; p = 0.6894; One Way ANOVA, Tukey's; **Figure 6B**). Increased sEPSC frequency was observed ipsilateral to the injury after CCI. Low-dose rapamycin treatment reduced, but did not eliminate this increase, whereas sEPSC frequency was



(A) Representative traces of field potential responses to antidromic hilar stimulation in control and CCI-injured mice. A secondary depolarization was often observed in slices ipsilateral to CCI injury (red box indicates area of secondary depolarization). (B) Percentage of slices ipsilateral to injury that displayed secondary depolarization from control, CCI lpsi, CCI + Rapa(3) lpsi, and CCI + Rapa(10) lpsi treatment groups. Error bars represent SEM; $^{\ast}p < 0.05$ for CCI lpsi relative to control or CCI + Rapa(10). $^{\#}$ Indicates p < 0.05 for CCI + Rapa(3) lpsi vs. control.

similar to controls in mice treated with 10 mg/kg rapamycin daily.

DISCUSSION

Multiple outcome measures associated with epileptogenesis after CCI have been established in the dentate gyrus, allowing for mechanistic investigation of cellular events subsequent to TBI. Within a few weeks after CCI trauma in mice, sprouting of dentate granule cell axons to proximal granule cell dendrites in the inner molecular layer of the dentate gyrus (i.e., mossy fiber sprouting) occurs, synaptic reorganization

of dentate granule cells is observed near the injury site, and mice develop spontaneous seizures after several weeks (Hunt et al., 2009, 2010; Guo et al., 2013). This study focused on the role of mTOR signaling in PTE development using the CCI model of TBI. Previous studies on the effects of rapamycin treatment in acquired epilepsy models have focused mainly on the anatomical phenotype of mossy fiber sprouting and the functional correlation with seizure frequency (Buckmaster and Lew, 2011; Guo et al., 2013). However, increased mossy fiber sprouting and seizure frequency were noted after cessation of rapamycin treatment, suggesting epileptogenic mechanisms that trigger mTOR activity and subsequent neurogenesis or other cellular activity post-injury may be sustained, although the activity itself is suppressed during rapamycin treatment. To avoid confounds associated with reemergence of mTOR activityrelated phenotypes after cessation of rapamycin treatment, we continued rapamycin treatment daily throughout the duration of our experiments. Here, we report that rapamycin treatment after CCI injury inhibits the progression of epileptogenesis after focal brain injury in a manner that involves effects on several cellular outcomes associated with development of spontaneous seizures after TBI, including post-injury neurogenesis, mossy fiber sprouting, and synaptic reorganization in the dentate gyrus ipsilateral to the injury. No differences were observed contralateral to injury, implying rapamycin alone had little effect compared to uninjured controls. Both doses of daily rapamycin treatment (3 and 10 mg/kg) were effective in reducing the proportion of mice that developed spontaneous seizures, consistent with effects of an intermediate dose (6 mg/kg) administered for four weeks after CCI injury (Guo et al., 2013). Both rapamycin doses also significantly reduced post-injury neurogenesis in the granule cell layer. Other outcomes, including mossy fiber sprouting and elevated synaptic excitation were reduced, but not abolished by the lower rapamycin dose, but were abrogated by the high-dose regimen. Granule cell layer area and FJB staining measurements indicated that neither rapamycin dose significantly reduced overall neuronal death after CCI, but cell death was reduced by the high dose regimen at a specific site along the septo-temporal hippocampal axis. The lack of effect on cell death is consistent with the reemergence of spontaneous seizures after cessation of rapamycin treatment in previous reports and suggests that important components of the underlying injury that triggers the eventual development of epilepsy are not abrogated by mTOR inhibition, even though several other cellular correlates of epileptogenesis are suppressed during high-dose rapamycin treatment.

Newborn Neurons

The continual adult generation of select neuron populations, including within the subgranular zone (SGZ) of the hippocampus (Altman and Das, 1965; Eriksson et al., 1998), remains one of the least well understood types of experience-dependent brain plasticity. Adult neurogenesis has been proposed to either decrease (Gould and Tanapat, 1997; Rola et al., 2006) or increase after TBI (Liu et al., 1998; Parent et al., 1998; Dash et al., 2001; Arvidsson et al., 2002; Chirumamilla et al.,

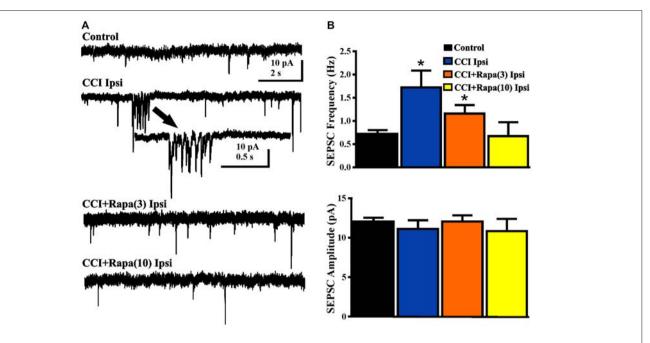


FIGURE 6 | Spontaneous EPSCs (sEPSCs) in dentate granule cells 8–13 weeks post-injury. (A) Representative traces of sEPSCs in dentate granule cells ipsilateral to injury from four treatment groups: sham-operated control, CCl injury + vehicle (CCl Ipsi), CCl injury + 3 mg/kg rapamycin (CCl + Rapa(3)), and CCl injury + 10 mg/kg rapamycin (CCl + Rapa(10)). All recordings were performed in the presence of nominally Mg^{2+} -free ACSF containing 30 μ M bicuculline. Arrow indicates expanded example of a burst of sEPSCs in a dentate granule cell from the vehicle-treated CCl group. (B) Mean sEPSC frequency and amplitude in the same treatment groups. Error bars indicate SEM; *p < 0.05.

2002), and markers of adult neurogenesis were diminished if rapamycin was administered prior to pilocarpine-induced status epilepticus (Zeng et al., 2009), suggesting effects of rapamycin on proliferation and/or survival of newborn neurons. Two weeks post-injury, we identified an increase in DCX-positive cell density in the dentate gyrus ipsilateral to the injury, consistent with previous reports linking seizures with increased adult neurogenesis (Parent et al., 1997, 1998, 2006).

Both rapamycin doses used in this study suppressed postinjury neurogenesis in association with diminished seizure prevalence, consistent with the hypothesis that mTOR inhibition is associated with decreased adult neurogenesis. Little is known about the role immature dentate granule cells play in the functional connectivity of hippocampal circuitry after brain injury, but several studies have linked seizure-associated synaptic reorganization to abnormal connectivity of newborn neurons. The hypothesis that newborn dentate granule cells contribute selectively to synaptic reorganization and epileptogenesis has been proposed (Kron et al., 2010). Genetic enhancement of the PI3K→AKT→mTOR pathway by deletion of PTEN (i.e., transgenic phosphatase and tensin homolog), specifically in neural progenitors, is sufficient to increase adult neurogenesis (Amiri et al., 2012) and cause development of spontaneous seizures (Pun et al., 2012; Hester and Danzer, 2013; Lasarge et al., 2015). Further, mTOR inhibition with rapamycin attenuates development of seizures in PTEN knockout mice (Sunnen et al., 2011), implicating the mTOR-mediated modulation of adult neurogenesis in the development of acquired epilepsy. Interestingly, and perhaps paradoxically, increased mTOR activation has been proposed as a means of diminishing injury and improving cognitive recovery after TBI in patients (Don et al., 2012), whereas use of rapamycin to suppress mTOR activity post-TBI has been proposed to prevent or suppress epileptogenesis (Guo et al., 2013). A better understanding of the contribution of newly-born neurons to adult brain function in healthy and disease states appears necessary in order to optimally utilize mTOR modulation after TBI for cognitive recovery and prevention of PTE.

Mossy Fiber Sprouting

Synaptic reorganization in the dentate gyrus after CCI injury was also reduced by rapamycin treatment. The relationship between modulation of post-injury synaptic reorganization and reducing the prevalence of spontaneous seizures in rapamycin treated mice is unclear, since the treatment did not completely eliminate either seizures or mossy fiber sprouting. This was most apparent at the low dose of rapamycin, where both mossy fiber sprouting and seizure prevalence were reduced but not eliminated. It is possible that even limited synaptic reorganization is sufficient for seizure expression (Hunt et al., 2010; Pun et al., 2012). The general failure of many studies to quantitatively link post-injury mossy fiber sprouting with spontaneous seizures, along with recent studies showing that seizures develop in the absence of robust mossy fiber sprouting in the pilocarpine-induced status epilepticus model of epilepsy after rapamycin treatment, have led to the suggestion that

mossy fiber sprouting and spontaneous seizures are not causally linked. However, even when quantitatively reduced in rapamycin treated mice, some degree of mossy fiber sprouting was observed in all mice that displayed spontaneous seizures here. While no proven causal relationship exists to date, the qualitative presence of post-injury mossy fiber sprouting suggests it cannot be excluded as a cellular correlate of epileptogenesis. Alternatively, mossy fiber sprouting and synaptic reorganization may represent a secondary change associated with epileptogenesis, since rapamycin prevented significant mossy fiber sprouting, but not spontaneous seizures, in rapamycin treated mice after pilocarpine-induced status epilepticus (Heng et al., 2013). It is also possible that the synaptically reorganized dentate gyrus reflects a relatively mature stage of epileptogenic circuit formation, whereas other factors that occur in earlier stages of epileptogenesis, including cell loss and adult neurogenesis, contribute to the eventual change in connectivity. The cellular triggers of epileptogenesis remain poorly-defined. However, the association of rapamycin treatment with a reduction in seizure prevalence and cellular markers of PTE is consistent with the hypothesis that activation of the mTOR pathway plays a role in development of PTE.

Mice receiving severe unilateral CCI injury begin to develop PTE after a latent period of \sim 6-10 weeks post-injury (Hunt et al., 2009; Guo et al., 2013). The percentage of mice reported to develop spontaneous behavioral or electrographically measured seizures varies from 36-50% (Hunt et al., 2009; Guo et al., 2013). Here 40% of mice receiving CCI without drug treatment developed spontaneous behavioral seizures, similar to previous reports. Rapamycin treatment (6 mg/kg) for 4 weeks after injury reduced seizure prevalence, with 13% of mice expressing electrographically identified seizures (Guo et al., 2013). The proportion of mice exhibiting spontaneous behavioral seizures observed here was similarly reduced in mice that received either low- or high-dose rapamycin treatment in the present study to 8 and 9%, respectively, representing a trend toward reduced seizure prevalence. Notably, our behavioral seizure measurements probably underestimate the total number of seizures in all groups, due to periodic observation. Rapamycin treatment therefore tended to reduce, but did not eliminate, development of generalized spontaneous seizures after CCI.

Network Excitability

Electrophysiological indices of network excitability are increased in the dentate gyrus after CCI, including evoked network responses and sEPSC frequency in dentate granule cells. Commensurate with diminished mossy fiber sprouting, synaptic excitability was suppressed in rapamycin treated CCI-injured mice, and was comparable to controls with the high dose regimen, although increased network excitability was observed in slices where mossy fiber sprouting was present. Others have reported effects of mTOR inhibition on mossy fiber sprouting, but assessment of synaptic or network activity has not been reported previously. Increased electrophysiological responses are hallmarks of synaptic reorganization in excitatory circuitry of

the dentate gyrus and are correlated with mossy fiber sprouting and development of spontaneous seizures in this and other epilepsy models (Dudek and Spitz, 1997; Patrylo and Dudek, 1998; Lynch and Sutula, 2000; Winokur et al., 2004; Hunt et al., 2009, 2010, 2011, 2012). Axon plasticity after injury or seizures is a feature of many neuron types, and these neurons could also contribute to increased sEPSC frequency in dentate granule cells. Although a causative link between synaptic reorganization and epilepsy remains controversial, these results are consistent with reduced functional synaptic reorganization after CCI injury in rapamycin-treated mice.

Cell Death

Another hallmark of CCI injury is selective cell loss, particularly in the hilus and dentate gyrus (Hicks et al., 1993; Graham et al., 2000; Maxwell et al., 2003; Anderson et al., 2005). The use of FJB as a marker to infer cell degeneration and necrotic cell death indicates peak cell loss within the first 3 days after CCI injury, with a gradual reduction in neuronal degeneration over time (Anderson et al., 2005; Ansari et al., 2008; Hall et al., 2008). Rapamycin was shown previously to reduce FJB staining in dentate gyrus, CA3, and CA1 regions of the hippocampus three days post-injury at a site ~1 mm posterior to epicenter (Guo et al., 2013). Here, we assessed the full septo-temporal axis of the hippocampus and found that neither low- nor high-dose rapamycin treatment attenuated FJB staining overall in the dentate gyrus and hilus. Although FJB labeling remained significantly greater than in controls, however, 10 mg/kg rapamycin treatment did attenuate FJB staining relative to vehicle treatment after CCI in the ipsilateral hemisphere in the same area (i.e., ~ 1 mm from injury epicenter) as previously reported (Guo et al., 2013). This region corresponds to the area of greatest cell death in this brain injury model. Together, these results suggest the possibility that rapamycin may moderately suppress post-injury neuronal death regionally, even if cell death overall in the dentate gyrus and hilus is unaffected by the treatment.

CONCLUSION

The findings of this study are consistent with the hypothesis that mTOR inhibition reduces synaptic reorganization among granule cells and inhibits post-traumatic epileptogenesis after CCI. Continuous rapamycin treatment reduced the percentage of mice expressing spontaneous seizures, inhibited measures of synaptic reorganization in the granule cell layer, and abrogated the increase in neurogenesis following CCI injury. Notably, even the highest dose of rapamycin failed to completely prevent PTE or specific cellular changes associated with epileptogenesis, including post-injury cell death, in a substantial number of injured mice. These findings suggest that mTOR inhibition alters disease progression, but may not prevent the initiation of epileptogenesis. The relationship between adult neurogenesis, excitatory synaptogenesis, and seizure susceptibility remains uncertain in the CCI and other models of acquired epilepsy, but we hypothesize that the inhibition of post-injury neurogenesis is a significant feature of the anti-epileptogenic effects of rapamycin

treatment following CCI injury. Effects of hormones and growth factors that cross the blood brain barrier after injury have been attributed to an increase in neurogenesis mediated by mTOR activity, and several studies have targeted this mechanism as a therapeutic option to restore cognitive function post-injury (Lu et al., 2005; Sun et al., 2009; Xiong et al., 2012; Carlson et al., 2014). However, the present results imply that potential benefits of increased mTOR signaling might be mitigated by the potentially detrimental epileptogenic effects over time. This study highlights the need for further work to understand how newly born dentate granule cells integrate and function in the injured hippocampus and how this integration is related to both functional recovery after TBI and the potentially increased risk of seizure susceptibility. Understanding mTOR's role in these processes may help define the critical features of epileptogenesis and recovery from TBI.

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AUTHOR CONTRIBUTIONS

CB, JB, and BS made substantial contributions to the conception and design of the work; acquisition and analysis were performed by CB and JB; interpretation of data was done by CB, JB, and BS. CB, JB, and BS drafted and revised the manuscript critically for important intellectual content. All authors approve the version of the manuscript to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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