

A Role for Myelin in Traumatic Brain Injury Deficits and Recovery

by

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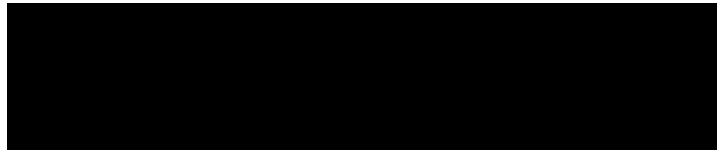
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DEDICATION

This work is dedicated to Winnifred S. Hartvigsen who was never afraid to break barriers in order to help those in need and to Fred “Wes” Marion who instilled in me the risks and rewards of dedicating your life to the work you love.

ABSTRACT

A Role for Myelin in Traumatic Brain Injury Deficits and Recovery

Christina M. Marion, Ph.D, 2019

Thesis directed by: Dr. Regina C. Armstrong

Impact-acceleration forces to the head can cause traumatic brain injury (TBI). In many cases, including mild injuries, TBI produces traumatic axonal injury (TAI) within long axons of white matter tracts. In addition to TAI, we have shown that experimental TBI causes distinct myelin pathologies in the corpus callosum (CC), a large white matter tract commonly affected in human TBI cases. These pathologies include demyelination, defined as myelin loss along intact axons. Little is known about how these myelin pathologies arise or how they may affect patient deficits and eventual recovery. We explored to what extent demyelination may persist in an experimental mouse model of TBI where axonal pathology is attenuated. SARM1 activation is essential for execution of the conserved axon death pathway through which axon damage progresses to full degeneration. We assessed axon and myelin pathology in mice with genetic deletion of *Sarm1* (*Sarm1*^{-/-}) using ultrahigh resolution structural analysis in the CC at three days after TBI. *Sarm1*^{-/-} mice had reduced axon degeneration as compared to littermate mice with the wild type gene. Furthermore, TBI-induced demyelination did not occur in *Sarm1*^{-/-} mice, suggesting a relationship between axon degeneration and demyelination. We next assessed disrupted axon-myelin interactions and how they may contribute to slowed processing speed caused by TBI. We identified, ultrastructurally and with molecular markers, TBI-induced abnormalities at the paranode where myelin attaches to

the axon. Action potential conduction is very sensitive to paranode abnormalities and demyelination, and indeed compound action potential (CAP) velocity was slowed specifically along myelinated axons at three days after TBI. Interestingly, CAP partially recovered by two weeks, which can occur with remyelination. To explore myelin dynamics after TBI, we fluorescently labeled oligodendrocyte progenitors in *NG2CreER;mTmG* mice. TBI increased new membrane synthesis along axons and extending to functionally relevant paranodes, indicative of remyelination. However, moving into later time points after injury, fewer axons contributed to the CAP at six weeks, suggesting axon loss. By eight weeks the CC exhibited significant atrophy in TBI mice. We also assessed *Sarm1*^{-/-} mice to determine if the axonal preservation observed at three days after TBI also extended to protection from CC atrophy at eight weeks. Indeed, CC atrophy was attenuated in *Sarm1*^{-/-} mice, as compared to mice with the gene. These studies identify a progression of axon-myelin pathology and related conduction deficits. Furthermore, we have identified potential therapeutic windows after TBI. Early SARM1 inactivation may reduce axon degeneration, demyelination, and late white matter atrophy caused by TBI. Additionally, we have identified an intervening recovery period corresponding to remyelination that is also an excellent target for therapeutic intervention. Together these studies underscore the importance of axon-myelin interactions throughout the time course following TBI.

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LIST OF ABBREVIATIONS

ANOVA:	Analysis of variance
BrdU:	Bromodeoxyuridine
β -APP:	Beta-amyloid precursor protein
CAP:	Compound action potential
CC:	Corpus callosum
CC1:	Anti-adenomatous polyposis coli clone
CNS:	Central nervous system
CT:	Computerized tomography
DAI:	Diffuse axonal injury
Caspr:	Contactin associated protein 1
CD11b:	Integrin alpha-M/beta-2
DAPI:	4',6-diamidino-2-phenylindole
DTI:	Diffusion tensor imaging
DKI:	Diffusion kurtosis imaging
EdU:	5-ethynyl-2'-deoxyuridine
EM:	Electron microscopy
FGF2:	Fibroblast growth factor 2
FA:	Fractional anisotropy
GFAP:	Glial fibrillar acidic protein
GFP:	Green fluorescent protein
GSTpi:	Glutathione S-transferase pi
Iba1:	Ionized calcium binding adaptor molecule 1
Ka:	Axial kurtosis
Kr:	Radial kurtosis
MD:	Mean diffusivity

mG: Membrane green fluorescence

MK: Mean kurtosis

MOG: Myelin oligodendrocyte glycoprotein

MRI: Magnetic resonance imaging

mT: Membrane tdTomato red fluorescence

mTBI: Mild traumatic brain injury

NAD⁺: nicotinamide adenine dinucleotide

NG2: Neural/glial antigen 2

Olig2: Oligodendrocyte transcription factor 2

OPC: Oligodendrocyte progenitor cell

PLP: Proteolipid protein

RD: Radial diffusivity

ROI: Region of interest

SARM1: Sterile alpha and Toll/interleukin-1 receptor motif-containing 1

s-mTBI: Single mild traumatic brain injury

s-sham: Single sham procedure

TAI: Traumatic axonal injury

TBI: Traumatic brain injury

TIR: Toll interleukin receptor

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

WM: White matter

YFP: Yellow fluorescent protein

CHAPTER 1: Introduction

Traumatic brain injury

Traumatic brain injury (TBI) is a major global health problem, with more than 27 million new cases annually. Concerningly, the global incidence rate has steadily increased over the past 20 years (83). In the United States, TBI is an increasingly recognized concern for both civilian and military populations. On the civilian side, TBIs lead to at least 1.7 million confirmed emergency room visits occur annually, not counting many additional mild TBIs that are diagnosed in doctors' offices or go undiagnosed entirely (48). Within the military, the incidence rate for deployed service members is estimated to be one in 30, or approximately double the rate of non-deployed personnel (4). The majority of TBIs, about 80%, are classified as mild, which the Department of Defense defines as loss of consciousness up to 30 minutes, a confused or disoriented state and/or memory loss lasting less than 24 hours, and normal structural brain imaging (1). The standard of care in emergency rooms is to use computerized tomography (CT) as the primary imaging modality for assessing brain injury, and generally only detects large hemorrhages and lesions in patients suffering moderate to severe TBI.

On the contrary, diagnosis of mild TBI (mTBI), sometimes referred to as concussion, is dependent on signs and symptoms rather than imaging. Patients often have physical symptoms like headaches, in addition to other issues like memory problems, changes in emotional stability or sleep patterns, and trouble with global function (48). Specifically, information processing speed is often slowed in TBI patients, which contributes to impaired executive function, slowed reaction times and difficulty with

attention and learning (42; 43; 103; 128; 191). Furthermore, while most patients recover from mTBI within a few weeks, 15-30% will develop post-concussion syndrome that generally includes ongoing symptoms (80).

Traumatic axonal injury

TBIs, especially acceleration-deceleration injuries, cause strain on specific brain regions. From mild through severe TBI, long axons within the white matter are particularly susceptible to compression, tension, and torsion forces (23; 85). These forces cause traumatic axonal injury (TAI) in the white matter. First discovered in patients with severe TBIs without overt imaging evidence of hemorrhages or lesions, TAI occurs in a pattern of damaged axons distributed among intact axons within the white matter. In more severe cases, this involves multiple brain white matter regions and is defined as diffuse axonal injury, or DAI. Originally, severity of DAI was assigned based on the white matter areas in which TAI was identified using post-mortem pathology (3). Recent developments in neuroimaging have also provided a similar magnetic resonance imaging (MRI) scale for patients (88).

It is important to note that for patients who have suffered a moderate to severe TBI, a common long-term consequence is white matter atrophy. Roughly 77% of severe TBI patients showed atrophy of the corpus callosum (CC), a major white matter structure (69). Additionally, patients with moderate to severe TBI were found to have structural atrophy of several brain regions, including the CC at approximately one year after injury. However, when these patients were followed for additional imaging eight years after TBI, the CC was found to be the only structure that had continued to progressively atrophy

beyond the initial findings (181). Patients with CC atrophy also tend to have cognitive impairments (77; 126; 193).

TAI has also been identified in mTBI and is associated with reduced white matter integrity that often corresponds to worsened outcomes for TBI patients (3; 28; 94). In mTBI, axonal injury is generally found in discrete white matter locations, rather than a diffuse pattern across many brain regions. The CC, a large white matter tract with a large number of axons, frequently shows abnormalities even in mTBI patients (129; 154; 157). The CC is also a common location for TAI in experimental TBI models (11). Within the CC, both unmyelinated and myelinated axons are vulnerable to cytoskeletal disruption due to initial injury forces (23; 179).

Myelin pathology in TBI

Though important, and generally well-recognized, TAI is not the only consequence of TBI in white matter. Many of the axons within the CC are long axons that are particularly susceptible to the forces of TBI. Long axons also tend to be larger and myelinated, and indeed there are many myelinated axons in the CC. In addition to TAI, TBI also induces myelin pathology. Myelin destruction was originally recognized along with DAI in some of the seminal post-mortem TBI analyses (132). Subsequent assessments directed at identifying myelin pathology have shown abnormal myelin structure and demyelinated axons even in milder forms TBI (23; 116; 171).

Demyelination, defined as loss of myelin around an otherwise intact axon, is of particular concern given normal myelin function. Myelin enables fast action potential conduction and provides trophic and metabolic support to axons (76; 118; 122). Demyelinating

diseases, such as multiple sclerosis, are stark examples of just how detrimental myelin pathology can be. Unlike these diseases, however, mTBI does not produce large regions of demyelination. Instead, myelin pathology follows a similar pattern as TAI, with individual demyelinated axons distributed among normally myelinated axons (Fig 1). Still, TBI-related myelin pathology could cause processing speed deficits and expose axons to further damage (93; 130; 189). Additionally, both axon and myelin pathologies are likely to influence the neuroinflammatory response after TBI. Neuroinflammation can persist for years in the CC after TBI, and experimental models have identified pro-inflammatory cell types in the CC in mTBI (84; 116; 171).

The white matter injury progression in TBI patients and experimental models is not well understood. Disrupted axon-myelin interactions likely contribute to impaired white matter function and atrophy but have not been directly explored in the context of TBI. Furthermore, understanding the relationship between axon and myelin pathologies helps inform potential interventions. Ideally, this would help differentiate between axon and myelin pathologies that are irreversible, and those through which axon preservation and remyelination are possible potential outcomes (Fig 1)(11).

Wallerian degeneration

Experimental models suggest that TAI does not necessarily lead to death of the corresponding neuronal cell body, particularly in mTBI (71). Axons that are irreversibly damaged after TBI, however, undergo Wallerian degeneration. By this mechanism, the distal process of the axon degrades through a conserved molecular axon death pathway (34; 74; 195). Most axon damage is not due to direct axonal shearing from the forces of

TBI, but rather occurs through this secondary pathway in the days and weeks after initial insult, making it a target for acute intervention after TBI (23; 106; 109).

Figure 1

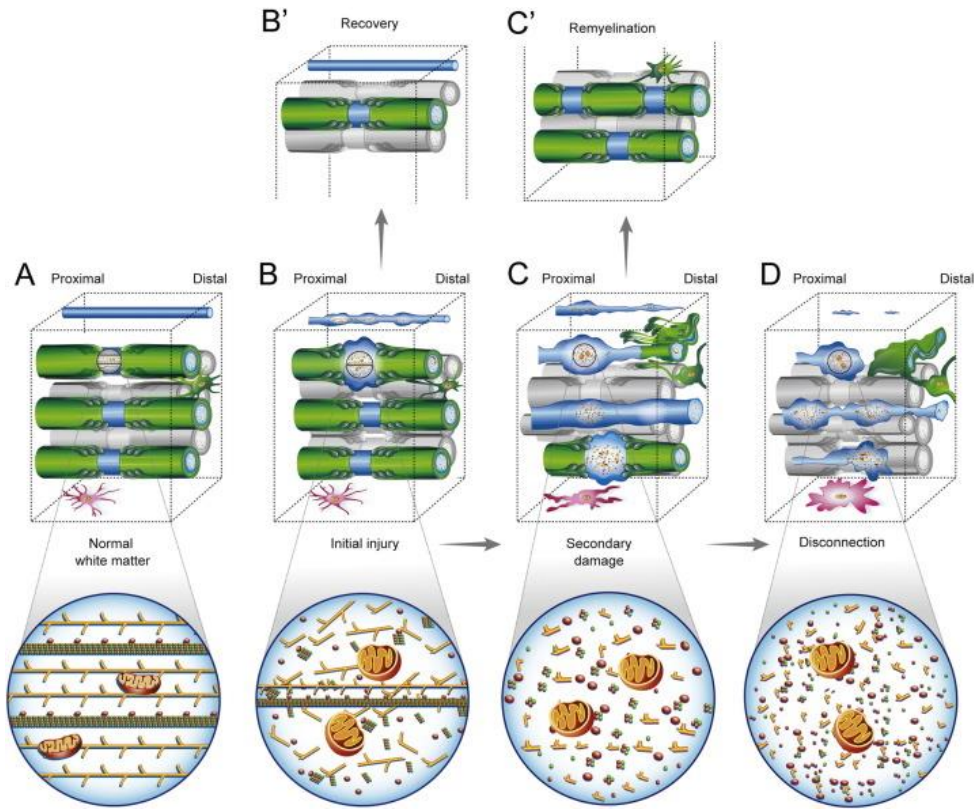


Figure 1: White matter axon and myelin pathology after mTBI. Both axon and myelin pathologies are evident in white matter after TBI. Under normal conditions (A), myelinated (green myelin around blue axon) and unmyelinated (small blue axon) fibers, myelinating oligodendrocytes (green) and resting microglial cells are present in white matter. After TBI, TAI can occur, initially disrupting axon microtubules (inset) and causing axonal swellings along the length of the axon (B). Importantly, this axonal damage has the potential to recover, rather than progress through an injury cascade where axon demyelination, myelin abnormalities, and axon degeneration can occur (C). Ideally, mechanisms to promote recovery, such as remyelination, are important factors in preventing full axonal disconnection, further axon damage, and persistent neuroinflammation (D). Schematic from Armstrong, Mierzwa, Marion, Sullivan, 2016 (11).

The process of Wallerian degeneration has an initiation phase, a potentially reversible latent period, and an execution phase during which the axon degrades completely. Intra-axonal calcium fluxes, mitochondrial dysfunction, and reactive oxygen species generation are thought to be contributing factors to axon death pathway initiation (32; 34; 192; 195). A highly conserved genetic modulator has been identified as the central executioner in the axon degeneration program. Axonal injury induces phosphorylation of sterile alpha and Toll/interleukin-1 receptor motif-containing 1 (SARM1) (133). SARM1 initiates execution of axon degeneration by hydrolyzation of nicotinamide adenine dinucleotide (NAD⁺). It has been shown that the Toll/interleukin-1 receptor (TIR) domain of SARM1 possesses an intrinsic function as a NADase (25; 47). Local NAD⁺ destruction triggers a cascade of ion imbalances and cytoskeletal destruction that lead to axon degeneration (60-62; 174). Importantly, *SARM1* is conserved across 107 species, including humans, with a highly regular sequence for the TIR domain in particular. While this strongly suggests evolutionary pressure to retain this sequence, it is important to note that SARM1 function differs from other proteins containing TIR domains that are generally associated with the innate immune response. To what extent SARM1 may be involved in the innate immune response is unknown (104).

Given the role for Sarm1 in initiating the conserved molecular axon death pathway, it has been explored as a potential intervention point for preventing axon degeneration. In the context of experimental nerve transection, genetic deletion of *Sarm1* provides lifelong protection against axon degeneration (58; 65). It is less clear how well *Sarm1* deletion would protect axons in the context of a complex injury response as is

observed after TBI, where axon damage is not the only pathology. Additionally, axon preservation may not prevent the myelin pathology, mitigate neuroinflammation, or reduce white matter degeneration and atrophy.

Myelin loss and remyelination

When thinking about potential interventions, it is important to recognize that axon preservation alone may not prevent functional deficits if myelin pathology is still present. Independent of axon damage, myelin pathology can have significant consequences. It has long been known that demyelination causes conduction deficits in the central nervous system (112; 188). Normally, myelinated axons have a distinct structure that allows for rapid signal propagation. Sodium channels are clustered in the nodes of Ranvier, between long stretches of unexcitable axon insulated by myelin internodes. At the edge of the myelin internode, referred to as the paranode, myelin loops are anchored to the axon by a protein called Contactin associated protein 1 (Caspr). The structure of these axon-myelin interactions allows the propagating signal to jump from node to node across the myelin internode (33).

In the context of demyelination, myelin internodes are lost, exposing the axon and underlying ion channels. This not only prevents action potential propagation but can disrupt normal ion influx within the axon. Eventually, sodium channels will no longer cluster within the node of Ranvier but spread out along the unmyelinated length of axon. This allows for the signal to again propagate, but conduction will be much slower (189). Ultimately, this is likely to desynchronize neural circuits, and in the context of TBI, could contribute to the slowed processing speed so often exhibited by patients.

Myelin is unique in the central nervous system in that it has a fairly robust regenerative capacity. Oligodendrocyte progenitor cells (OPCs) are the most common mitotic cell in the adult brain, and there is evidence myelin plasticity is a contributing factor in strengthening neural circuits throughout life (50; 127; 142). Importantly, OPCs have the capacity to respond to demyelination. Remyelination, through OPCs maturing into myelinating oligodendrocytes or through already present oligodendrocytes extending new myelinating processes, has the capacity to restore axon conduction. Remyelination also protects axons from further damage, which is particularly important in TBI given the ongoing biochemical cascades that can lead to axon degeneration (21; 82).

When thinking of potential therapeutic strategies for TBI, it is important to consider the axon-myelin interactions. Axon degeneration and demyelination are both significant pathologies after TBI leading to white matter alterations that have an impact on patient outcomes. Preventing axon degeneration alone may preserve axon integrity, but without proper myelin support, the axon would still not function properly. On the other hand, remyelination will not be fully effective unless axon structural integrity is preserved, and the axon remains healthy enough to initiate the proper myelinating signaling. A better understanding of how axon-myelin interactions are impacted by TBI is essential to inform future interventions.

Purpose of research

This project explores the hypothesis that myelin changes play an important role in initial deficits and eventual recovery following mild TBI. The first aim of this project was to genetically delay axon degeneration to distinguish primary versus secondary myelin

pathology after TBI. The second aim was to demonstrate that demyelination corresponds to conduction velocity deficits after TBI and subsequent myelin remodeling parallels functional recovery.

Chapters two and three of this dissertation present the experimental findings that identified the myelin and CC changes that we hypothesize contribute to deficits and potential recovery after TBI. Chapter two shows that in addition to axon damage, TBI produces distinct myelin pathologies. Furthermore, there was evidence of remyelination after injury, which may support recovery. Chapter three emphasizes the importance of focusing on the CC for analysis of the single injury model and the advantages of *Thy1-YFP-16* mice to visualize axon damage.

Dissertation chapters four and five directly address aims one and two respectively. In chapter four we use *Sarm1* gene deletion to attenuate TBI-induced axon degeneration. This allows us to determine whether early TBI-generated myelin pathologies are directly related to axon degeneration or occur through other injury mechanisms. Furthermore, blocking axon degeneration may change the trajectory of injury progression versus recovery after TBI. In chapter five we explore conduction across the entire CC after TBI to identify both deficits and evidence of functional recovery. Furthermore, we utilize a transgenic mouse model to visualize new myelin forming in the CC after injury to better understand myelin plasticity and potential remyelination after TBI. Longer time point studies also demonstrate chronic CC atrophy, which is an important clinical correlate for patient outcomes that we show involves *Sarm1*.

CHAPTER 2:

Components of Myelin Damage and Repair in the Progression of White Matter Pathology after Mild Traumatic Brain Injury

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Statement of Contributions:

Christina Marion was responsible for the electron microscopy assessment of the TBI and sham mice at all time points in this chapter. Specifically, she helped derive the axon degeneration classifications. She was the blinded researcher who classified and counted each axon. She also measured the myelin surrounding each axon and the length of all excessive myelin figures. Christina also contributed to editing the manuscript.

ABSTRACT

White matter tracts are highly vulnerable to damage from impact-acceleration forces of traumatic brain injury (TBI). Mild TBI is characterized by a low density of traumatic axonal injury (TAI) while associated myelin pathology is relatively unexplored. We examined the progression of white matter pathology in mice after mild TBI with TAI localized in the corpus callosum. Adult mice received a closed-skull impact and were analyzed from 3 days to 6 weeks post-TBI/sham surgery. At all times post-TBI, electron microscopy revealed degenerating axons distributed among intact fibers in the corpus callosum. Intact axons exhibited significant demyelination at 3 days followed by evidence of remyelination at 1 week. Accordingly, BrdU pulse-chase labeling demonstrated the generation of new oligodendrocytes, identified by PLP mRNA expression, at 3 days post-TBI. Overall oligodendrocyte populations, identified by CC1 and/or GSTpi, were similar between TBI and sham mice by 2 weeks. Excessively long myelin figures, similar to redundant myelin sheaths, were a significant feature at all post-TBI time points. At 6 weeks post-TBI, microglial activation and astrogliosis were localized to areas of axon and myelin pathology. These studies show that demyelination, remyelination, and excessive myelin are components of white matter degeneration and recovery in mild TBI with TAI.

Key words: axon damage, traumatic axonal injury, demyelination, remyelination, oligodendrocyte, redundant myelin, neuroinflammation

INTRODUCTION

Traumatic brain injury (TBI) affects millions of people worldwide, many of whom experience a broad range of neurological deficits (150). TBI often results from impact-acceleration forces and is most commonly classified as mild TBI. However, even a mild TBI will leave approximately 33% of patients below full functional status at 3 months post-injury and these deficits will continue through at least 1 year in 22% of patients (114). TBI resulting from impact-acceleration forces leads to traumatic axonal injury (TAI) within white matter tracts. Long axonal projections that traverse the brain in white matter tracts are damaged from forces of torsion, tension, and compression in impact-acceleration injuries to the head (160). The corpus callosum is a relatively common site of high strain from impact and frequent abnormalities are identified by diffusion tensor imaging even in mild TBI (17; 18; 44; 111). The progression of white matter pathologic features that underlie prolonged deficits experienced following mild TBI is poorly understood.

Although myelin is a major component of white matter, the significance of myelin pathology has been relatively unexplored in mild TBI. Studies using a relatively mild fluid percussion model have shown that unmyelinated fibers are particularly vulnerable to damage (146; 147). However, the majority of pre-clinical TBI studies of oligodendrocyte populations relative to myelin pathology have used models that involve focal axon loss due to white matter hemorrhage or neuron cell body death with axon loss due to cortical cavitation (2; 52; 99). In these models, focal axon loss causes myelin degeneration due to lack of viable axons to ensheath. This overt loss of tissue, involving axons and myelin, is distinct from demyelination of intact axons. In a model with TAI in the corpus callosum,

demyelination was observed that did not correlate with axon degeneration, indicating separate pathological processes after TBI (171). Demyelination of viable axons impairs saltatory conduction and renders the axons more vulnerable to further damage. Slow conduction along demyelinated axons desynchronizes neural circuitry, which may contribute to deficits in information processing speed following TBI (37; 41). Damage to myelin may also cause myelin debris that can stimulate microglial activation (30). This progression could contribute to chronic neuroinflammation localized in white matter tracts, which is observed in models of single and repetitive mild TBI with dispersed TAI (119; 171).

Subsequent to demyelination, remyelination of viable axons may be particularly relevant to recovery after mild TBI but has not yet been sufficiently studied. As with demyelination, analysis of remyelination requires models of TBI that do not incur extensive focal tissue loss from cavitation or hemorrhage. Studies in demyelinating disease models have shown that remyelination can both enable recovery of function and protect denuded axons from progressive degeneration and disconnection, which results in permanent loss of function (21; 82). Furthermore, viable axons may exhibit myelin remodeling, which is a form of adult plasticity that can be modified by cortical activity and experience (63; 95).

A marked feature of the myelin pathology in our initial study of TAI in the corpus callosum was abnormal myelin sheaths with excessive myelin folding back onto itself (171). These excessive myelin figures with double-layered sheaths resembled “redundant” myelin observed during CNS development (151). Recent three-dimensional reconstruction analysis revealed excess myelin outfoldings as part of normal myelin

sheath formation in the developing CNS (163). In the context of TBI, further evaluation is needed to determine whether excessive myelin figures are a component of white matter damage and/or myelin remodeling.

The purpose of the current study was to determine the progression of myelin pathology and repair in a model of mild TBI that exhibits localized TAI in the corpus callosum, without tissue loss from hemorrhage or cavitation. We examine the corpus callosum pathology across a time course from 3 days to 6 weeks post-TBI. Electron microscopy captures the ultrastructural detail required to quantify the spectrum of pathological features indicative of degenerating axons relative to myelin changes, including demyelination, excessive myelin figures, and remyelination. Interpretation of these ultrastructural studies is further supported by analysis of cellular responses associated with the specific myelin findings. These experiments clarify the contribution of both axon and myelin components of white matter to the progression of pathology after mild TBI.

MATERIALS AND METHODS

Traumatic Brain Injury

All procedures were performed with 8-week-old male C57BL/6 mice (Jackson Laboratories; Bar Harbor, ME). Mice were housed in pairs according to the guidelines of the National Institutes of Health and the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences.

Mice received a mild TBI using a previously characterized model of impact onto the closed skull to produce TAI in the corpus callosum over the lateral ventricles (115; 171). Impact to the skull was centered at bregma using an Impact OneTM Stereotaxic

Impactor (Leica; Buffalo Grove, IL) device with a 3-mm diameter flat tip set to a depth of 1.5 mm, velocity of 4 m/s, and a dwell time of 100 ms. Isoflurane was administered continuously at 2% via a nose cone until immediately after impact. Video analysis of the impact velocity and depth verified an average impact velocity of 4.3 ± 0.2 m/s and depth of 1.8 ± 0.1 mm. Sham mice received anesthesia as well as a scalp incision. Naïve mice did not receive either anesthesia or an incision. These impact parameters resulted in post-surgical apnea of 17.93 ± 3.35 sec (s.e.m). Immediate post-impact visual inspection of the skull revealed a minimal extent of typically linear fracture near bregma in approximately 62% of mice. Mice with unexpectedly severe skull fracture, including depressed fractures, were excluded from further analysis. Post-surgery, mice exhibited a righting reflex of 6.65 ± 0.48 min in TBI mice and 1.27 ± 0.34 min in sham mice. Mice sacrificed at 2 hrs after injury did not exhibit blood-brain barrier leakage based on immunohistochemistry to detect mouse IgG (data not shown). At the time of brain dissection, i.e. 3, 7, 14, or 42 days post-surgery, there was no visible evidence of fractures on the skull nor contusion on the surface of the brain.

Tissue Preparation for Electron Microscopy

For electron microscopy, mice were anesthetized with ketamine/xylazine prior to transventricular cardiac perfusion with 2% paraformaldehyde and 3% glutaraldehyde at 3 days, 1 week, 2 weeks, or 6 weeks after surgery and stored in fixative overnight prior to dissection. Dissected brains were post-fixed in fixative overnight. Brains were hemisected along the midline. One hemisphere was sectioned (40 μ m) in coronal slices and the other in sagittal slices using a vibrating VT-1000 vibratome (Leica; Buffalo Grove, IL). Sections were selected for further processing based on localization at the

coronal level of the anterior commissure and at parasagittal levels to include the corpus callosum over the lateral ventricles. Samples were immersed in 2% osmium tetroxide in PBS (OsO₄; Electron Microscopy Sciences; Hatfield, PA) for 1 hour. Samples were then washed again in PBS, dehydrated in ethanol, and infiltrated with Spurr's epoxy resin (Electron Microscopy Sciences). Samples were then polymerized at 70°C for 11 hours and thin sections (~70 nm) were cut on a Leica Ultracut UC6 ultramicrotome (Leica). Copper grids containing thin sections were post-stained for 20 minutes in 2% aqueous uranyl acetate (Electron Microscopy Sciences) and 5 minutes in Reynold's lead citrate.⁽¹⁴⁹⁾ Grids were reviewed on a JEOL JEM-1011 transmission electron microscope (JEOL USA, Inc.; Peabody, MA). Images were captured on an AMT XR50S-A digital camera (Advanced Microscopy Techniques; Woburn, MA).

Tissue Preparation for Light Microscopy

Mice for histopathology, immunohistochemistry or in situ hybridization were anesthetized with ketamine/xylazine prior to transventricular cardiac perfusion with 3% paraformaldehyde. Brains were post-fixed overnight, infiltrated with sucrose, and embedded in OCT. Coronal cryostat sections (14 µm) were selected at the level of the anterior commissure.

Cryosections were processed for Prussian blue histological stain for hemosiderin combined with pararosaniline nuclear stain according to the manufacturer's instructions (Sigma, St. Louis, MO).

Cryosections were immunostained with primary antibodies against glial fibrillary acidic protein (GFAP, rabbit polyclonal, 1:500; DAKO; Carpinteria, CA) as a marker for astrocytes, CD11b (rat monoclonal, 1:100; AbCam; Cambridge, MA) for microglia, and

glutathione S-transferase (GSTpi, rabbit polyclonal, 1:200; MBL International; Woburn, MA) and CC1 (mouse monoclonal, 1:200; Millipore; Billerica, MA) for oligodendrocytes. Secondary antibodies included goat anti-rabbit IgG conjugated with Alexa Fluor 488 (1:200; Life Technologies; Grand Island, NY) to detect GFAP and GSTpi, goat anti-rat IgG conjugated with Alexa Fluor 555 (1:200; Life Technologies) to detect CD11b, and donkey anti-mouse IgG conjugated with Cy3 to detect CC1. Sections were counterstained with DAPI (Sigma; St. Louis, MO) before mounting with Vectashield (Vector Laboratories; Burlingame, CA).

In situ hybridization was performed using a previously described riboprobe to hybridize to proteolipid protein (PLP) mRNA transcripts as a marker of oligodendrocytes (10). After hybridization, labeling was detected with alkaline phosphatase-conjugated sheep anti-digoxigenin and reaction with substrate solution (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate [NBT/BCIP]; DAKO; Carpinteria, CA). To detect apoptotic cells, after completion of the in-situ hybridization procedure, TUNEL staining was performed using ApopTag^R Plus Peroxidase *In Situ* Apoptosis Detection Kit (S7101 EMD Millipore Corporation; Temecula, CA). To examine the conversion of progenitor cells into oligodendrocytes, progenitor cells were pre-labeled with a pulse of bromodeoxyuridine (BrdU). BrdU administration prior to TBI/Sham surgery labels endogenous cycling cells and minimizes post-injury concerns of labeling reactive astrocytes, activated microglia, or cells with DNA damage. Mice received intraperitoneal injections with 200 mg/kg of BrdU (Sigma, St. Louis, MO) at 24 and 22 hours before surgery and were subsequently perfused on day 3 after TBI or sham surgery. After completion of the in-situ hybridization sections, were treated with 2M HCl and incubated

overnight with anti-BrdU antibody conjugated to fluorescein (Roche Diagnostics, Indianapolis, IN).

Quantification of immunohistochemistry and in situ hybridization

Digital images were captured on an Olympus IX70 microscope using a Spot CCD camera (Diagnostic Instruments; Sterling Heights, MI). The area of the corpus callosum was measured using Spot Advanced software. The area within the corpus callosum immunostained for GFAP or CD11b was estimated based on a cutoff for pixel intensity values that were above background levels using the Metamorph software thresholding function (Molecular Devices; Downingtown, PA). Immunostaining intensity across sections was controlled for by comparison of regions outside the corpus callosum. CD11b cells within the corpus callosum were also counted individually to differentiate activation stage as resting (fine processes), activated (hypertrophic with thickened processes), or ameboid (retracted processes) as previously characterized (171). All other cell phenotypes were counted manually within the area of the corpus callosum. CC1 and GSTpi cells were counted as single or double labeled phenotypes. Cells with cytoplasmic PLP mRNA hybridization signal were counted based on nuclear DAB reaction for either BrdU or TUNEL. In all conditions, quantification in the corpus callosum included at least 3 sections per mouse and included 24 mice (BrdU and TUNEL with PLP, n = 3 naïve, n = 3 TBI; CC1/GSTpi, n = 5 sham, n = 3 TBI; CD11b/GFAP, n = 5 sham, n = 5 TBI).

Corpus Callosum Width

The width of the corpus callosum was measured in osmicated coronal sections (40 μ m) at the level of the anterior commissure. Measurements were taken in four locations—the midline, the peak of the cingulum, the lateral edge of the ventricle, and 2

µm lateral to the midline. Quantification included 19 mice (3 day, n = 3, n = 4 TBI; 1 week, n = 3 TBI; 2 week, n = 5 TBI; 6 week, n = 4 sham, n = 4 TBI).

Electron Microscopy Image Quantification

Images of sagittal sections through the corpus callosum were acquired at 5,000X magnification for measurements of axon diameter and myelin thickness along with manual counting of degenerating axons, demyelinated axons, and excessive myelin figures. Analysis included 3-5 images per animal (at least 120 axons per image) and 3-6 animals per condition were used. Degenerating axons were defined as axons with cytoskeletal changes (microtubule and neurofilament density extremely low or high with irregular spacing), axons filled with vesicles, or axons with swollen mitochondria (filling over 50% of the axon cross section). Demyelinated axons were manually counted based on a lack of detectable compact myelin and a diameter larger than 0.3 µm. Axons smaller than 0.3 µm were excluded from analysis as potentially unmyelinated fibers (169). An excessive myelin figure was identified as a myelin sheath that was not tightly enwrapping an axon but instead folded back on itself, i.e. “redundant” myelin (151; 171). These figures can be seen surrounding and extending from an intact or degenerating axon, as well as without an axon. Within each counting frame, all apparently intact axons were measured to determine axon diameter and myelin thickness using Metamorph software (Molecular Devices), as previously detailed (180; 194). These values were also used to calculate the g-ratio (axon diameter divided by myelinated fiber diameter) as an indicator of remyelination. Excessive myelin figures were measured along the lumen of the myelin sheaths and that length was doubled to determine the length of each excessive myelin figure. Additional images were taken at 10,000X for illustration of pathology.

Quantification included 26 mice (3 day, n = 3 sham, n = 4 TBI; 1 week, n = 5 TBI; 2 week, n = 5 TBI; 6 week, n = 4 sham, n = 5 TBI).

Electron Microscopy in Cuprizone Model of Corpus Callosum Demyelination

C57BL/6 male mice administered cuprizone have been previously characterized by electron microscopy during acute and chronic stages of demyelination.(194) In addition, electron microscopy has shown that the absence of fibroblast growth factor 2 (FGF2) dramatically increases remyelination in *Fgf2* null versus wild type mice after chronic cuprizone demyelination (180). Images from each of these prior studies were analyzed as described above to now quantify the frequency of excessive myelin figures in the cuprizone model for comparison with the TBI model. Quantification included 15 C57 mice (no cuprizone, n= 5; 4 week cuprizone, acute demyelination, n = 3; 12 week cuprizone, chronic demyelination, n = 7) and 18 *Fgf2* mice (n = 3 for each of the three time point for both null and wild type genotypes).

Statistics

Prism 6.0 (GraphPad Software) was used for statistical analyses and graphing of quantitative data. One-way ANOVA was performed to determine significant differences across multiple post-injury time points compared to each sham time point, with post-hoc multiple comparisons using Dunnett's test or Kruskal-Wallis test, if D'Agostino and Pearson normality test failed. Two-way ANOVA was used to compare *Fgf2* and wild type genotypes across time points and to compare oligodendrocyte populations (CC1 +/- GSTpi) between sham and TBI conditions. Significant differences between 2 groups for a given time point or condition were determined using the Student's t-test. Slopes were

calculated using linear regression and analyzed using Pearson correlation. Statistical significance was determined as $p < 0.05$.

RESULTS

Overview of corpus callosum pathology in mild TBI model with TAI

The current studies of corpus callosum pathology take advantage of our previously characterized TBI model of impact onto the skull at bregma, which focuses TAI over the lateral ventricles at the coronal level of the anterior commissure (171). Brains were examined at multiple time points after injury for evaluation at the gross level for overall pathology and by electron microscopy for subcellular ultrastructure (Figure 2). The current impact protocol produced an injury categorized as mild TBI based on the absence of overt pathology such as contusion with cavitation in the cortex, or corpus callosum hemorrhage (Figure 2A-C). Corpus callosum thinning, along with ventricular enlargement, was observed in some TBI mice (Figure 2C), but this was not significantly different from sham mice (Figure 2D). Cortical regions under the impact site maintained appropriate cytoarchitecture with evidence of prior bleeding observed only rarely along the cortical surface (Figure 2E, F). The injury did not produce hemorrhage or cavitation within the corpus callosum (data not shown) (171). Ultrastructural examination of the corpus callosum demonstrated axon and myelin pathological features across all post-TBI time points (Figure 2H-M). Degenerating axons are dispersed among intact axons consistent with a pattern of TAI. Myelin abnormalities include demyelination, collapsed myelin, and excessive myelin figures that are not tightly enwrapping an axon and so are

similar to “redundant myelin”. These features and the associated cellular responses were further examined to evaluate the progression of myelin pathology in this model of TAI.

Persistent axon degeneration after TAI

A distributed pattern of degenerating axons among intact fibers within white matter tracts is characteristic of TAI (3; 88; 148; 161). In our previous characterization of this model, we have detected axon damage that was highest early during the first week post-TBI using immunohistochemistry for β -amyloid precursor protein (β APP) (117; 171). We now examined axon degeneration at the definitive ultrastructural level (Figure 3) to detect the full range of pathological features, and thus avoid the technical caveats of histological stains and immunohistochemical markers that often detect specific stages of axon damage within the post-injury time course. With the high structural resolution of electron microscopy (Figure 3A-I), degenerating axons exhibited breakdown of the cytoskeleton with abnormally high cytoplasmic density and vesicle accumulation (51.1 - 59.9%) or with abnormally low cytoplasmic density (23.7 - 33.1%). Mitochondrial swelling was also noted (13.8 - 25.2% of degenerating axons) as a subtler indicator of axon damage. At each time point examined, degenerating axons were significantly increased in TBI mice, with a range from 2.4 – 3.8 fold increase over sham values (Figure 3J). The 2.9 fold increase of degenerating axons at 6 weeks post-TBI indicates a prolonged pathological response to injury.

Prominent excessive myelin figures

After loss of an axon, the associated myelin sheath may collapse to a double-layered myelin figure prior to degradation. Also, during myelin biogenesis, the myelin

can extend out beyond the axon circumference and fold back onto itself. For analysis across these potential categories of myelin structures, double-layered myelin sheaths extending out from an axon or lacking a visible axon were categorized as excessive myelin figures. We quantified excessive myelin figures relative to axon pathology across a range of post-injury time points. Excessive myelin figures were associated with intact axons (Figure 4A) or degenerating axons (Figure 3B), as well as without an axon (Figure 4C). Among the excessive myelin figures observed, the frequency across time points ranged from 3.0-4.2% surrounding intact axons, 29.0-35.5% associated with degenerating axons, and 60.3-67.8% without an axon. All three configurations were present at similar frequencies across time points. Excessive myelin figures were significantly increased at 3 days (62.6 fold), 1 week (45.9 fold), and 2 weeks (23.2 fold) compared to sham mice (Figure 4D). The presence of excessive myelin figures correlated positively with the increasing frequency of degenerating axons in TBI mice (Figure 4E). Thus, mild TBI pathology in the corpus callosum results in both axon damage and abnormal myelin sheaths.

Demyelination and remyelination of intact axons

Given the extent of myelin abnormalities associated with degenerating axons, we next explored whether TBI may also result in myelin remodeling along intact axons through demyelination and remyelination. Demyelinated axons were significantly increased at 3 days (1.8 fold) and 6 weeks (1.5 fold) post-TBI but comprised only a small proportion (< 5%) of total axons (Figure 5A-C). The frequency of demyelinated intact axons did not correlate with the presence of degenerating axons (Figure 4D).

Remyelination is indicated by myelin that is thinner than normal for a given diameter of axon (24; 101; 152). Among TBI mice, the thickness of myelin among intact fibers was reduced at 1, 2, and 6 weeks in comparison with sham time points (Figure 6A). Comparison of sham and TBI mice across the full range of axon diameters showed a shift in the overall distribution (Figure 6B-C) and a significant reduction of the mean axon diameter in TBI mice at both 3 days (10.2%) and 6 weeks (16.2%). The g-ratio (calculated as axon diameter divided by myelinated fiber diameter) indicates remyelination more specifically than myelin thickness by accounting for axon size (152). The slope of the g-ratio relative to axon diameter is increased for TBI animals, indicating that myelin is thinner for a given axon diameter (Figure 6D-G). Starting at 1 week, remyelination among the population of intact fibers is indicated by both thinner myelin (Figure 5A) and an increased slope in the g-ratio plots (Figure 6E). Furthermore, demyelination that was significant at 3 days returned to normal levels by 1 week (Figure 5C), which is consistent with remyelination progressing during this interval.

Oligodendrocyte response to injury

Remyelination is carried out by newly generated oligodendrocytes. We pre-labeled cycling progenitor cells with BrdU 1 day prior to injury and perfused the mice at 3 days post-TBI to examine oligodendrogenesis. BrdU detection was combined with in situ hybridization for proteolipid protein (PLP) to identify newly generated oligodendrocytes. Both naïve and TBI mice showed BrdU incorporation in the subventricular zone, a germinal zone that serves as an internal positive control (Figure 7A-B). Within the corpus callosum, newly generated oligodendrocytes (PLP+ BrdU+) were readily detected by PLP mRNA transcript expression in the cytoplasm and BrdU

incorporation in the nucleus (Figure 7C-E). PLP+ BrdU+ cells were relatively rare but significantly more prevalent in TBI mice compared to naïve mice (Figure 7F). This increase of newly generated oligodendrocytes at 3 days post-TBI supports the potential for subsequent remyelination of demyelinated axons between 3 days and 1 week post-TBI.

To further explore the basis of demyelination observed at 3 days post-TBI (Figure 4), oligodendrocyte apoptosis was examined using TUNEL detection combined with PLP in situ hybridization. TUNEL labeled cells were readily detected but rare in the corpus callosum (Figure 7G-H). Cells double labeled for PLP and TUNEL were even less frequent, with only 1-2 cells in the corpus callosum per section from either naïve (2 ± 0 cells) or TBI mice (1 day = 1.0 ± 0.3 , 3 days = 1.4 ± 0.9). Although TUNEL may not detect the full extent of apoptosis at a given point in time, this low level of TUNEL labeling during and prior to demyelination indicates that demyelination is not likely due to oligodendrocyte loss in this mild TBI model.

To more broadly examine the overall oligodendrocyte population, we quantified mature oligodendrocytes based on immunolabeling with two markers, CC1 and GSTpi (Figure 7I-M). In both sham and TBI mice, more cells were immunolabeled by CC1 than by GSTpi. In addition, all cells labeled for GSTpi were double labeled for CC1. No significant difference was observed in the total population of oligodendrocytes labeled with CC1 or the subpopulation labeled by GSTpi following injury (Figure 7M). Therefore, this mild TBI does not produce a prolonged detrimental effect on the oligodendrocyte population in the corpus callosum.

Excessive myelin figure frequency in model of widespread demyelination and remyelination

The excessive myelin figures in TBI are difficult to interpret relative to processes of demyelination and remyelination. Therefore, we compared the current results in TBI with cuprizone ingestion, which exhibits distinct stages of demyelination and remyelination (Figure 8). Acute cuprizone treatment (4 weeks) causes death of oligodendrocytes with strong microglial activation and prevalent axon beading (194). This cuprizone time point had the highest frequency of excessive myelin figures, but the levels were only approximately one-tenth of the levels observed in mice with mild TBI (Figures 4, 8A). Prolonged cuprizone treatment (12 weeks) induced chronic demyelination throughout the corpus callosum, which resulted in lower levels of excessive myelin figures (Figure 8A). Excessive outfoldings of myelin can occur during myelin formation in CNS development (163). Therefore, we examined excessive myelin figures during the spontaneous remyelination that occurs after removal of cuprizone from the diet (Figure 8B). This analysis was performed with chronic cuprizone demyelination followed by a recovery period on normal chow (12 + 6 weeks) during which partial spontaneous remyelination occurs. To specifically test whether the frequency of excessive myelin figures corresponds with the extent of remyelination, wild type mice were compared with fibroblast growth factor 2 (*Fgf2*) null mice that undergo significantly more remyelination (9; 180). Excessive myelin figures were not more frequent in *Fgf2* null mice versus wild type mice (Figure 8B). Notably, in comparison with the mild TBI model, excessive myelin figures were observed at much lower frequencies in the cuprizone study and were typically associated with intact axons (Figure 8C-F).

Myelin load

Additional characterization of excessive myelin figures was performed to interpret the relevance of this pathological feature to axon degeneration and collapse of the remaining myelin sheath. We measured the length of each excessive myelin figure and analyzed the frequencies at each time point (Figure 9). The average length of myelin sheaths enwrapping intact axons was calculated from data in Figure 5 and is marked by a dotted line (Figure 8). Abnormal myelin figures shorter than this dotted line are likely myelin that collapsed and folded onto itself due to loss of the associated axon. The vast majority of abnormal myelin figures are longer than the average length predicted from simple axon collapse and many exceed the length (6.12 μm) predicted for ensheathing the largest axon quantified. The overall distribution of the lengths of excess myelin contributed by these abnormal myelin figures can be compared from the area under the curve (Figure 9 table). This calculation cannot accurately estimate the volume of excessive myelin but can serve as a surrogate measure of myelin load. This comparison indicates that the myelin load from excessive myelin figures is highest early after injury, up to 34.8 fold increased, and is still elevated 18.9 fold at 6 weeks post-TBI.

Persistent neuroinflammation

Myelin debris can stimulate microglial activation (30). Therefore, excessive myelin figures could contribute to persistent neuroinflammation in this model of mild TBI. Previous characterization of this model through 1 week post-TBI showed significantly increased astrogliosis and microglial activation at 3 days and 1 week post-injury that was localized within the corpus callosum (171). We now demonstrate both

ultrastructural and immunohistochemical evidence of neuroinflammation persisting in the corpus callosum through at least 6 weeks post-TBI (Figure 10). Using electron microscopy, a normal astrocyte and microglia cell are shown in a sham mouse (Figure 10A). In TBI mice, astrocytes exhibited cytoplasmic changes indicative of cytogenic edema (Figure 10B) while microglia often contained debris indicative of phagocytic activity (Figure 10C). Immunostaining for GFAP (astrocytes) and CD11b (microglia) detected neuroinflammation more broadly than is possible with electron microscopic sampling and demonstrated localization to the corpus callosum (Figure 10D-E). Astrocytes and microglial undergo morphological changes indicative of reactive and activated stages (Figure 10F-H). Evidence of astrogliosis or microglial activation was not observed in cortical regions (data not shown). Quantification of immunostaining confirmed that GFAP staining is significantly increased in the corpus callosum at 6 weeks post-TBI (Figure 10I). Similarly, there is also an increase in CD11b immunofluorescence in the corpus callosum of TBI mice (Figure 10J) due to an increase in activated microglia (Figure 10K). Thus, reactive neuroinflammatory cells persist in the corpus callosum for at least 6 weeks after injury in this model of mild TBI.

DISCUSSION

The current study demonstrates distinct features of myelin pathology and plasticity that influence white matter integrity following mild TBI. Closed skull impact in adult mice resulted in damaged axons dispersed among intact fibers of the corpus callosum. Regions that exhibited this pattern of TAI also showed significant demyelination among the population of intact axons. We provide evidence of

oligodendrogenesis and subsequent remyelination among the population of intact axons. Degenerating axons continued to be observed through 6 weeks post-TBI and were strongly correlated with the presence of excessive myelin sheaths. Neuroinflammation was also observed at 6 weeks post-injury and specifically localized to the corpus callosum, indicating that these features of axon and myelin pathology may lead to a chronic neurodegenerative environment.

Reduced white matter integrity is a common component of TBI, including blast exposures and mild TBI (196; 198). Axon damage is the major pathological feature associated with reduced white matter integrity after TBI (18). However, demyelination of intact axons is a potential factor underlying desynchronized or slowed neural circuitry due to impaired saltatory conduction. Even in the normal adult CNS, gaps in myelination along cortical projection axons modify circuit function (182). Abnormal gaps in myelination can slow information processing speed, which is a common deficit in TBI patients (37; 41). Social isolation has also been shown to alter myelination in the prefrontal cortex of adult mice (95). Importantly, optogenetic modulation of neuron activity in mouse cortex can modulate myelination of the associated axons and gait of the associated limb (63). These studies have led to the recognition of myelin remodeling as a form of adult plasticity. Therefore, myelination may be a neurobiological substrate of specific neurological deficits after TBI that could potentially be modified to improve the function of neural circuits.

The closed-skull impact model used in the current study did not cause overt gross brain pathology or evidence of contusion or hemorrhage, which is appropriate for categorization as mild TBI among pre-clinical models. The impact results in pathology

localized in the corpus callosum over the lateral ventricles. In contrast to our initial studies in this model, the current experiments minimized skull fractures. Previously, axon damage was detected at intervals throughout the first week post-TBI using immunohistochemistry for β APP (171). While β APP immunostaining detects impaired fast axonal transport associated with early phase axon damage, it is not sensitive to later stage axon pathology and neurofilament compaction (39; 57; 166). Electron microscopy can detect axon damage based on mitochondrial swelling, accumulation of vesicles, and disruption of the cytoskeleton. Using these parameters, our ultrastructural analysis demonstrated significant axon damage at 3 days post-injury that remained through 1, 2, and 6 weeks (Figure 3). Degenerating axons were dispersed among intact fibers as is characteristic for TAI (18; 148; 160). Mechanical injury and secondary insults result in Wallerian degeneration of axons, which involves mitochondrial dysfunction and a conserved axon death signaling pathway (34; 186). Stretch injury causes rapid dislocation of myelin paranodal junctions that may contribute to calcium changes associated with initial TAI and possibly continued axon damage (108). Our data cannot accurately quantify axons damaged from the initial injury relative to continued axon degeneration from secondary processes since Wallerian degeneration can be a prolonged process in the CNS (18; 184). Furthermore, stretch injury of the optic nerve shows that axons continue to initiate degeneration days to weeks after injury (109).

Our ultrastructural analysis demonstrates demyelination of intact axons that is not correlated with axon degeneration, indicating distinct pathological mechanisms (Figure 5). At 3 days post-TBI, demyelination was observed without ongoing apoptosis of oligodendrocytes (Figure 7). These findings of mild TBI in mice contrast with studies of

the corpus callosum after moderate TBI in rats. Moderate fluid percussion injury resulted in loss of oligodendrocytes, identified by CC1, by 3 days and a marked increase of TUNEL labeled cells in white matter by 12 hours which continued to increase through 1 week post-injury (35; 99). Myelin loss and oligodendrocyte apoptosis, detected by CC1 combined with activated caspase-3, were significantly increased at 2-21 days after fluid percussion injuries with hemorrhages in the corpus callosum (52). In our analysis, additional studies will be required to determine the mechanism of demyelination, which appears to be subtler than in the above studies since electron microscopy is required to identify intact axons without myelin. Demyelinated axons were dispersed among adjacent myelinated fibers and were not readily quantified at the light level using histological or immunohistochemical technique (171 and current study Figure 1). Distributed demyelinated axons were also observed at 6 weeks post-TBI which may be due to detrimental effects of persistent neuroinflammation and/or a lack of sufficient remyelination to compensate for continued demyelination.

Several findings indicate that demyelinated axons can remain viable and undergo remyelination between 3 days and 1 week post-TBI. Demyelination was observed at 3 days but not at 1 week post-injury (Figure 5). Among the population of intact axons evaluated after TBI, remyelination was indicated by higher g-ratio values in conjunction with overall reduction in myelin thickness between 3 days and 1 week post-injury (Figure 6). Studies of demyelinating disease models, such as cuprizone toxicity in the mouse corpus callosum, have shown that remyelination requires oligodendrocyte progenitor cells to proliferate and differentiate into new oligodendrocytes which form the new myelin sheaths (82; 121). The cellular responses required for remyelination were found in

the corpus callosum after TBI. Newly generated oligodendrocytes, co-labeled for PLP and BrdU, were increased at 3 days in the corpus callosum of TBI mice (Figure 7). Furthermore, our prior studies showed that proliferation of oligodendrocyte progenitors was significantly increased in the corpus callosum by 3 days post-TBI and continued to be elevated at 1 week (171). During this first week post-injury, oligodendrocytes also exhibited a shift to more active transcription of PLP, which is the most abundant myelin protein (171).

Excessive myelin sheaths were prominent in the corpus callosum at all post-TBI time points and correlated with the presence of degenerating axons (Figure 4). However, the majority of excessive myelin figures were much longer than would be expected from simple collapse of myelin sheaths around degenerating axons (Figure 9). These excessive myelin figures may form from processes associated with axon loss and/or myelin synthesis. In the developing CNS, double-layered myelin figures have been referred to as “myelin outfoldings” or “redundant myelin” (151; 163). During myelin biogenesis, excess myelin membrane extends outward radially from the growing axon as loose layers that progressively expand around the axon and laterally along the axon (163). These extensions of excess myelin generate double-layered myelin figures. Therefore, after TBI, excessive myelin may reflect productive myelin synthesis to remyelinate viable axons and/or aberrant myelin synthesis around degenerating axons due to dysregulation of myelin-axon signaling. The current study is the first to characterize excessive myelin figures in TBI or relative to demyelination and remyelination in a demyelinating disease model. Further studies are needed to interpret the processes leading to the generation of these excessive myelin figures in adults and the pathological implications.

Dorsal rhizotomy studies of ascending spinal cord tracts also support a role for myelin synthesis in the formation of excessively long myelin figures. Adult cats with dorsal rhizotomy exhibit myelin figures of the lengths expected for collapsed myelin sheaths that remain after the axon degenerates (53; 184). In contrast, after dorsal rhizotomy in kittens, tracts with degenerating axons exhibit double-layered myelin figures that are much longer than in adult cats (54). In addition, excessively long double-layered myelin figures without an associated axon were observed extending around cell bodies in the lesioned tracts of kittens (54). In our adult TBI model, the excessively long myelin figures exhibit similarity to structures observed after rhizotomy in the developing CNS. Adult white matter may exhibit more excessive myelin figures only when axon damage is dispersed, as in our TBI model. Consistent with this hypothesis, similar excessive myelin figures have been illustrated in the corpus callosum associated with relatively subtle axon damage in mouse models of mild, repetitive closed-skull TBI (16; 158).

Specific to mild TBI, the dispersed white matter axons damaged by TAI may be a subset of only one or two axons among a cohort of 20 or more axons ensheathed by a surviving oligodendrocyte. This mismatch of axon integrity among the cohort may dysregulate myelin maintenance signals to the oligodendrocyte, resulting in aberrant myelin synthesis. Comparison of the TBI data with the cuprizone data of corpus callosum demyelination (Figure 7) argues against productive remyelination accounting for the majority of excessive myelin figures observed after TBI. Cuprizone demonstrates similar excessive myelin figures that are present at a much lower frequency (Figure 8) even though remyelination is much more extensive (9; 180). Similarly, the cuprizone example

argues against the frequency of axon damage solely accounting for the abundance of excessive myelin figures after TBI. During acute cuprizone demyelination, axon damage as detected by β APP or electron microscopy is higher than in this model of TBI (164; 171) yet excessive myelin figures are much fewer (Figure 8). However, cuprizone elicits strong microglial phagocytosis of damaged myelin and the majority of axon damage does not progress to transection (79; 180; 194). Interestingly, axon damage is consistently observed with demyelination in cuprizone while axon damage is dramatically reduced with genetic manipulations that enhance remyelination (180; 200). Further studies would be important to more specifically demonstrate the extent to which demyelination after TAI may render axons more vulnerable to further damage from secondary processes, such as neuroinflammation, and to what extent remyelination may be neuroprotective.

In this model of TBI, we have shown neuroinflammation localized within the corpus callosum during the first week after injury (171) and after a prolonged 6 week survival period (Figure 10). Localization of microglial activation in the corpus callosum has been observed in a spectrum of TBI from repetitive mild TBI models in mice (119; 158) to chronic cases of single moderate-severe TBI in humans.(84) Myelin changes, including excessive or redundant myelin, are increased in aged non-human primate white matter and potentially related to microglial senescence and reduced efficiency of clearance of myelin debris with aging (124; 137). The relationship between excessive myelin figures and neuroinflammation will be important to delineate in future studies. Activated microglia and reactive astrocytes synthesize increased levels of growth factors and cytokines that regulate oligodendrocyte progenitor proliferation, differentiation, and myelin synthesis (56; 123). On the other hand, myelin debris can stimulate microglial

activation (30). Therefore, in addition to axon damage, myelin degradation may be a key element stimulating neuroinflammation and propagating chronic neurodegeneration in white matter tracts.

Our findings highlight the need to evaluate changes in both axon integrity and myelination throughout the post-injury time course after TBI. Magnetic resonance imaging studies in mild TBI patients demonstrated that diffusion tensor imaging of white matter is a significant predictor of outcome (198). Characteristics of the injury and host factors, including the immune response, may contribute to the progression of white matter damage and repair after TBI. These complex interactions warrant further attention to identify strategies to prevent white matter degeneration and enhance recovery.

Acknowledgements

We thank Tuan Le and Dr. Fengshan Yu for technical assistance. We thank lab members, Dr. Kimberly Byrnes and Dr. Aviva Symes for providing critical comments on the manuscript. This work was supported by the U.S. Department of Defense in the Center for Neuroscience and Regenerative Medicine.

FIGURES

Figure 2

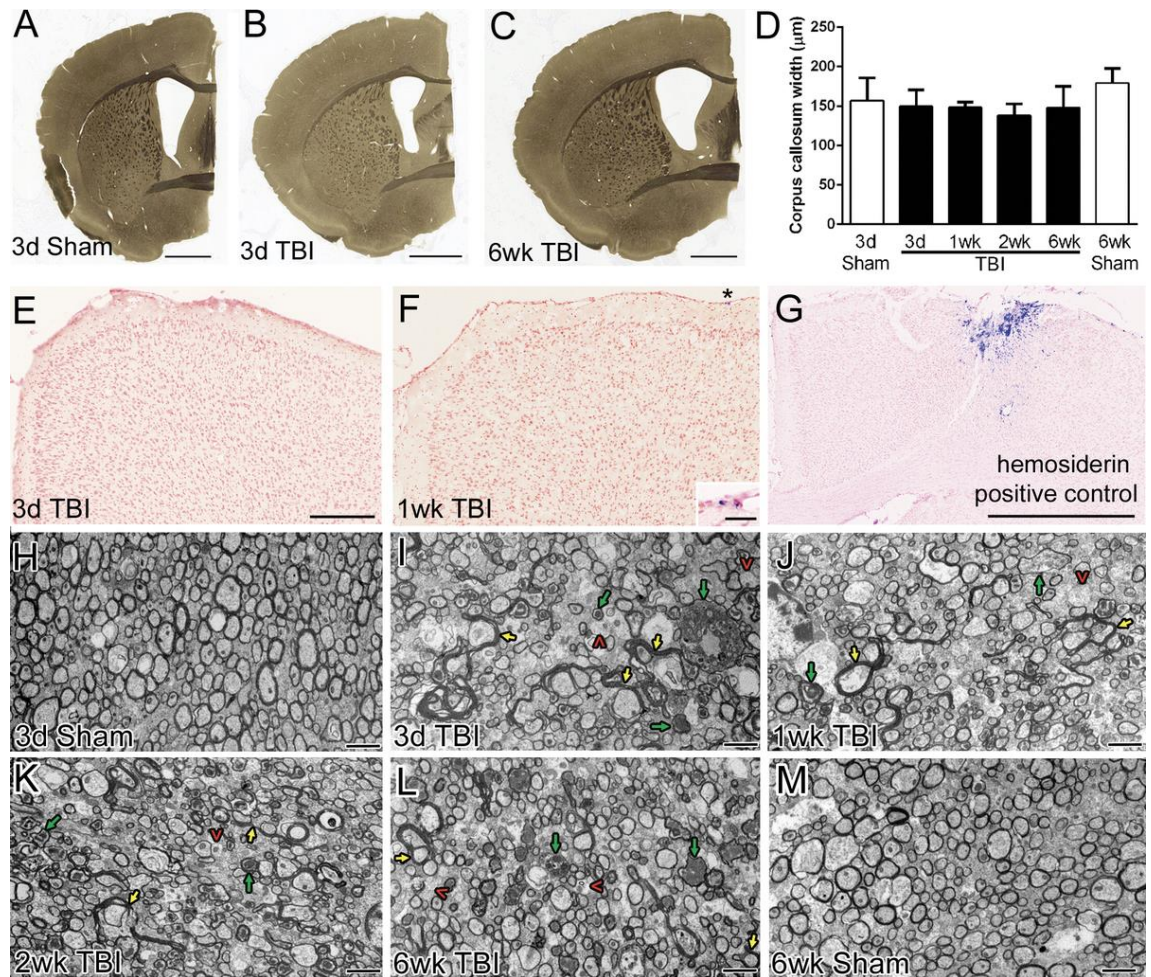


Figure 2. Overview of pattern of axon and myelin pathology in corpus callosum. **A-C:** Osmicated 40 µm sections show little overt pathology at the coronal level of the anterior commissure where the injury was centered. In some injured animals, there appears to be thinning of the corpus callosum with ventricular enlargement (C). **D:** The width of the corpus callosum does not differ significantly across injury conditions. **E-G:** Prussian blue histological stain to detect hemosiderin laden macrophages indicating areas of prior blood extravasation. In mice representative of those included in this study (E, F), the impact site showed appropriate cortical cytoarchitecture (pink pararosaniline nuclear counterstain) without cavitation. Rare hemosiderin deposits (blue) were located superficially (F, inset shows area below asterisk). In contrast, a mouse excluded from the study due to atypical skull fracture is shown as a positive control for the Prussian blue detection of hemosiderin (G). **H-M:** Sagittal sections (axons cut in cross section) were examined by electron microscopy at multiple time points in sham (H, K) and TBI (I-M) mice. Degenerating axons (green arrows), demyelinated axons (red caret symbols) and excessive myelin figures (yellow arrows) are each prominent pathological findings in TBI mice. These features are dispersed among a majority of normal appearing axons.

Ultrastructural changes are shown in more detail in subsequent figures. Scale bars A-C = 1 μ m; E-G = 250 μ m; F inset = 25 μ m; H-M = 2 μ m.

Figure 3

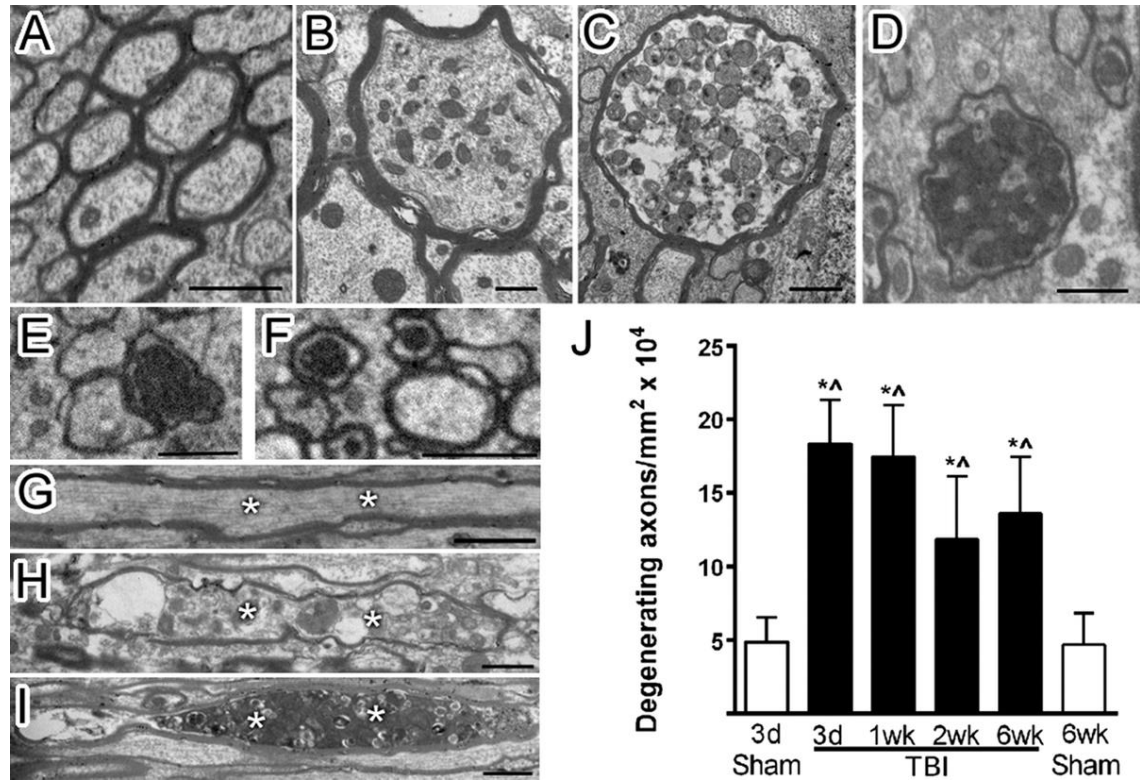


Figure 3. Multiple stages of degenerating axons are observed post-injury. A-I: After TBI, corpus callosum axons exhibit multiple stages of degeneration as shown in cross section (A sham; B-F TBI) and in longitudinal sections (G sham; H-I TBI). Sham axons (A) have a uniform cytoskeletal structure. In TBI animals, axon degeneration involves swelling and varying densities of accumulated vesicles (B-D). Axons with very dense cytoplasm (E) and swollen mitochondria (F) were also scored as degenerating. Longitudinal views also demonstrate uniform cytoskeletal structure in sham axons (G; asterisks) and accumulation of vesicles in degenerating axons after TBI (H, I; asterisks). **J:** Degenerating axons are increased across all time points in TBI mice. *p ≤ 0.0001 compared to 3 day sham; ^p ≤ 0.0001 compared to 6 week sham; Image time points – A, G: 3 day sham; B, C, E, F, I: 3 day TBI; D, H: 1 week TBI. Scale bars = 1 μ m.

Figure 4

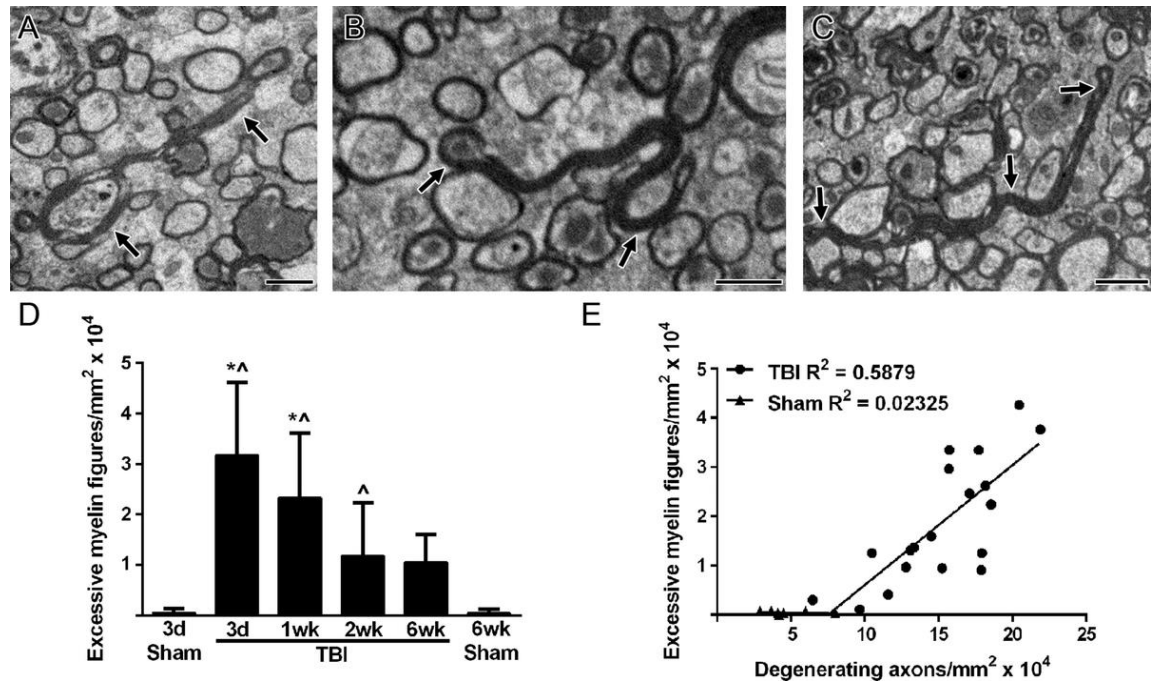


Figure 4. Excessive myelin figures correlate with degenerating axons. **A-C:** Sagittal sections of corpus callosum show examples of excessively long myelin folding back onto itself rather than tightly wrapping around an axon. Excessive myelin figures (black arrows) are associated with intact axons (A) and degenerating axons (B) or remain without an axon (C). **D:** Excessive myelin figures are prevalent at 3 days post-TBI and continue to be elevated through at least two weeks following injury. * $p \leq 0.0001$ compared to 3 day sham; ^ $p \leq 0.0001$ compared to 6 week sham for TBI at 3 day and 1 week but $p \leq 0.05$ for 2 week TBI. **E:** Excessive myelin figure values are graphed relative to degenerating axons. Each value represents a single mouse. All time points (3 days-6 weeks) are included. A strong correlation is observed within individual animals (Pearson $r = 0.7667$, $p < 0.0001$). Image time points - A: 6 week TBI; B: 1 week TBI; C: 3 day TBI. Scale bars = 1 μm .

Figure 5

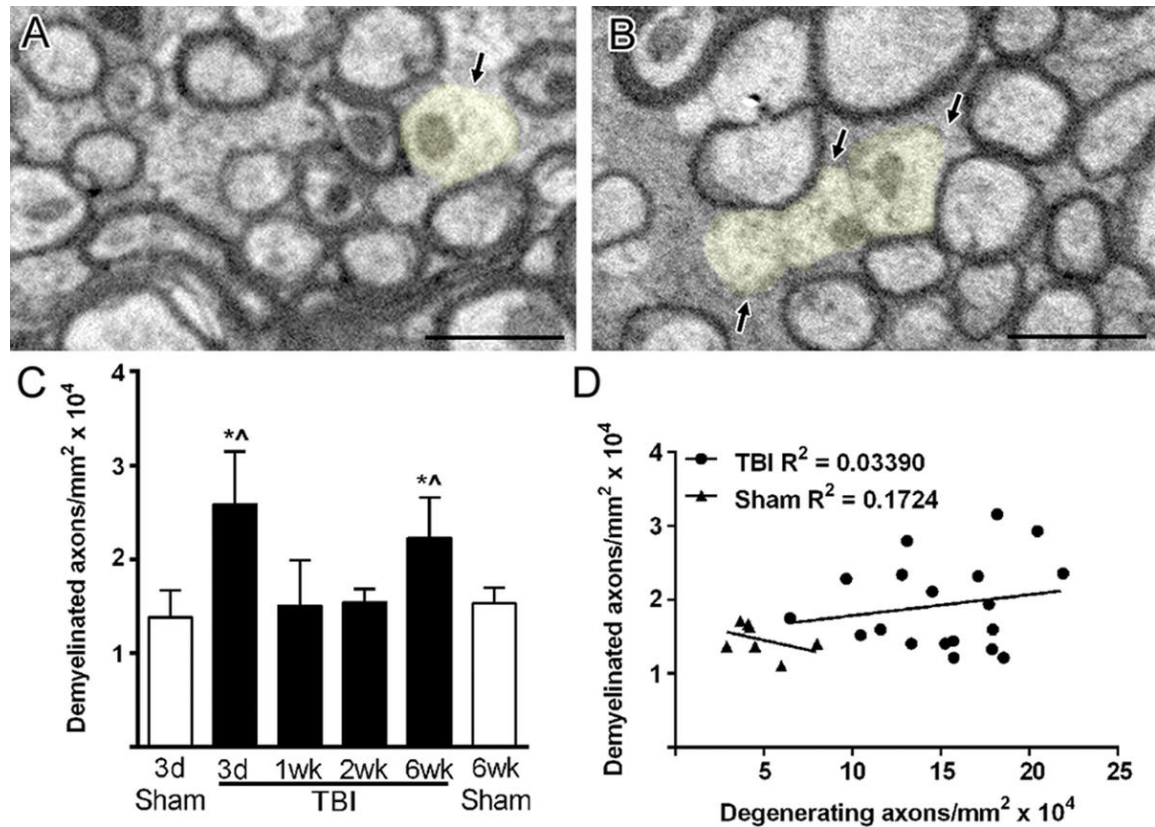


Figure 5. Intact axons undergo demyelination after TBI. **A-B:** Demyelinated axons (light yellow) have normal mitochondria and cytoskeletal structure but lack a myelin sheath. Only axons of $> 0.3 \mu\text{m}$ in diameter were counted as demyelinated in order to avoid potential inclusion of unmyelinated axons. **C:** Demyelinated axons are significantly increased after TBI but only at 3 days and 6 weeks. * $p \leq 0.01$ compared to 3 day sham, ^ $p \leq 0.05$ compared to 6 week sham. **D:** Values for demyelinated axons are not correlated with the values for degenerating axons within individual animals across the post-TBI time course (3 days - 6 weeks). Image time points – A: 3 day TBI; B: 6 week TBI. Scale bars = $1 \mu\text{m}$.

Figure 6

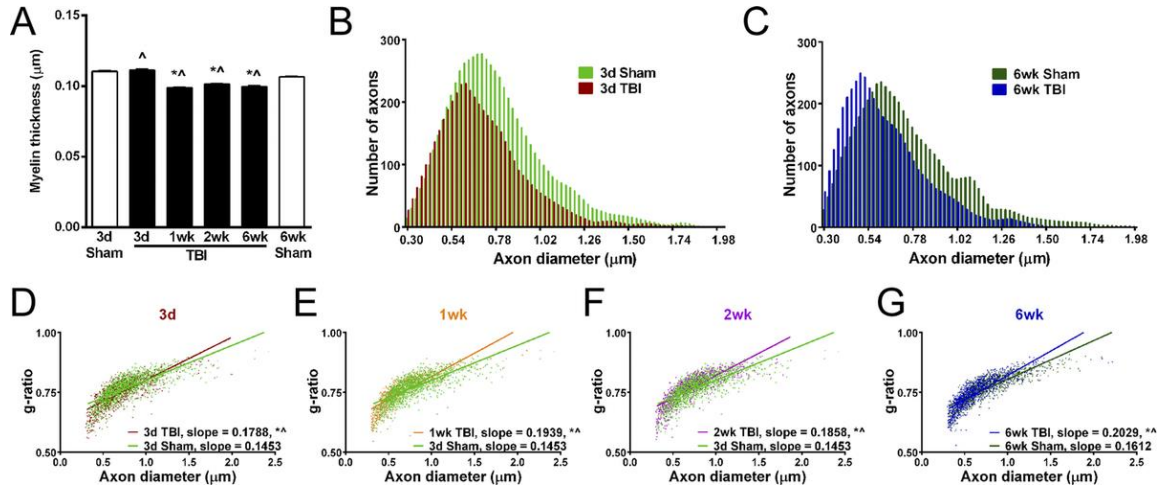


Figure 6. Reduction in myelin thickness after TBI. A-G: Quantitative analysis of axon diameter and myelin sheath thickness from sagittal electron micrographs of the corpus callosum. **A:** Myelin thickness is significantly reduced by 1 week post-TBI and continues to be thinner through 6 weeks. **B-C:** Among the total myelinated fiber populations examined, axon diameters are reduced across the range of fiber sizes at both early (B, 3 days) and late (C, 6 weeks) time points. The mean axon diameter is significantly reduced at 3 days (B; sham = 0.78 ± 0.26 ; TBI = 0.70 ± 0.23 ; $p \leq 0.001$) and 6 weeks (C; sham = 0.74 ± 0.28 ; TBI = 0.62 ± 0.22 ; $p \leq 0.001$). **D-G:** Analysis of the g-ratio (axon diameter divided by myelinated fiber diameter) facilitates more specific analysis of myelin thickness relative to a given axon size. Consistent with remyelination, the g-ratio slope is increased at each time point post-TBI. * $p \leq 0.0001$ compared to 3 day sham, [^] $p \leq 0.0005$ compared to 6 week sham.

Figure 7

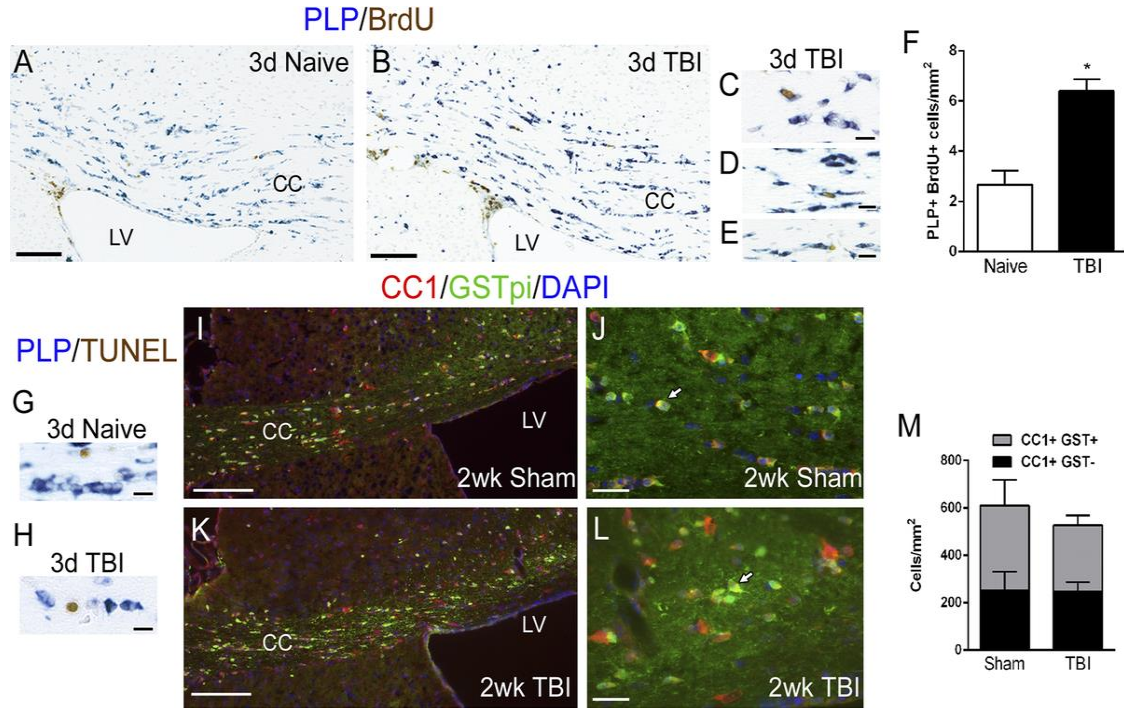


Figure 7. Characterization of the oligodendrocyte population post-TBI. **A-E:** The generation of new oligodendrocytes was examined by labeling endogenous cycling cells with a pulse of BrdU one day before TBI followed by a chase period through 3 days post-TBI to allow time for cell differentiation. Naïve mice were run in parallel. Oligodendrocytes were identified by in situ hybridization for PLP mRNA combined with immunostaining to detect nuclei with BrdU incorporation. **F:** Cells double labeled for PLP and BrdU, i.e. newly differentiated oligodendrocytes, are significantly increased in the corpus callosum after TBI (* $p = 0.0068$). **G-H:** To examine apoptosis of oligodendrocytes in the corpus callosum, in situ hybridization for PLP mRNA was combined with TUNEL staining at 3 days post-TBI or in age-matched naïve mice. TUNEL labeled cells were rare (1-2 cells/corpus callosum) and the labeled cells did not express PLP. **I-L:** The overall population of mature oligodendrocytes was identified by double immunostaining for CC1 and GSTpi. GSTpi is expressed in a subset of CC1 labeled oligodendrocytes (white arrows). **M:** The CC1 immunolabeled populations, with or without GSTpi, are not significantly different between sham and TBI mice. Scale bars A, B = 250 μ m; I, K = 100 μ m; J, L = 50 μ m (J, L); C-E, G, H = 5 μ m. CC = corpus callosum, LV = lateral ventricle.

Figure 8

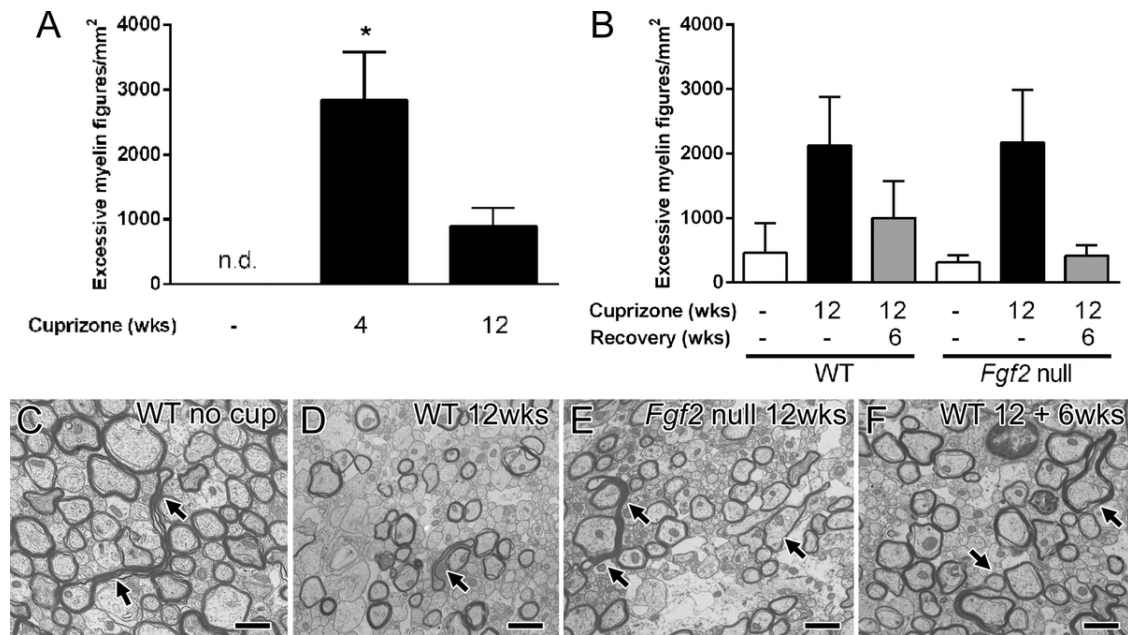


Figure 8. Excessive myelin figures during cuprizone induced demyelination and remyelination. **A:** In C57BL/6 mice, cuprizone ingestion produces widespread demyelination of the corpus callosum. Cuprizone treatment for 4 weeks results in oligodendrocyte loss, initial demyelination, and axon damage. Prolonged cuprizone administration through 12 weeks produces chronic demyelination. Excessive myelin figures were not detected (n.d.) in 8 week old mice without cuprizone but were significantly increased during the period of initial demyelination (* $p = 0.0009$). **B-F:** Excessive myelin figures were also analyzed relative to the extent of remyelination. Removal of cuprizone from the diet allows for endogenous progenitor cells to attempt to generate new oligodendrocytes and initiate remyelination. Remyelination is more extensive in fibroblast growth factor 2 (*Fgf2*) null mice in comparison with wild type (WT) mice (180). Excessive myelin figures were similar between WT and *Fgf2* null mice without cuprizone, during chronic demyelination (12 weeks), and after a subsequent 6 week period on a normal diet to allow for remyelination (12 + 6 weeks). **C-F:** Arrows indicate examples of excessive myelin figures present in all genotypes and time points. Scale bars = 1 μ m.

Figure 9

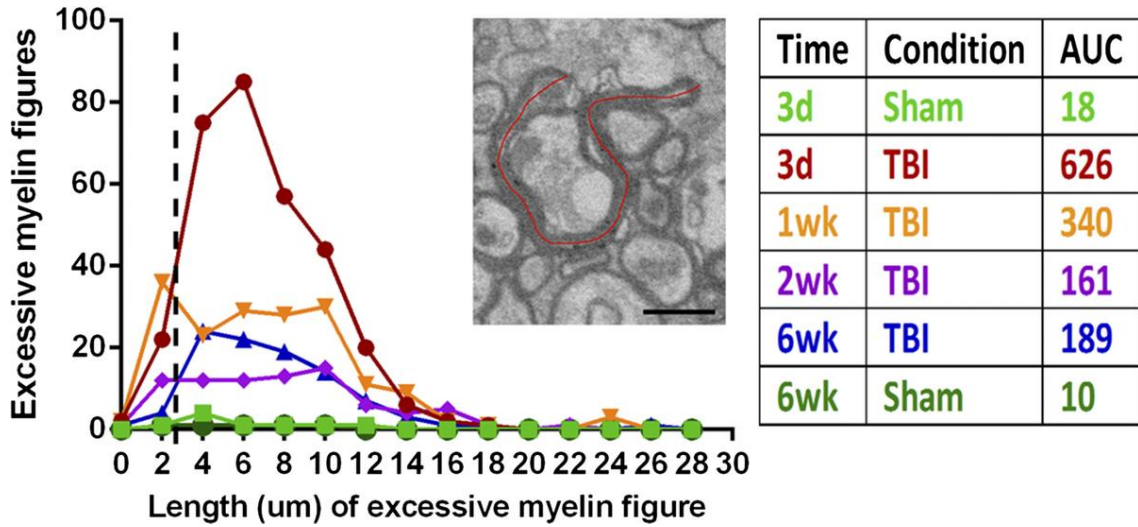


Figure 9. Quantification of myelin associated with excessive myelin figures. Excessive myelin figures were measured along the lumen of the myelin sheaths as shown on the micrograph (red line) and then doubled to determine the length of each excessive myelin figure. The frequency of excessive myelin figures is plotted relative to length at each sham or TBI time point. The dotted line shows the expected average length of a myelin sheath as calculated from the average myelin thickness and axon circumference. Thus, myelin figures to the left of the dotted line are of the length expected from simple axon loss and collapse of the remaining myelin sheath. Myelin figures longer than expected, i.e. to the right of the dotted line, are present at each post-TBI time point and markedly increased in length and frequency at 3 day post-TBI. The calculated area under the curve (AUC; table) quantifies the amount of myelin associated with excessive myelin figures. Scale bar = 1 μ m.

Figure 10

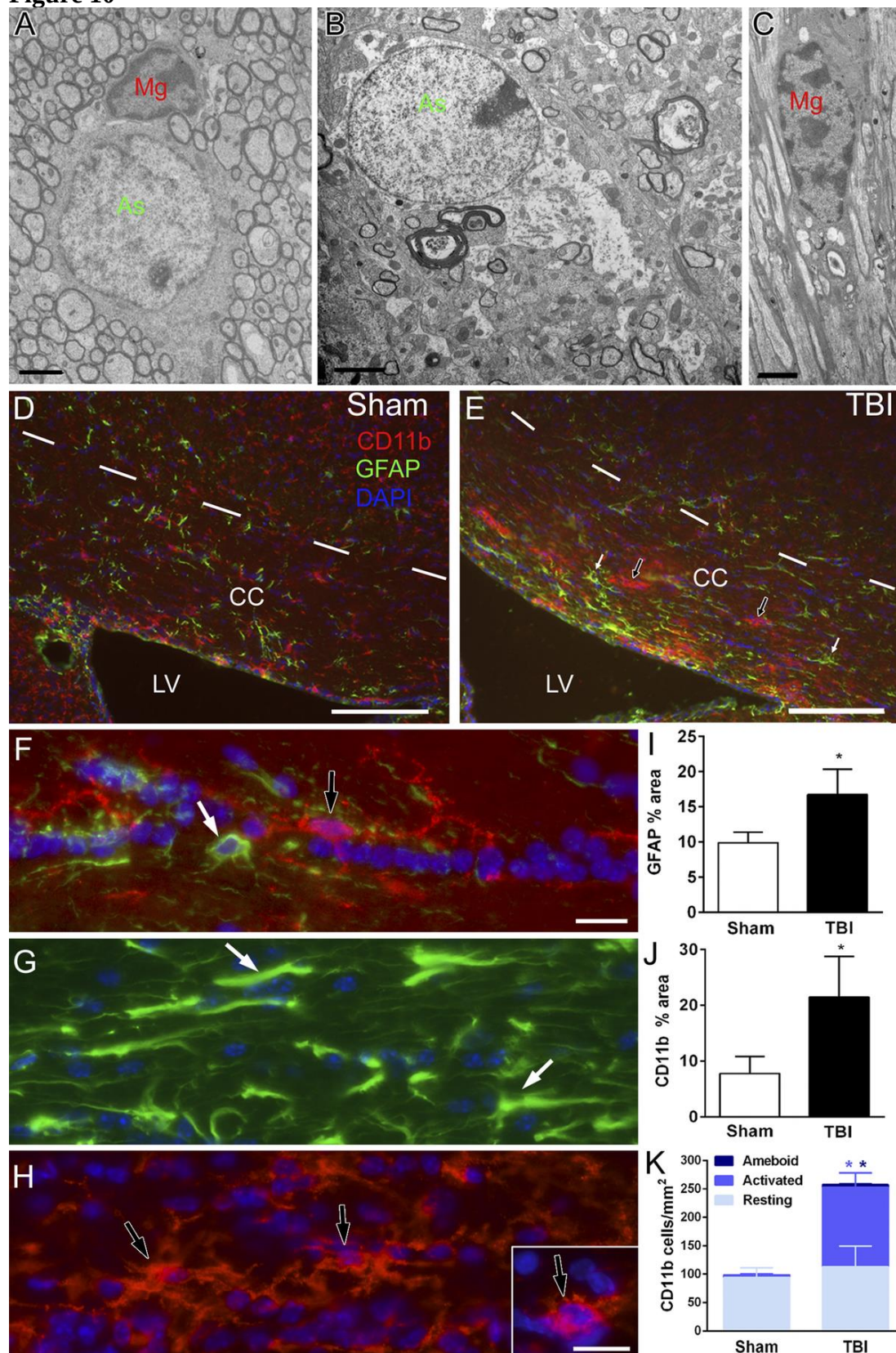


Figure 10. Mild TBI with TAI results in prolonged neuroinflammation. A-C: Electron microscopy of the corpus callosum shows normal astrocytes (green, As) and microglia (red, Mg) in sham mice (A) in contrast with images acquired in TBI mice, which exhibited hypertrophic astrocytes (B; green, As) and microglia that often contained debris (C; red, Mg). **D-H:** Immunohistochemistry for GFAP detects reactive astrocytes (E, white arrows) and CD11b shows activated microglia (E, black arrows) indicative of neuroinflammation localized to the corpus callosum after TBI (E; 6 week TBI), as compared to normal adult phenotypes in the sham mice (D; 6 week sham). Superior border of corpus callosum (CC) marked by dashes with lateral ventricle (LV) along inferior border. **F, G:** Higher magnification of GFAP immunolabeling of normal (F, white arrow) and reactive (G, white arrows) astrocytes. **F, H:** Higher magnification of CD11b of normal (F, black arrow), activated (H, black arrows), and ameboid (H, inset) microglia. **I:** At 6 weeks, astrogliosis persists in TBI animals (*p = 0.0040). **J:** CD11b staining of microglia is also increased at 6 weeks (*p = 0.0286). **K:** Resting microglia are similar between sham and TBI at 6 weeks. However, activated microglia and ameboid microglia are significantly increased (activated *p = 0.0015, ameboid *p = 0.0026). Image time points – A: 3 day sham; B, C: 3 day TBI; D: 6 week sham; E: 6 week TBI. Scale bars A-C = 2 μ m; D, E = 100 μ m; F, G, H and inset = 25 μ m.

CHAPTER 3:

Repetitive Model of Mild Traumatic Brain Injury Produces Cortical Abnormalities Detectable by Magnetic Resonance Diffusion Imaging (DTI/DKI), Histopathology, and Behavior

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Statement of Contributions:

Christina Marion started the in-house breeding for the Thy1-YFP-16 line used throughout the paper. She initially characterized aspects of the single TBI model in these Thy1-YFP-16 mice. Furthermore, she contributed to the single-injury Thy1-YFP-16 tissue analyzed. She also contributed to editing the full manuscript.

CHAPTER INTRODUCTION

In this chapter, we present supplementary materials and figures from “Repetitive Model of Mild Traumatic Brain Injury Produces Cortical Abnormalities Detectable by Magnetic Resonance Diffusion Imaging (DTI/DKI), Histopathology, and Behavior.” The paper characterized a repeat-injury TBI model, while the supplemental figures showed the corresponding experiments in a single-injury so that direct comparisons could be made between the TBI models. The entire supplement is presented here, as it characterizes the single-injury in the Thy1-YFP-16 mice that are used extensively in the projects presented in Chapters 4 and 5. Furthermore, the imaging, pathology, and behavioral findings included here also informed the experimental approaches used in those chapters.

SUPPLEMENTARY MATERIALS

Single-injury methods

A single-impact (s-mTBI) model has been previously characterized (116). Mice and methods for anesthesia and stereotaxic procedures were the same as described for the repetitive impact model. For s-mTBI, the scalp was incised along the midline to expose the skull, and a 3-mm-diameter tip was used to impact the skull at bregma (velocity set at 4.0 m/sec; depth of 1.5 mm; dwell time of 100 ms). Sham mice underwent identical procedures without receiving impacts. The single-injury study included 27 s-mTBI mice and 27 s-sham mice.

FIGURES

Figure 11

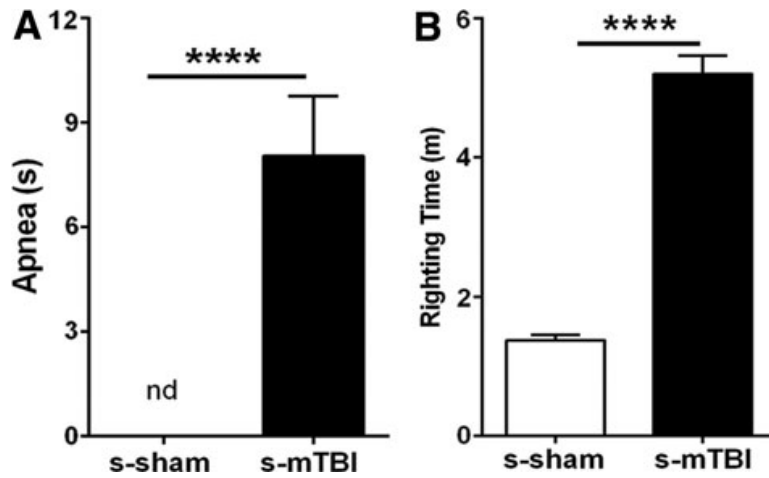


Figure 11. Post-surgical data for single mild traumatic brain injury (s-mTBI). Apnea was not detected (nd) after the sham procedure, but apnea duration was significantly increased after the impact in s-mTBI mice (A). The righting reflex time is significantly delayed in injured versus sham mice (B). Values are mean – standard error of the mean; n = 27; ****p < 0.0001.

Figure 12

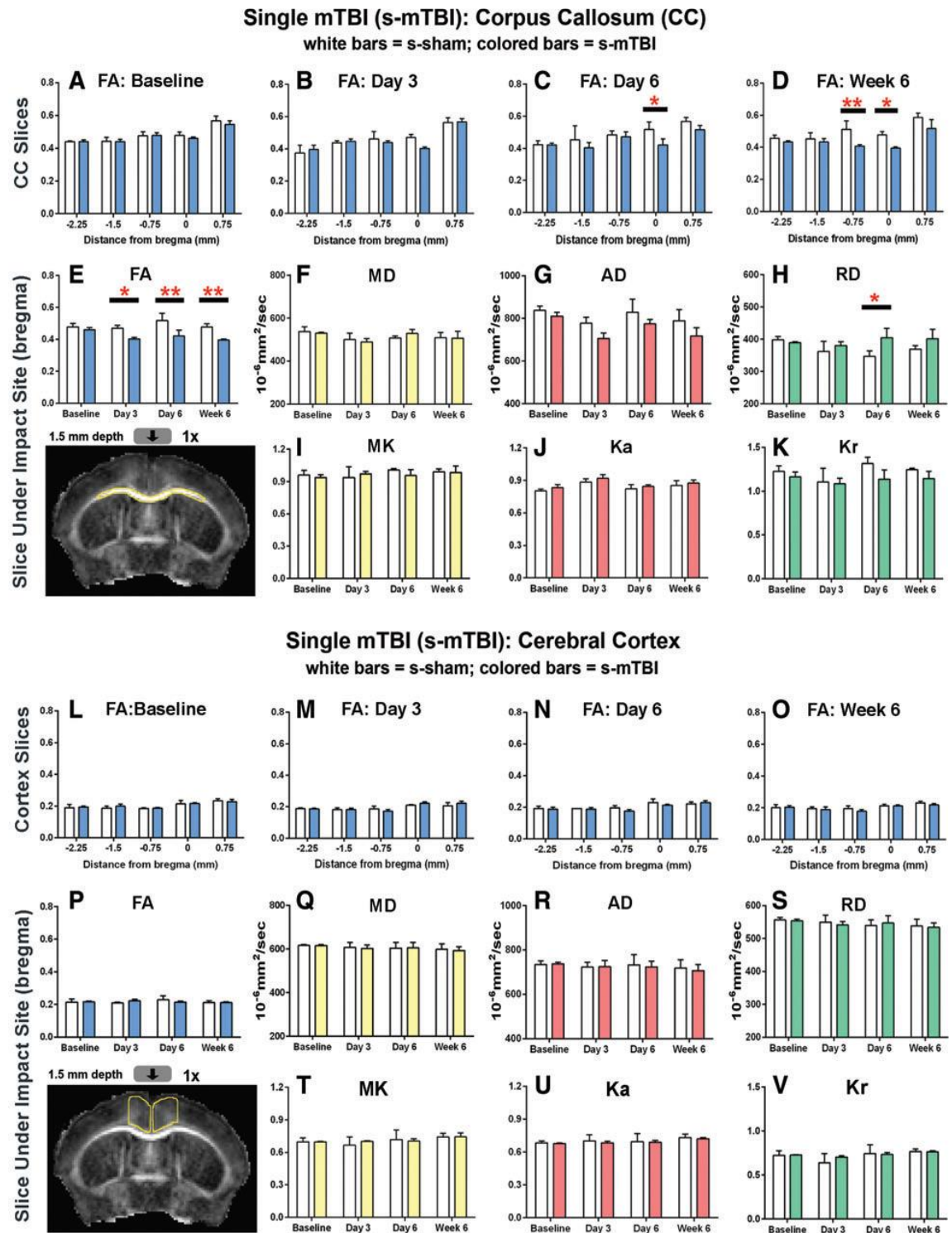
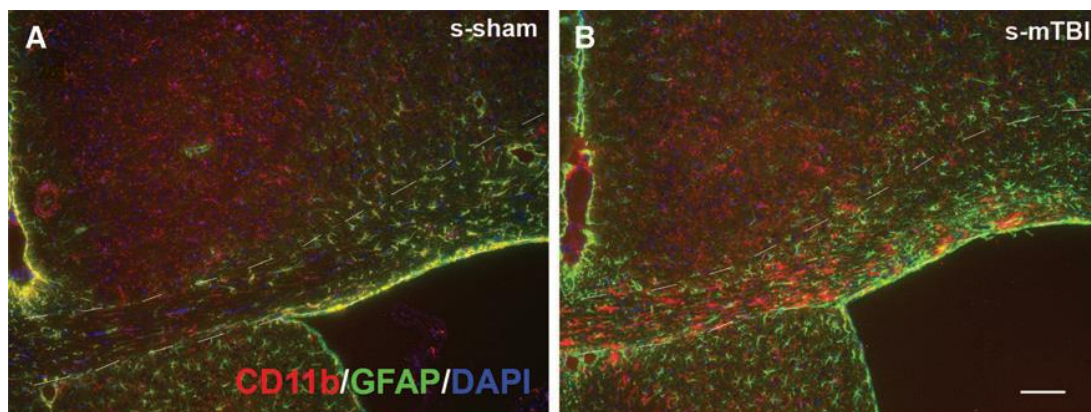
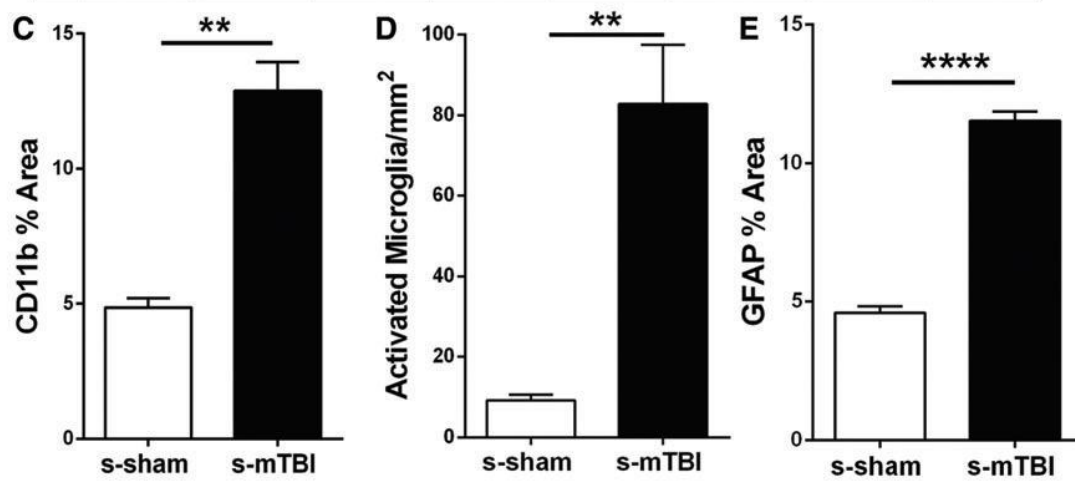


Figure 12. Diffusion imaging results for single mild traumatic brain injury (s-mTBI). (A–K) DTI (A–H) and DKI (I–K) analysis of corpus callosum (CC) region outlined in yellow on upper image. (L–V) DTI (L–S) and DKI (T–V) analysis of medial cerebral cortex region outlined in yellow on the lower image. Each region is shown at the coronal level containing the anterior commissure, which is under the impact site at bregma. s-sham and s-mTBI mice were scanned as yoked pairs with all mice being scanned at four time points—baseline (BL) and post-injury at 3 days, 6 days, and 6 weeks. Five coronal slices (one rostral to bregma, one at the coronal level of bregma = 0, and three caudal to bregma) were analyzed. (A–D) FA values in the CC showing all five coronal slices with locations noted as relative to bregma. A significant reduction in FA was noted in s-mTBI mice only at the bregma level at day 6 (C) and spread to include the adjacent caudal slice at week 6 (D). (E–K) CC values in the individual coronal slice at bregma are shown across time points for the full set of parameters analyzed—diffusion tensor imaging (FA, MD, AD, and RD) and diffusion kurtosis imaging (MK, Ka, and Kr). FA was significantly lower in the CC of s-mTBI mice compared to matched sham mice at each post-injury time point (E). Injury decreased AD values, but no time point reached significance on post-hoc analysis (G). RD values also show an overall injury effect and a significant increase at 6 days (H). (L–V) Matching analysis of the medial cortex shows a lack of effect from the single injury. Values are mean – standard deviation, n = 3 for each condition and time point; *p < 0.05; **p < 0.01. AD, axial diffusivity; FA, fractional anisotropy; Ka, axial kurtosis; Kr, radial kurtosis; MD, mean diffusivity; MK, mean kurtosis; RD, radial diffusivity; s-sham, single sham.

Figure 13



Corpus Callosum



Cerebral Cortex

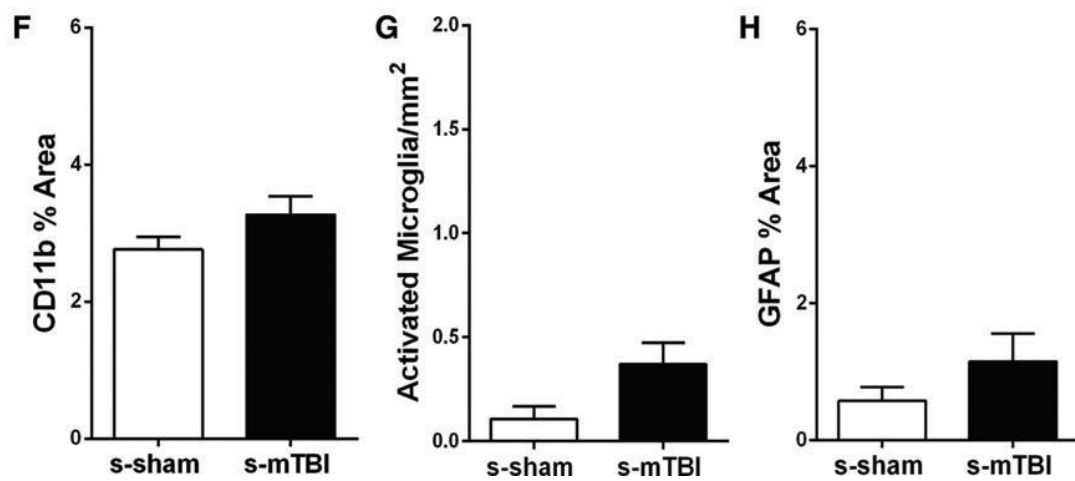


Figure 13. Neuroinflammation in the corpus callosum and cortex for single mild traumatic brain injury (s-mTBI). (A and B) Immunohistochemistry was used to detect microglia/macrophage cells with CD11b and astrocytes with GFAP in mice perfused after the final imaging scan, that is, 6 weeks post-injury or sham procedure. Microglia/macrophage activation and astrogliosis was most evident in corpus callosum after s-mTBI, as shown by the extent of GFAP immunolabeling and the increased intensity of CD11b immunolabeling in cells with a hypertrophic cell body and thickened processes (B) compared to the corpus callosum in s-sham mice (A). (C–E) Quantitative analysis shows that s-mTBI mice exhibit a significant increase in microglial activation in the corpus callosum relative to sham control (C and D). Similarly, astrogliosis in the corpus callosum was significantly increased after s-mTBI (E). (F–H) In contrast, the cortex did not show an increase of microglia activation (F and G) or astrogliosis (H) after s-mTBI. Dashed lines outline the corpus callosum. Values are mean – standard error of the mean; n = 3; scale bar = 50 μ m; **p < 0.01; ****p < 0.0001. DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; s-sham, single sham.

Figure 14

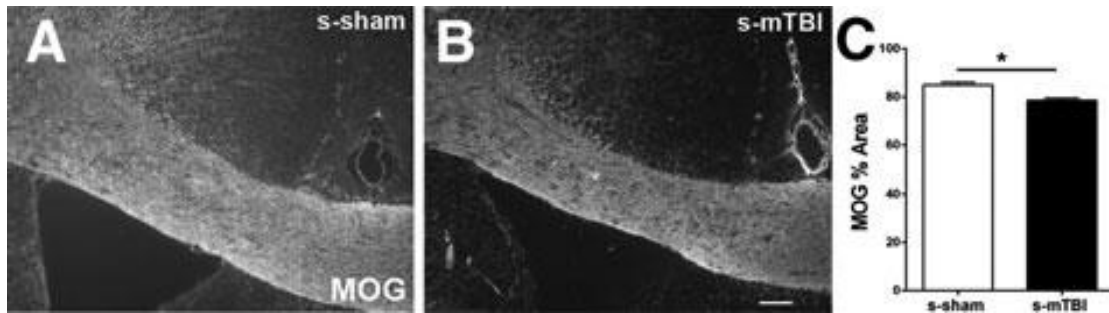


Figure 14. s-mTBI (single mild traumatic brain injury) resulted in demyelination in the corpus callosum at 6 weeks post-injury. (A and B) Immunohistochemistry for myelin oligodendrocyte glycoprotein (MOG) was used to study myelination status post-imaging after s-mTBI (B) or sham procedure (A). (C) Quantification of MOG showed a small, but significant, decrease in the area of MOG immunolabeling in the corpus callosum after s-mTBI. Values are mean – standard error of the mean; n = 3; scale bars = 50 μ m; *p < 0.05. s-sham, single sham.

Figure 15

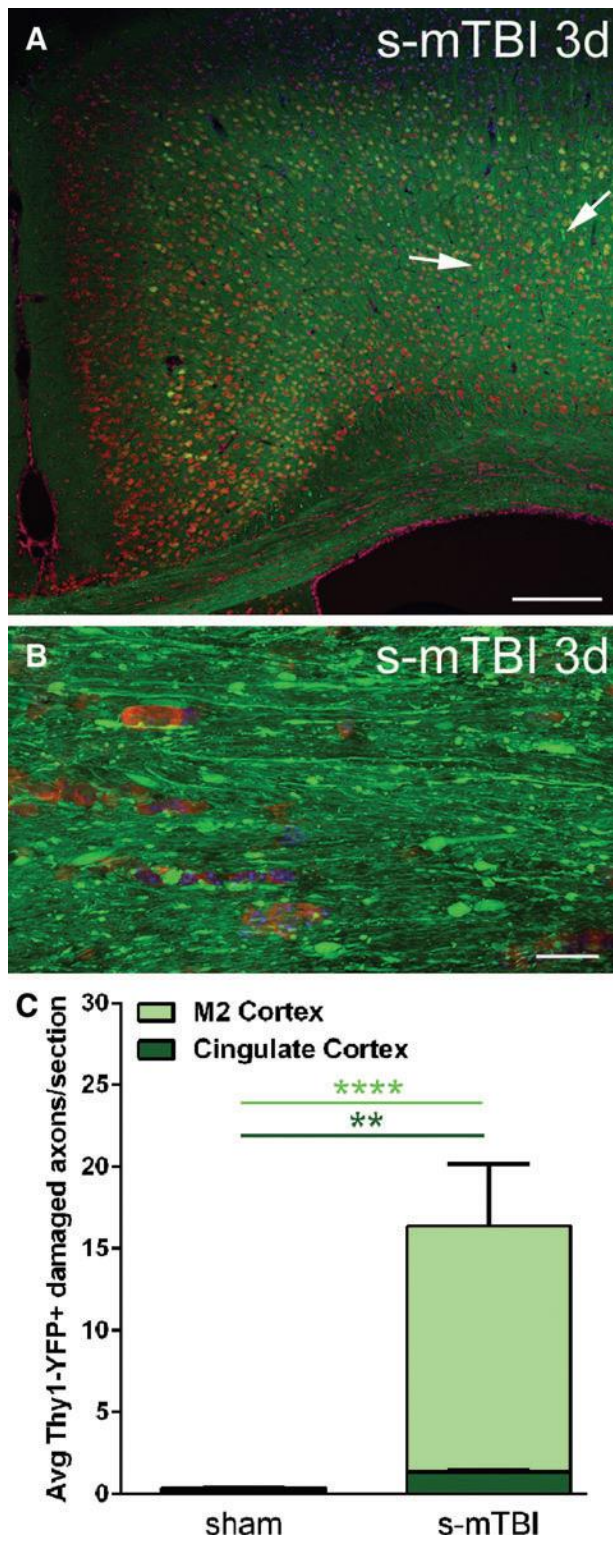


Figure 15. *Thy1-YFP-16 mice show axonal pathology in the cortex and corpus callosum after single mild traumatic brain injury (s-mTBI).* (A) Thy1-YFP (green) shows labeling of neurons throughout the cortex along with axons projecting through the corpus callosum in mice perfused 3 days after s-mTBI. Nissl staining (red) of the cytoplasmic rough endoplasmic reticulum along with DAPI nuclei counterstaining (blue) shows the overall cortical cytoarchitecture, which is not markedly disrupted in smTBI mice. Arrows point to examples of swollen segments of damaged axons. (B) Thy1-YFP (green) labeled the numerous swollen axonal segments observed in the corpus callosum of smTBI mice. Red = Nissl; blue = DAPI. (C) Quantification of damaged axonal profiles (swollen, axonal bulbs, and varicosities) labeled with Thy1-YFP in the cingulate and M2 motor cortices that comprise the region of interest in the medial cortex for DTI/DKI analyses. A significant increase of damaged axons was observed in the s-mTBI group compared to sham control. Values are mean – standard error of the mean; n = 3; 3–9 sections per animal. **p < 0.01; ****p < 0.0001. Scale bars A = 200 μ m; B = 20 μ m. Avg, average; d, days; DAPI, 4',6-diamidino-2-phenylindole; DKI, diffusion kurtosis imaging; DTI, diffusion tensor imaging.

Figure 16

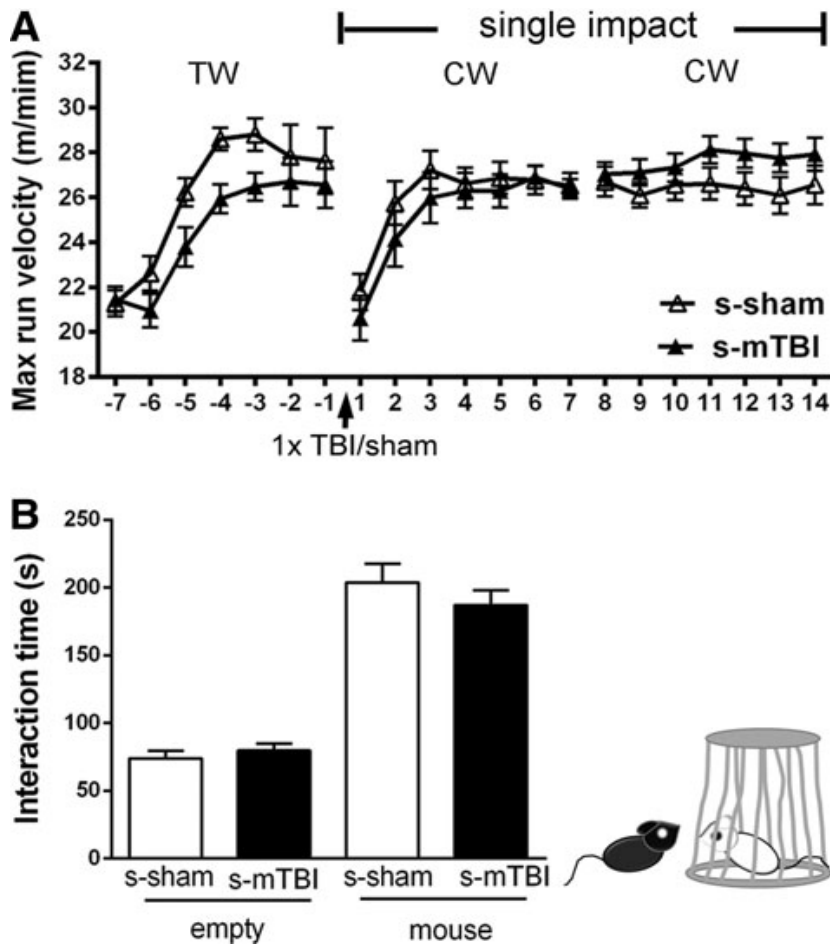


Figure 16. No social deficits were observed after single mild traumatic brain injury (s-mTBI). The mice were assessed for motor and social tasks related to functions of cortical regions under the impact site at bregma and corresponding areas of the corpus callosum. (A) The cohorts ($n = 12$ per condition) did not show differences in any phase on the training or complex wheel after s-mTBI. (B) A three-chamber sociability test was further employed to test social behavior at 3 weeks after mTBI. The s-sham mice spend the majority of time interacting with the carrier containing a stranger mouse rather than the empty carrier. No difference was observed after s-mTBI. Values are mean – standard error of the mean; $n = 12$. s-sham, single sham.

CHAPTER 4

***Sarm1* Deletion Reduces Axon Degeneration, Demyelination, and White Matter Atrophy After Experimental Traumatic Brain Injury**

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Statement of Contributions:

Christina Marion contributed to the experimental design of all experiments and performed all experimental procedures, with the exception of the electron microscopy processing, sectioning, and some imaging, which was handled by Dr. Dennis McDaniel.

ABSTRACT

Traumatic brain injury (TBI) often damages axons in white matter tracts and causes corpus callosum (CC) atrophy in chronic TBI patients. Injured axons encounter irreversible damage if transected, or alternatively may maintain continuity and subsequently either recover or degenerate. Secondary mechanisms can cause further axon damage, myelin pathology, and neuroinflammation. Molecular mechanisms regulating the progression of white matter pathology are important potential targets for developing therapeutics. SARM1 is essential for execution of the conserved axon death molecular pathway. We examined the contribution of SARM1 to axon degeneration and chronic white matter pathology by producing mild TBI with CC traumatic axonal injury in mice with genetic deletion of *Sarm1* (knockout mice; *Sarm1*^{-/-}). Ultrahigh resolution structural analysis at 3 days post-TBI revealed dramatically reduced axon degeneration in *Sarm1*^{-/-} mice, as compared to wild-type controls (C57BL/6 and *Sarm1*^{+/+}). TBI induced demyelination, i.e. myelin loss along otherwise intact axons, was reduced in *Sarm1*^{-/-} mice, while excessive myelin figures remained elevated. We next assessed *Sarm1* involvement in white matter neurodegeneration at 8 weeks post-TBI in *Sarm1*^{-/-} mice crossed to Thy1-YFP reporter mice. Cortical neurons under the impact site did not exhibit apoptosis or overt pathology, while occasional swellings in CC axons indicated continued axon damage. Importantly, TBI produced significant CC atrophy in Thy1-YFP/*Sarm1*^{+/+} mice that was attenuated in Thy1-YFP/*Sarm1*^{-/-} mice. Surprisingly, astrogliosis and microglial activation persisted in the CC of Thy1-YFP/*Sarm1*^{-/-} mice. This study demonstrates that *Sarm1* inactivation has promising acute and chronic benefits of reducing axon degeneration, demyelination, and white matter atrophy after TBI.

Figure 17

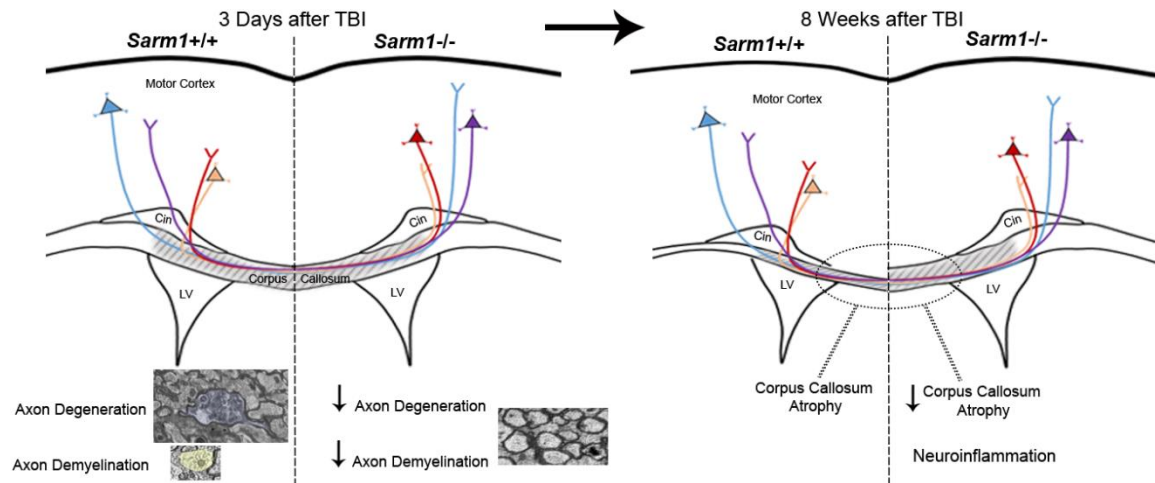


Figure 17. Graphical abstract for *Sarm1* Deletion Reduces Axon Degeneration, Demyelination, and White Matter Atrophy After Experimental Traumatic Brain Injury.

Highlights:

- *Sarm1* deletion has a protective effect against axon degeneration after TBI.
- *Sarm1* deletion prevents TBI induced demyelination of otherwise intact axons.
- *Sarm1* deletion reduces corpus callosum atrophy in late stage TBI.
- *Sarm1* deletion increases astrogliosis and microglial activation in late stage TBI.
- SARM1 inactivation is a promising target to prevent long term white matter injury.

Keywords: SARM1, Sterile alpha and TIR containing motif 1, axon, myelin, TBI, white matter, corpus callosum, myelin outfoldings, demyelination, traumatic axonal injury

INTRODUCTION

Traumatic brain injury (TBI) is a massive global health issue, with more than 27 million new cases annually and an increasing incidence rate (83). Across the TBI spectrum, long axons within the white matter are especially susceptible to compression, tension, and torsion forces resulting in diffuse axonal injury (23; 39; 40; 85; 160). Reduced white matter integrity associated with diffuse axonal injury, particularly in the corpus callosum (CC), is often observed in TBI patients and corresponds to worsened outcomes (3; 11; 28; 94; 129; 154). Neuroinflammation and white matter degeneration can persist for years after TBI, and unfortunately CC atrophy is common in patients with chronic symptoms (77; 84; 125; 181; 191).

The progression of white matter injury after TBI is poorly understood. Within white matter tracts, both unmyelinated and myelinated axons are vulnerable to damage by TBI. In addition, TBI induced myelin pathology includes demyelination, which is the loss of myelin around intact axons, and abnormal myelin structure (23; 105; 116; 171). Myelin enables rapid impulse firing and fast conduction of action potentials and also provides trophic and metabolic support to axons (76; 118; 122). Therefore, myelin pathology could cause processing speed deficits and expose axons to further damage (93; 130; 134).

After TBI, axons that are irreversibly damaged undergo Wallerian degeneration, by which the distal process degrades following a conserved molecular axon death pathway (32; 34; 74). This molecular pathway is of particular interest as a target for acute interventions that could mitigate axon degeneration after TBI. Still, axon preservation alone may not be sufficient to prevent functional deficits after TBI. Myelin pathology,

such as demyelination, must also be addressed to enable proper axon function. Additionally, an acute intervention must also mitigate chronic neurodegeneration and reduce atrophy of vulnerable white matter tracts to alter a patient's recovery trajectory and improve TBI outcome.

Traumatic axonal injury-induces phosphorylation of sterile alpha and Toll/interleukin-1 receptor motif-containing 1 (SARM1) to initiate execution of axon degeneration. SARM1 hydrolyzes nicotinamide adenine dinucleotide (NAD⁺) and loss of NAD⁺ leads to a cascade of ionic imbalance and cytoskeletal/structural degeneration of the axon (25; 60-62; 173). *SARM1* has a highly regular and conserved sequence across 107 species, including humans, indicating potential evolutionary pressure to regulate function (104). Specifically, genetic selection has highly purified the *SARM1* Toll interleukin receptor (TIR) domain, which functions as a NADase to execute axon degeneration (47; 104). However, SARM1 differs from other TIR domain containing proteins and so involvement in the innate immune response is not yet clear (104).

In experimental primary axonal injury, such as nerve transection, genetic deletion of *Sarm1* delays axon degeneration throughout the lifespan (58; 65; 133). However, the role of SARM1 is not yet understood *in vivo* in complex lesions with continued exposure to multiple sources of potential insults to vulnerable axons. In the context of acute TBI in mice, *Sarm1* deletion attenuates TBI-induced behavioral deficits and neurometabolic dysfunction, which was primarily observed at 2 hours after injury (78). Furthermore, knocking out *Sarm1* reduces the number of axonal lesions associated with damage between 2 and 48 hours after TBI.(78; 202) The majority of axon damage occurs after the initial TBI event and results from secondary mechanisms of damage (23; 106). Studies in

an optic nerve stretch model of traumatic axonal injury indicated that axons continue to initiate Wallerian degeneration days and weeks after the initial mechanical insult (109). This continued initiation of axonal injury offers a therapeutic window for intervention in *Sarm1* execution of axon degeneration.

The objective of the current study was to test whether *Sarm1* genetic deletion can significantly mitigate TBI induced white matter pathology in both axons and myelin. Additionally, at longer post-injury time points, we sought to test whether *Sarm1* deletion is sufficient to attenuate neuroinflammation and chronic CC atrophy. We used a single-impact TBI in mice that produces traumatic axonal injury in the CC, which reflects the susceptibility of long myelinated axons in the human CC after TBI (105; 116; 171; 197). This TBI model also results in an innate immune response with persistent astrogliosis and microglial activation. This white matter injury leads to chronic CC atrophy (105; 116). Within the CC, ultrahigh resolution electron microscopy identifies a range of TBI-induced axon pathology, including disrupted fast axonal transport, compacted cytoskeletal structure, and swollen mitochondria (116). Electron microscopy also identifies distinct myelin pathologies that can only be detected and quantified at the ultrastructural level (105; 116). Therefore, the current experiments accurately delineate the effect of *Sarm1* deletion on axonal pathology and the corresponding myelin pathology after TBI. In addition, this study examines the capacity for *Sarm1* deletion to mitigate CC atrophy and evaluates the relationship to neuroinflammation in the CC and cerebral cortex. Together these experiments provide a genetic proof-of-concept that blocking activation of the conserved axon death pathway would be beneficial for preserving axonal integrity, preventing demyelination, and reducing chronic white matter atrophy after TBI.

METHODS

Mice

All mice were treated in accordance with guidelines of the Uniformed Services University of the Health Sciences and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were socially housed with 2- 5 mice per 35 cm × 16.5 cm × 18 cm cages containing enrichment objects. Mice were maintained on a standard 12 hour cycle of daytime light (6:00–18:00) during which all procedures took place. The following mouse strains were obtained from The Jackson Laboratory: C57BL/6J (RRID: IMSR_JAX:000664), B6.129X1-*Sarm1*^{tm1Aidi}/J (RRID:IMSR_JAX:018069), and B6.Cg-Tg(Thy1-YFP)16Jrs/J (RRID:IMSR_JAX:003709). The B6.129X1-*Sarm1*^{tm1Aidi}/J and was generated so that exons 3-6 were replaced with a neomycin resistance gene in reverse orientation and null mice were backcrossed to a C57BL/6 strain (89). Pathological evaluation, focused on the brain where *Sarm1* is normally highly expressed, did not identify abnormalities at the gross or microscopic level, and confirmed no *Sarm1* protein is produced in null mice (64; 89). A cohort of C57BL/6 (C57 (J)) and B6.129X1-*Sarm1*^{tm1Aidi}/J (*Sarm1*^{-/-} (J)) mice were purchased and acclimated for at least 3 d before use in experiments. For subsequent experiments, additional *Sarm1* knockout mice were crossed to either C57BL/6J mice or B6.Cg-Tg(Thy1-YFP)16Jrs/J (Thy1-YFP) mice and bred as in-house colonies to generate the experimental littermate mice (*Sarm1* littermates or Thy1-YFP/*Sarm1* littermates). The overall number of mice of each strain was C57 (J) (*n* = 13), *Sarm1*^{-/-} (J) (*n* = 14), *Sarm1*^{+/+} littermates (*n* = 9), *Sarm1*^{-/-} littermates (*n* = 10), Thy1-YFP/*Sarm1*^{+/+} littermates (*n* = 12) and Thy1-YFP/*Sarm1*^{-/-} littermates (*n* = 9).

TBI and Sham Procedures

The TBI procedure followed protocols previously described (105; 116; 197). This TBI model results in axon pathology in the CC that is most extensive at coronal levels beneath the impact site and is also present in axons in the overlying cingulum (105; 171; 197). Mice received TBI or sham procedures at 8–10 weeks of age. Under isoflurane anesthesia, the scalp was incised along the midline to expose the skull. TBI mice received an impact using a Leica stereotaxic impactor (Leica Biosystems, Buffalo Grove, IL) and 3-mm-diameter tip with velocity set at 4.0 m/s; depth of 1.5 mm; dwell time of 100 ms while sham mice were treated exactly the same but did not receive the TBI impact. Depressed skull fracture and impactor malfunction were predetermined exclusion criterion leading to exclusion of 2 mice. Apnea and righting reflex times were recorded for all mice. Mice were randomly allocated to TBI or sham procedures using the random number generator function in Microsoft Excel. Investigators were blinded to animal injury group allocation and genotype until after data analysis.

The righting reflex was used as a measure of the time the mouse was unresponsive after surgery and serves as an indicator of loss of consciousness. The righting reflex was recorded as the time from the end of anesthesia to the return to the upright position. Righting reflex time did not differ between male and female littermates, so the sexes were combined for analysis by genotype. The righting reflex was significantly slower after TBI as compared to the sham procedure for each genotype in the electron microscopy cohort (ANOVA One Way; $F(7, 35) = 22.92$; $p < 0.0001$) and for the Thy1-YFP crosses (ANOVA One Way; $F(3, 17) = 43.82$; $p < 0.0001$). For the electron microscopy cohort, the Sidak's adjusted values for each sham/TBI comparison

were C57 (J) ($p < 0.0001$), *Sarm1*^{-/-} (J) ($p = 0.0001$), *Sarm1*^{+/+} ($p < 0.001$), *Sarm1*^{-/-} ($p < 0.0001$), while the *Sarm1*^{+/+} and *Sarm1*^{-/-} mice had similar righting reflex delays after TBI ($p = 0.8046$). The adjusted values for the Thy1 crosses for sham/TBI comparisons were Thy1-YFP/*Sarm1*^{+/+} ($p < 0.0001$) and Thy1-YFP/*Sarm1*^{-/-} ($p < 0.001$) while TBI produced a similar righting reflex delay in both genotypes ($p = 0.9802$). These data indicate that the concussive TBI caused a significant loss of consciousness. *Sarm1* genotype did not alter this indicator of injury severity, in agreement with a prior study using a different concussive model (78).

Electron Microscopy

For ultrahigh resolution imaging of ultrastructure, mice were anesthetized with ketamine/xylazine before transventricular cardiac perfusion with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA; Cat #19210) and 2.5% glutaraldehyde (Electron Microscopy Sciences; Cat #16210) in 1M PO₄ at 3 days after surgery. The heads were post-fixed in the fixative solution for 3 hours before brains were dissected, hemisected along the midline, and left in fixative overnight. One hemisphere was sectioned (40 μ m) as sagittal slices using a Leica vibrating VT-1200 vibratome (Leica Biosystems) and left in fresh fixative overnight before washing with 1M PO₄. Sections were selected for further processing based on localization at the coronal level of the anterior commissure and at parasagittal levels to include the corpus callosum over the lateral ventricles. Samples were immersed in 2% (OsO₄; Electron Microscopy Sciences; Cat #19100) in cacodylate buffer (0.1M, pH 7.4) for 1 hour, then washed again in in cacodylate buffer, dehydrated in ethanol, and infiltrated with Spurr epoxy resin (Electron Microscopy Sciences; Cat #14300). Samples were then polymerized at 70 °C for 11

hours, and thin sections (~70 nm) were cut on an Ultracut UC6 ultramicrotome (Leica Biosystems). Copper grids containing thin sections were poststained for 20 minutes in 2% aqueous uranyl acetate (Electron Microscopy Sciences; Cat #22400) and for 5 minutes in Reynolds lead citrate (149). Grids were reviewed on a JEOL JEM-1011 transmission electron microscope (JEOL USA Inc, Peabody, MA). Images from 5,000-15,000x were captured using an AMT XR50S-A digital camera (Advanced Microscopy Techniques, Woburn, MA).

Immunohistochemistry

Thy1-YFP/*Sarm1* mice were analyzed for pathology at 8 weeks after TBI or sham procedures. Mice were deeply anesthetized and transcardially perfused with 0.1M PB then 4% paraformaldehyde (PFA) before brains were excised, dissected, and post-fixed overnight in fixative. After 24 hours in 30% sucrose at 4 °C for cryoprotection, brains were embedded in OCT compound (Sakura Finetek, Torrance, CA), and sectioned coronally at 14 µm thickness on a Leica CM 1900 UV cryostat (Leica Biosystems, Buffalo Grove, IL). Myelin was immunolabeled with a primary mouse monoclonal recognizing myelin oligodendrocyte glycoprotein (MOG) (1:100; Millipore, Burlington, MA; Cat# MAB5680, RRID: AB_1587278) followed by incubation with AlexaFluor-647-conjugated secondary antibody (1: 300, Jackson ImmunoResearch Laboratories, West Grove, PA; 715-605-151, RRID: AB_2340863) and counterstaining with DAPI (Sigma-Aldrich, St. Louis, MO; D9542) before mounting with Vectashield (Vector Laboratories, Burlingame, CA; H-1000, RRID: AB_2336789). Astrocytes were immunostained with monoclonal mouse antibody against glial fibrillary acidic protein (GFAP) (1:500, Millipore; Cat# MAB3402, RRID: AB_94844) with secondary

incubation using AlexaFluor-647-conjugated secondary antibody (1: 300, Jackson ImmunoResearch Laboratories; 715-605-151, RRID: AB_2340863).

Microglia/macrophages were identified using polyclonal rabbit antibody against ionized calcium binding adaptor molecule 1 (Iba1) (1:1000; Wako, Richmond, VA; Cat# 019-19741, RRID: AB_839504) followed by incubation with AlexaFluor-594-conjugated secondary antibody (1:100, Jackson ImmunoResearch Labs Cat# 711-585-152, RRID: AB_2340621). For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), we utilized ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore; Cat# S7100, RRID: AB_2661855). To assess cytoarchitecture of the cerebral cortex, slides were incubated with NeuroTrace 530/615 red fluorescent Nissl stain (Thermo Fisher Scientific, Waltham, MA; Cat# N21482, RRID: AB_2620170).

Quantification of Electron Microscopy

In-house bred littermate cohorts consisted of *Sarm1*^{+/+} sham (n = 5; 3 female, 2 male) and TBI (n = 4; 2 female, 2 male) and *Sarm1*^{-/-} sham (n = 5; 3 female, 2 male) and TBI (n = 5; 1 female, 4 male). Cohorts used directly as purchased from Jackson Laboratories consisted of C57 (J) sham (n = 7 males) and TBI (n = 6 males) and *Sarm1*^{-/-} (J) sham (n = 7 males) and TBI (n = 7 males) run in parallel. Mice were sacrificed at 3 days after sham or TBI procedure.

Prepared grids of sagittal sections through the CC were imaged at 5,000× magnification. Five or more images per animal were quantified. Within each image a 17 µm x 12.5 µm region was defined as the counting frame, within which more than 120 axons were quantified. All axons within the counting frame were classified as intact

axons, demyelinated axons, degenerating axons, or axons with abnormal mitochondria by an investigator blinded to the TBI/sham condition and genotype. Degenerating axons were defined as axons with cytoskeletal changes (microtubule and neurofilament density extremely low or high with irregular spacing) or axons with accumulated vesicles. Axons with mitochondria filling >50% of the axon cross-section were defined as having abnormal mitochondria. Demyelinated axons were manually counted based on a lack of detectable compact myelin and an axon diameter larger than 0.3 μm . Axons smaller than 0.3 μm were excluded from analysis as potentially unmyelinated fibers that are present in healthy adults (169). Excessive myelin figures were manually counted and identified as myelin sheaths folding back onto one another while extending away from an axon. Excessive myelin figures were measured along the lumen between the two sides of the myelin sheath, and that length was doubled to determine the length of each excessive myelin figure. Additional images were taken at 10,000-15,000x for illustration of pathological features.

Quantification of immunohistochemistry

Cohorts of Thy1-YFP/*Sarm1* littermates underwent either TBI or sham surgery and were perfused for analysis 8 weeks after the procedure for *Sarm1*^{+/+} sham (n = 6; 3 female, 3 male) and TBI (n = 6; 1 female, 5 male) along with *Sarm1*^{-/-} sham (n = 4; 1 female, 3 male) and TBI (n = 5; 2 female, 3 male).

Fluorescent images were acquired on an Olympus IX70 microscope using a SPOT RT3 camera (Diagnostic Instruments). Intensity values of immunolabeled pixels above background levels were thresholded for quantification using ImageJ (National Center for

Microscopy and Imaging Research: ImageJ Mosaic Plug-ins, RRID: SCR_001935). Myelin, astrocyte, and microglial fluorescence thresholding to estimate the immunolabeled area was performed as previously detailed (9). For TUNEL quantification, tissue sections were digitized on a Zeiss Axio Scan Z.1 (Carl Zeiss) and the number of TUNEL+ cells was counted in the CC and cortex for each section using Zen software (Carl Zeiss, ZEN Digital Imaging for Light Microscopy, RRID: SCR_013672). For all images quantified, the CC region-of-interest (ROI) extended from the midline laterally to under the peak of the cingulum at coronal levels between +0.5 and -0.5 mm relative to bregma. The CC width (superior-inferior thickness) was calculated as the average of measurements taken at the midline, ~200 μ m lateral to the midline, under the peak of the cingulum, and over the lateral ventricle using MOG staining (105; 116). The cerebral cortex ROI extended from the midline, laterally above the CC and cingulum, and extending from the peak of the cingulum upward to the cortical surface (197). Quantification for MOG, Iba1, and GFAP included three or more images collected from at least two tissue sections per mouse. TUNEL+ quantification included at least three full sections.

Statistical Analysis

All investigators responsible for data collection and analysis were blinded to the sham or TBI condition and to genotype. Data was analyzed and graphed using GraphPad Prism 7.0 software (RRID: SCR_002798). Sample size was predetermined based on prior experiments, so that an *n* of 5 or 6 mice per condition were used for electron microscopy quantification or immunohistochemical analysis (detailed above). Statistical comparisons for sham and TBI conditions were made within genotype and across genotypes for

littermate cohorts (*Sarm1*^{+/+}, *Sarm1*^{-/-}). Experiments using C57BL/6 mice and *Sarm1*^{-/-} mice were compared only within each genotype for sham versus TBI and were not compared across genotypes. Therefore, two-way ANOVA was not appropriate for both data sets. One-way ANOVA was used to determine statistically significant differences between the means with correction for multiple comparisons using Sidak's test. Accordingly, this same analysis was used for the Thy1-YFP/*Sarm1* experiments. A p value less than 0.05 was considered statistically significant. For clarity, only statistically significant differences are provided.

RESULTS

***Sarm1* genetic deletion dramatically reduces axon degeneration after TBI but does not prevent mitochondrial swelling.**

We first asked whether *Sarm1* deletion delays the diverse forms of axon pathology resulting from TBI. We used ultrahigh resolution electron microscopy to interrogate axonal structure in *Sarm1*^{-/-} knockout and *Sarm1*^{+/+} wild type littermate controls after sham or TBI (Fig 1). A time point of 3 days was selected to capture ongoing TBI-induced Wallerian degeneration in the CC (105; 116; 171). In sham mice, intact axons have organized cytoskeletal structures and healthy mitochondrial morphology (Fig 18A). After TBI, *Sarm1*^{+/+} axons show a range of pathological features associated with injury, including swollen axons with accumulated vesicles (Fig 18B), axons with collapsed cytoskeleton and condensed cytoplasm (Fig 18C), and axons with swollen mitochondria (Fig 18D). Sagittal sections through the CC region-of-interest

show healthy axons of varied diameter in sham *Sarm1*^{+/+} mice (Fig 18E). In contrast, after TBI, dispersed degenerating axons and axons with swollen mitochondria are present in *Sarm1*^{+/+} mice (Fig 18F). *Sarm1*^{-/-} white matter axons have normal ultrastructure in sham mice (Fig 18G). After TBI, *Sarm1*^{-/-} mice have less overall axonal pathology (Fig 18H), which was borne out upon quantitative analysis. The density of intact axons is significantly reduced after TBI in *Sarm1*^{+/+} mice, with improved survival of intact axons in *Sarm1*^{-/-} mice (Fig 18I). TBI induced significant axon degeneration in mice of both genotypes, however the *Sarm1*^{-/-} mice had significantly less axon degeneration compared to *Sarm1*^{+/+} mice (Fig 18J). Importantly, genetic deletion of *Sarm1* did not alter the density of axons with swollen mitochondria after TBI (Fig 18K), suggesting mitochondrial abnormalities are upstream or independent from *Sarm1* execution of the axon death pathway. To demonstrate reproducibility, compare to prior studies that used C57BL/6 mice to assess axonal ultrastructure, and ensure against changes (e.g. genetic drift, microbiome, etc.) during our in-house breeding, we repeated these analyses in C57BL6/J mice and *Sarm1*^{-/-} mice purchased directly from the Jackson Laboratories. We again found that after TBI *Sarm1* deletion preserves intact axons (Fig 18L), and results in fewer degenerating axons (Fig 18M) yet does not prevent axons with swollen mitochondria (Fig 18N).

***Sarm1* genetic deletion reduces TBI induced demyelination.**

Traumatic axonal injury in white matter tracts often involves myelinated axons (105; 116). Therefore, we next asked whether the protective effect of *Sarm1* deletion on axons could extend to preservation of myelin, which is critical for maintaining proper axon function. TBI leads to two major myelin pathological features (Fig 19). TBI causes

demyelination, or loss of myelin, from otherwise intact axons (Fig 19A) (105; 116). TBI also results in excessive myelin figures (Fig 19B), which are long outfoldings of myelin membrane that extend away from intact or degenerating axons (116). We assessed these myelin pathological features at 3 days after sham or TBI procedures, when they have previously been found to be most prevalent (116). Sham *Sarm1*^{+/+} mice have a high density of myelinated axons in the CC ROI (Fig 19C). Comparatively, TBI in *Sarm1*^{+/+} mice causes demyelination of otherwise intact axons and excessive myelin figures (Fig 19D). In *Sarm1*^{-/-} sham mice have a typical and healthy pattern of myelination (Fig 19E). However, there is less overall TBI-related myelin pathology in the *Sarm1*^{-/-} mice (Fig 19F).

Quantitative analysis shows that as compared to sham mice, TBI significantly increases the density of demyelinated axons in *Sarm1*^{+/+}, but not *Sarm1*^{-/-} mice (Fig 19G). These data demonstrate that *Sarm1* deletion not only attenuates axon degeneration but also prevents myelin loss along intact axons. In contrast, TBI increases excessive myelin figures to a similar extent in both *Sarm1*^{+/+} and *Sarm1*^{-/-} mice, as compared to each sham condition (Fig 19H). However, the excessive myelin figures are distinctively shorter in *Sarm1*^{-/-} mice than in *Sarm1*^{+/+} mice (Fig 19I). Excessive myelin figures extended several microns in length and did not appear to be simply collapsed myelin sheaths of degenerating axons (Fig. 19J).

Analysis of myelin pathology in independent experiments, using C57 and *Sarm1*^{-/-} mice, directly purchased from Jackson Laboratories, also demonstrated that TBI increases demyelinated axons in C57 mice but not *Sarm1*^{-/-} mice (Fig 19K). In addition, TBI induces excessive myelin figures to similar levels in both genotypes (Fig 19L).

These findings indicate that demyelination and excessive myelin involve two distinct processes relative to the progression of axon degeneration after TBI, and that *Sarm1* deletion prevents demyelination.

***Sarm1* genetic deletion reduces corpus callosum atrophy in chronic TBI.**

We next asked whether these benefits of *Sarm1* deletion in acute TBI could extend to reduce the progression of white matter injury to chronic neurodegeneration and CC atrophy. We crossed *Sarm1*^{-/-} mice to Thy1-YFP mice that express yellow fluorescent protein (YFP) in a subset of cortical neurons, including those that extend axons across the CC. The distribution of YFP in axons is a very sensitive indicator of damage since YFP readily fills axonal swellings.(105; 197) The CC of Thy1-YFP/*Sarm1*^{+/+} and Thy1-YFP/*Sarm1*^{-/-} littermates was examined at 8 weeks after the sham or TBI procedure (Fig 20). The CC in Thy1-YFP/*Sarm1*^{+/+} sham mice is clearly identified by YFP labeling of axons and immunolabeling of myelin for MOG (Fig 20A). The CC appears thinned after TBI in Thy1-YFP/*Sarm1*^{+/+} mice (Fig 20B). The CC morphology in Thy1-YFP/*Sarm1*^{-/-} sham mice appears similar to sham Thy1-YFP/*Sarm1*^{+/+} littermates (Fig. 20C). Thy1-YFP/*Sarm1*^{-/-} mice do not have marked pathology in CC morphology or myelination after TBI (Fig 20D). Rare axonal swellings can still be detected by YFP labeling at 8 weeks after TBI in Thy1-YFP/*Sarm1*^{+/+} (Fig 20E) and Thy1-YFP/*Sarm1*^{-/-} (Fig 20F) mice. Thus, axon damage is not fully resolved and persists chronically after TBI. Quantification of MOG immunolabeling shows that a similar proportion of the CC area is myelinated in the sham and TBI groups, and in each genotype (Fig 20G). This finding is in agreement with our electron microscopic findings that TBI induced demyelinated axons dispersed within overall areas of myelinated white

matter (Fig 19). Importantly, the thickness of the CC is significantly reduced after TBI in Thy1-YFP/*Sarm1*^{+/+} mice (Fig 20H). This CC thinning is attenuated in Thy1-YFP/*Sarm1*^{-/-} littermates (Fig 20H). Therefore, *Sarm1* genetic deletion is sufficient to reduce late stage white matter atrophy after TBI.

***Sarm1* genetic deletion does not suppress CC neuroinflammation in chronic TBI.**

This *Sarm1* role in CC atrophy led us to examine neuroinflammation, which has been implicated as contributing to chronic white matter atrophy after TBI (84; 91; 100; 116; 171). Thy1-YFP/*Sarm1*^{+/+} and Thy1-YFP/*Sarm1*^{-/-} cohorts used to evaluate CC atrophy (Fig 20) were further analyzed by immunohistochemistry to determine if *Sarm1* deletion alters astrogliosis and microglial activation after TBI (Fig 21). Thy1-YFP/*Sarm1*^{+/+} sham mice show low levels of immunoreactivity for both GFAP in astrocytes and Iba1 in microglia (Fig 21A). Both markers are noticeably increased after TBI, indicating astrogliosis and microglial activation (Fig 21B). The Thy1-YFP/*Sarm1*^{-/-} mice also show low immunolabeling in sham mice that increases after TBI (Fig 21C-D). The more intense immunolabeling in both astrocytes and microglia corresponds with shorter, thicker processes indicative of reactive phenotypes after TBI (21E-G). Quantification supports the illustrated results of increased GFAP that is actually more extensive in Thy1-YFP/*Sarm1*^{-/-} mice than in Thy1-YFP/*Sarm1*^{+/+} mice after TBI (Fig 21H). Similarly, Iba1 immunolabeling was significantly increased after TBI, as compared to sham, only in Thy1-YFP/*Sarm1*^{-/-} mice (Fig 21I). This data demonstrates that white matter astrogliosis and microglial activation persists for months after TBI and is not attenuated in parallel with reduced CC atrophy in mice with *Sarm1* deletion.

***Sarm1* deletion effect in CC does not require overt cortical pathology.**

Finally, we explored the effect of *Sarm1* deletion in the overlying cortical areas that extend axons through regions of CC atrophy after TBI (Fig 22). The cortical cytoarchitecture in the Thy1-YFP/*Sarm1* cohort was examined in the premotor/motor cortex areas that lie beneath the impact site and extend axons in the CC ROI (63; 72). Thy1-YFP/*Sarm1*^{+/+} mice do not show marked changes within the cerebral cortex between sham and TBI procedures (Fig 22A-C). Similarly, Thy1-YFP/*Sarm1*^{-/-} mice also do not show overt differences in cortical cytoarchitecture after either sham or TBI procedures (Fig 22D-F). More specifically, TBI did not induce cortical atrophy (Fig 22G), myelin loss (Fig 22H), astrogliosis (Fig 22I), or microglial activation (Fig 22J) in either genotype. Additionally, TUNEL staining for apoptotic cells revealed only rare TUNEL⁺ cells within this cortical region for all conditions (*Sarm1*^{+/+} sham = 2.29, *Sarm1*^{+/+} TBI = 1.07, *Sarm1*^{-/-} Sham = 1.37, and *Sarm1*^{-/-} TBI = 1.81 per section in ROI). These negative pathology findings in the cortex reduce the likelihood that *Sarm1* effects in the CC are secondary to effects on apoptosis or neuroinflammation associated with the neuronal cell bodies. Additionally, this data localizes the protective effects of *Sarm1* genetic deletion to the white matter, through preventing TBI-induced axonal degeneration, demyelination, and chronic CC atrophy.

DISCUSSION

The current findings demonstrate that genetic deletion of *Sarm1* attenuates axon degeneration, demyelination, and white matter atrophy after TBI. This data supports

inactivation of the axon death pathway, and potentially SARM1, as a promising therapeutic target to mitigate white matter injury after concussive TBI. More directly, the current data yields important insights of axon-myelin pathology relative to molecular regulation of axon degeneration and development of chronic pathology after TBI.

The ultrahigh resolution structural detail afforded by electron microscopy revealed the effect of *Sarm1* deletion relative to multiple features of axon and myelin pathology induced by TBI. Prior neuropathological studies in human and in animal models have recognized distinct features of degenerating axons after TBI (39; 86; 116). *Sarm1* deletion reduced the extent of axon degeneration which was detected by electron microscopy as either impaired fast axonal transport in swollen axons filled with accumulated vesicles or as compaction of the axon cytoskeleton (Fig 18). This result is in agreement with *Sarm1* deletion preventing execution of the conserved axon death pathway and Wallerian degeneration.

These studies also demonstrated that *Sarm1* deletion did not prevent TBI induced mitochondrial swelling in axons (Fig 18). Mitochondrial swelling has long been recognized as an early feature of Wallerian degeneration (185; 190). Mitochondrial dysfunction contributes to SARM1 execution of the axon death pathway, possibly at multiple stages. SARM1 is phosphorylated and activated under oxidative stress conditions but SARM1 also inhibits mitochondrial respiration (120). However, *Sarm1* deletion that blocks calcium elevation and axon death does not prevent early mitochondrial dynamic changes (98). In addition, *Sarm1* deletion protects neurons from accumulation of reactive oxygen species (172). Therefore, our results after TBI, together

with these findings in simpler systems, indicate that TBI induced mitochondrial changes may occur upstream of SARM1 activation.

Evaluation of *Sarm1* deletion relative to myelin pathology has never before been reported, and particularly gained from an electron microscopy approach (Fig 19). Proper axon function is highly dependent on intact ensheathing myelin structure and axon-myelin molecular interactions (122; 131). In our current (Fig 19) and prior studies of this concussive model, TBI resulted in demyelination of dispersed axons within the white matter in wild type mice (116). In a prior study, additional techniques also identified abnormal nodes of Ranvier and adjacent paranodes, where myelin attaches to the axon (105). A corresponding functional deficit was demonstrated using *ex vivo* electrical recordings of CC axons at this same time point of 3 days post-TBI (105). Therefore, the current finding that TBI did not induce axon demyelination in mice with *Sarm1* deletion may be important for maintaining myelin for axon function along with effects on preserving axon integrity.

Sarm1 deletion did not prevent a second form of TBI induced myelin pathology referred to as excessive myelin figures (Fig 19). This formation of myelin sheaths extending away from a degenerating, or intact, axon is very rare in the sham condition yet dramatically increased after TBI in the current results and in our prior studies (116; 171). These excessive myelin figures appear similar to “redundant” myelin or myelin “outfoldings” reported in development and in aging (116; 139; 151; 163; 168). Myelin “outfoldings” have also been observed in mutations of myelin proteins or scaffolding proteins involved in axon-myelin interactions (46; 136). It is not clear why *Sarm1* deletion reduced the length of the excessive myelin figures but did not prevent their

formation after TBI. One possibility is that disrupted axon-myelin interactions may be an early response to axon damage, which would be consistent with the mitochondrial swelling that was not blocked by *Sarm1* deletion. Importantly, the specific effect of TBI induced formation of excessive myelin figures in white matter injury will require more study. Myelin outfoldings in some contexts can slow nerve conduction velocity (46; 136). In addition, myelin debris can activate microglia so that excessive myelin figures could fuel processes leading to chronic neuroinflammation (30; 53).

An important finding of the current study is that *Sarm1* deletion significantly reduced atrophy of the CC after chronic TBI. This promising result raises the possibility that intervening to reduce early axon degeneration could have a long-term benefit, and potentially prevent poor patient outcomes. A surprising contrast was that *Sarm1* deletion did not reduce astrogliosis and microglial activation in these same tissues (Figs 20, 21). Our prior TBI studies in this model have characterized the development of astrogliosis and microglial activation during the first week and at 6 weeks (116; 171). Therefore, this neuroinflammation begins early and likely persists in the CC throughout the time course to be observed now as late as 8 weeks post-injury (Fig 21). In contrast, CC atrophy is not significant at 4 or 6 weeks post-injury and then develops by 8 weeks (105; 116). Chronic TBI studies have often focused on microglia/macrophage activation as contributing to white matter neurodegeneration and atrophy (97; 143; 158). The current results indicate more specific studies are required to predict the relationship of astrogliosis and microglial activation in the progression of white matter injury, including the complex phenotyping of beneficial versus detrimental effects of this innate immune response in a given lesion environment (51; 67).

Certain limitations of the current study are important to the interpretation of the data. The *Sarm1* knockout employed is not restricted to neurons and so cannot definitively identify reduced axon degeneration as the mechanism underlying the other protective effects on demyelination and atrophy, which could involve indirect effects and non-neuronal cells. However, this model of genetic deletion in all cell types is relevant to understanding the potential effects of a typical broad pharmacological exposure. More importantly, this genetic deletion is not induced after TBI and so does not have a clinically practical time post-injury for the initiation of treatment. Further studies with additional approaches, such as gene therapy or small molecule inhibitors relevant to this pathway, may help determine an effective treatment window (59; 202). The interaction of SARM1 with related molecular pathways also warrants much more investigation to modify axon degeneration without unacceptable effects on restricting neuroplasticity and neuroregeneration (159). Additional limitations are inherent in our use of an experimental model that reflects specific aspects of human TBI but cannot recapitulate the full human pathology. This model reflects human TBI pathology based on the pattern of traumatic axonal injury of concussive TBI, reduced white matter integrity detected using diffusion tensor imaging, and late stage CC atrophy (3; 105; 116; 125; 129; 181; 197). This concussive model can be considered mild in severity since the mice experience loss of consciousness but do not show evidence of overt tissue damage or microhemorrhage on neuroimaging or neuropathology (116; 171; 197). Studies in additional experimental TBI models would be of interest to test the effects of *Sarm1* deletion on additional pathological features given the heterogeneity of human TBI.

Conclusion

This study shows that *Sarm1* genetic deletion reduces white matter pathology at early and late time points after experimental TBI. SARM1 is being examined as a promising therapeutic target for diverse forms of axonal injury, including chemotherapy-induced peripheral neuropathy, neurodegenerative disorders, and a subset of traumatic injuries (159). The current findings underscore the important role of SARM1 in the progression of white matter injury after TBI and provide evidence for TBI as a potential indication for therapeutics targeting this axon death pathway.

Acknowledgements

The authors thank Tuan Le and Xiaomei Zi for their technical assistance. These studies were funded by the U.S. Department of Defense Center for Neuroscience and Regenerative Medicine (CNRM703386; CNRM702720) and Defense Health Agency (ST7434918).

FIGURES

Figure 18

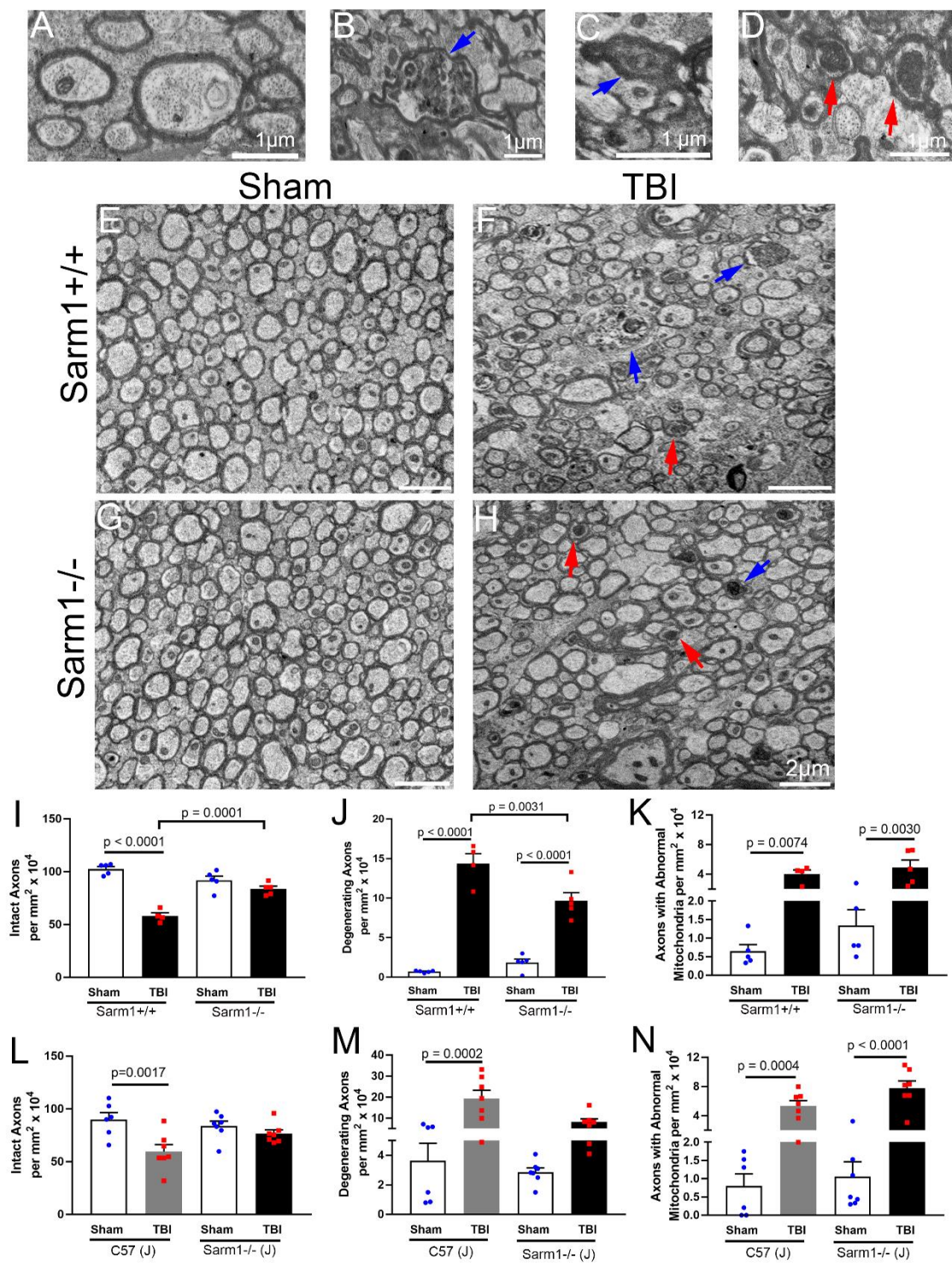


Figure 18. Axon degeneration at 3 days after TBI. **A-D**, Ultrahigh resolution electron microscopic examples of axonal ultrastructure in sham (A) and TBI (B-D) *Sarm1*^{+/+} mice. **A**, Intact axons with organized cytoskeletal structure, healthy mitochondrial morphology and compact myelin. **B**, Degenerating axon (blue arrow) swollen with accumulated vesicles indicative of disrupted fast axonal transport. **C**, Degenerating axons (blue arrow) with collapsed cytoskeleton and condensed cytoplasm. **D**, Axons (red arrows) with swollen mitochondria, which were scored as abnormal if enlarged to more than 50% of the axon cross section. **E-H**, Representative images of sagittal sections through the corpus callosum region-of-interest. **E**, Example of healthy white matter region in *Sarm1*^{+/+} sham mice showing axons of varied diameter. **F**, TBI results in dispersed degenerating axons (blue arrows) and axons with swollen mitochondria (red arrows) in *Sarm1*^{+/+} mice. **G**, *Sarm1*^{-/-} white matter has typical healthy axonal ultrastructure in sham mice. **H**, *Sarm1*^{-/-} mice exhibit less overall pathology after TBI, although examples of degenerating axons (blue arrow) and dispersed axons with swollen mitochondria (red arrows) are still present. **I-K**, Quantitative analysis of axon pathology in *Sarm1*^{-/-} mice and *Sarm1*^{+/+} mice bred in-house for analysis as littermates. **I**, The density of intact axons decreases significantly after TBI in *Sarm1*^{+/+} mice. More intact axons are preserved after TBI in *Sarm1*^{-/-} mice. **J**, TBI strongly induces axon degeneration (accumulated vesicles or compacted cytoskeleton) relative to sham procedures. Axon degeneration after TBI is significantly reduced in *Sarm1*^{-/-} mice, as compared to *Sarm1*^{+/+} littermates. **K**, Axons with abnormal mitochondria are increased after TBI in both *Sarm1*^{+/+} and *Sarm1*^{-/-} mice. **L-N**, This axon pathology was reproducible as shown in cohorts of *Sarm1*^{-/-} (J) mice and the background control C57BL/6 (J) mice, with (J) designating use directly from Jackson Laboratories. Statistical analysis was not made between C57 (J) and *Sarm1*^{-/-} (J) genotypes. TBI induces statistically significant loss of intact axons and formation of degenerating axons only in C57 (J) mice. TBI increased the axons with abnormal mitochondria both in C57 (J) and in *Sarm1*^{-/-} (J) mice.

Figure 19

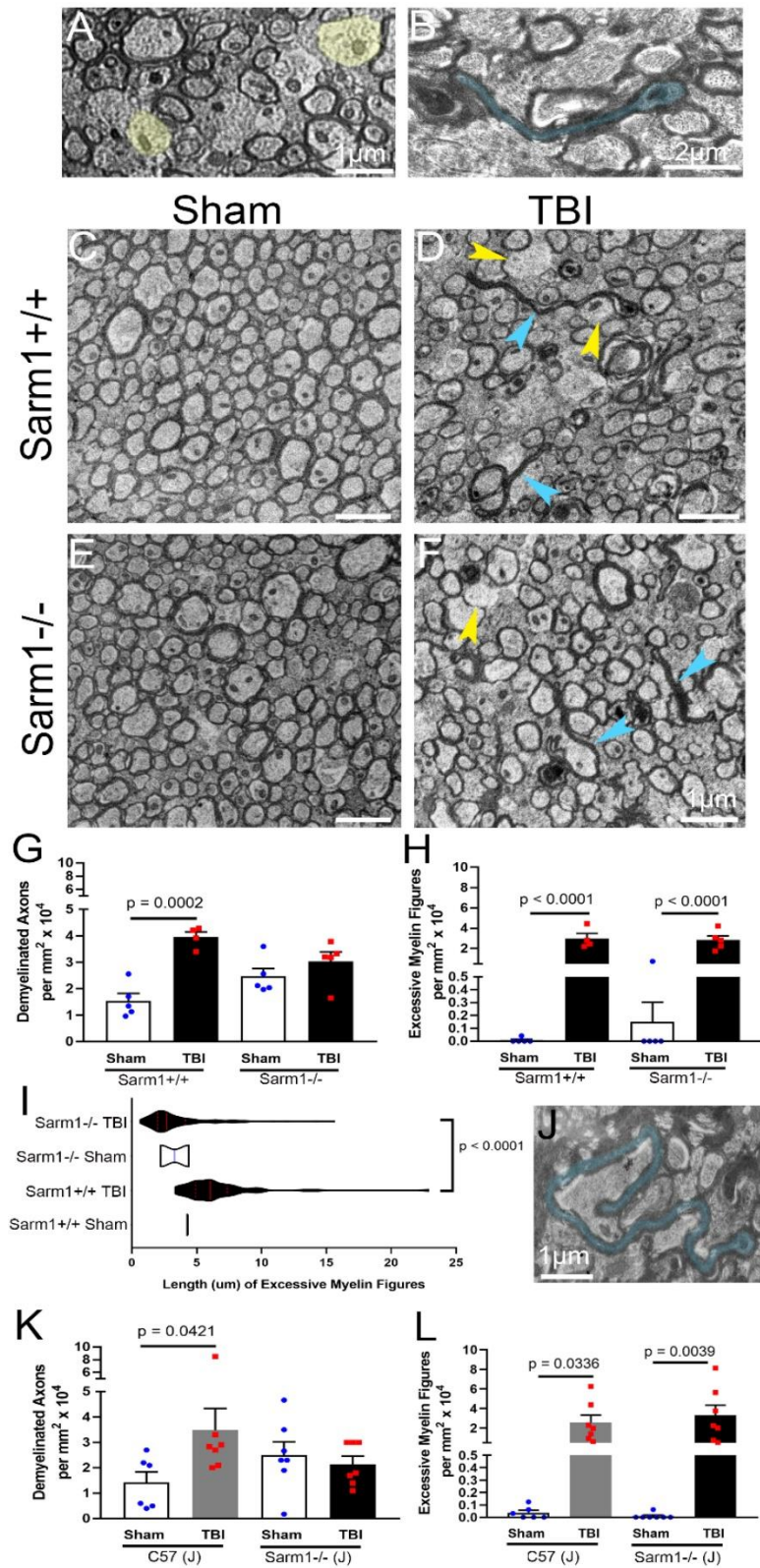


Figure 19. Myelin pathology induced by TBI. **A, B,** Ultrahigh resolution electron microscopic examples of two major forms of myelin pathology observed at 3 days after TBI in *Sarm1*^{+/+} mice. **A,** Demyelinated axons (yellow fill) with intact axonal ultrastructure yet lacking myelin sheaths. **B,** Excessive myelin figure (blue fill) comprised of compact myelin extending away from an axon, which is a condensed degenerating axon in this example. **C-F,** Representative images of sagittal sections through the corpus callosum region-of-interest. **C,** Example of healthy white matter region in *Sarm1*^{+/+} sham mice showing high density of myelinated axons. **D,** TBI causes demyelination of otherwise intact axons (yellow arrowheads) and excessive myelin figures (blue arrowheads) in *Sarm1*^{+/+} mice. **E,** *Sarm1*^{-/-} white matter has typical healthy myelination pattern similar to *Sarm1*^{+/+} littermates for the sham condition. **F,** *Sarm1*^{-/-} mice exhibit less overall white matter pathology, with relatively sparse demyelinated axons (yellow arrowhead) although excessive myelin figures (blue arrowheads) are still readily observed. **G-I,** Quantitative analysis of myelin pathology in *Sarm1*^{-/-} mice and *Sarm1*^{+/+} mice bred in-house for analysis as littermates. **G,** The density of demyelinated axons increases after TBI in *Sarm1*^{+/+} mice but not in *Sarm1*^{-/-}. **H,** *Sarm1*^{+/+} and *Sarm1*^{-/-} mice exhibit excessive myelin figures after TBI. **I,** The length of excessive myelin figures is reduced in *Sarm1*^{-/-} mice, as compared to *Sarm1*^{+/+}. **J,** Example of one of the longest excessive myelin figures (blue shading) from a *Sarm1*^{+/+} mouse. **L-K,** This myelin pathology was reproducible as shown in cohorts of *Sarm1*^{-/-} (J) mice and the background control C57BL/6 (J) mice. TBI induced demyelination is observed only in C57 (J) mice. TBI increased excessive myelin figures in both C57 (J) and *Sarm1*^{-/-} (J) mice.

Figure 20

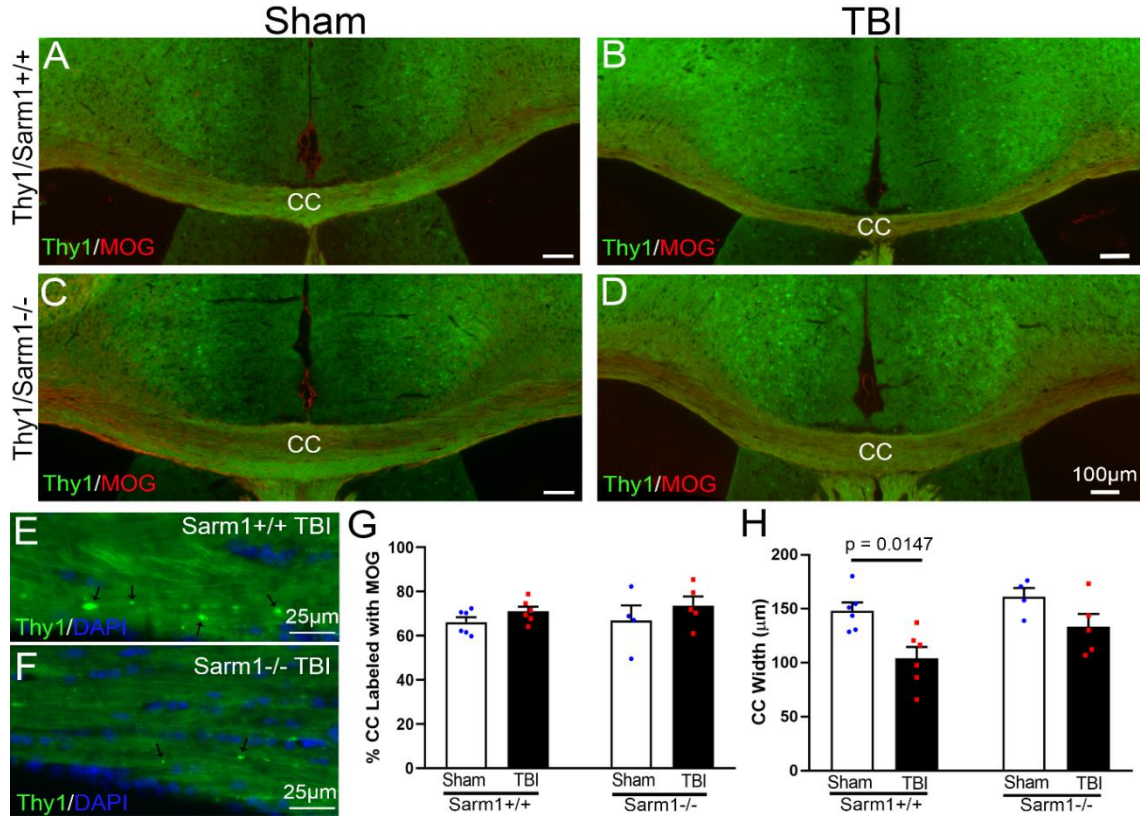


Figure 20. Corpus callosum atrophy at 8 weeks after TBI. **A-D**, Representative coronal images of Thy1-YFP/*Sarm1*^{+/+} mice (**A**, **B**) and Thy1-YFP/*Sarm1*^{-/-} mice (**C**, **D**) at 8 weeks after sham (**A**, **C**) or TBI (**B**, **D**) procedures. Yellow fluorescent protein (YFP; displayed as green) is present in cortical neurons and the associated axons that extend across the corpus callosum. Myelin is detected by immunohistochemistry for MOG (red). **E**, **F**, Higher magnification examples of YFP showing axonal swellings (arrows), a marker of axon damage, at 8 weeks post-TBI in Thy1-YFP/*Sarm1*^{+/+} mice (**E**) and in Thy1-YFP/*Sarm1*^{-/-} mice (**F**). **G**, The proportion of the corpus callosum (CC) with MOG immunolabeling does not differ between groups. **H**, TBI significantly reduced the corpus callosum width. This CC atrophy was attenuated in Thy1-YFP/*Sarm1*^{-/-} mice.

Figure 21

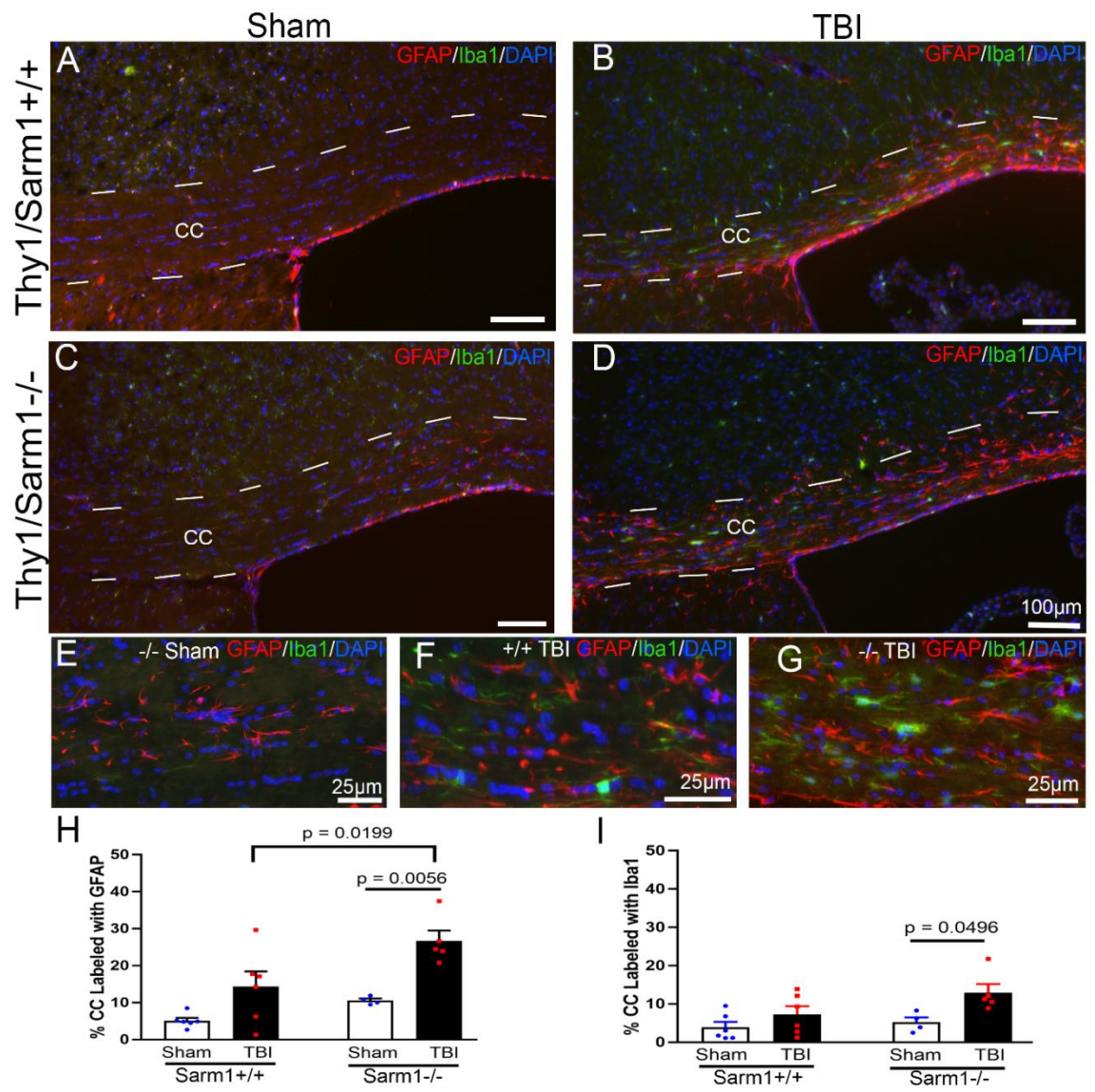


Figure 21. White matter neuroinflammation in chronic TBI. **A-D**, Representative coronal images of Thy1-YFP/*Sarm1* mice immunostained for the astrocyte marker GFAP (red) and microglial marker Iba1 (green) with DAPI nuclear stain (blue) at 8 weeks after sham (A, C) or TBI (B, D) procedures. Thy1-YFP/*Sarm1*^{+/+} mice (A, B) have low levels of immunoreactivity for GFAP or Iba1 in the corpus callosum (CC) of sham mice (A) while both markers are increased after TBI (B). In Thy1-YFP/*Sarm1*^{-/-} mice (C, D) immunolabeling for GFAP and Iba1 is also low in sham mice (C) and notably increased after TBI (D). **E-G**, Higher magnification images to illustrate morphological changes of astrocyte (GFAP) and microglia (Iba1) between the homeostatic morphologies in the sham condition (E) and the reactive phenotypes of shorter, thicker processes with more intense immunolabeling after TBI in Thy1-YFP/*Sarm1*^{+/+} mice (F) or Thy1-YFP/*Sarm1*^{-/-} mice (G). **H-I**, In Thy1-YFP/*Sarm1*^{-/-} mice immunolabeling within the corpus callosum (CC) was significantly increased for GFAP (H) and for Iba1 (I). Note that immunolabeling is displayed in assigned colors that were detected in distinct spectral channels (Iba1 collected in red and GFAP in far-red) that separated from the axonal YFP fluorescence (not shown).

Figure 22

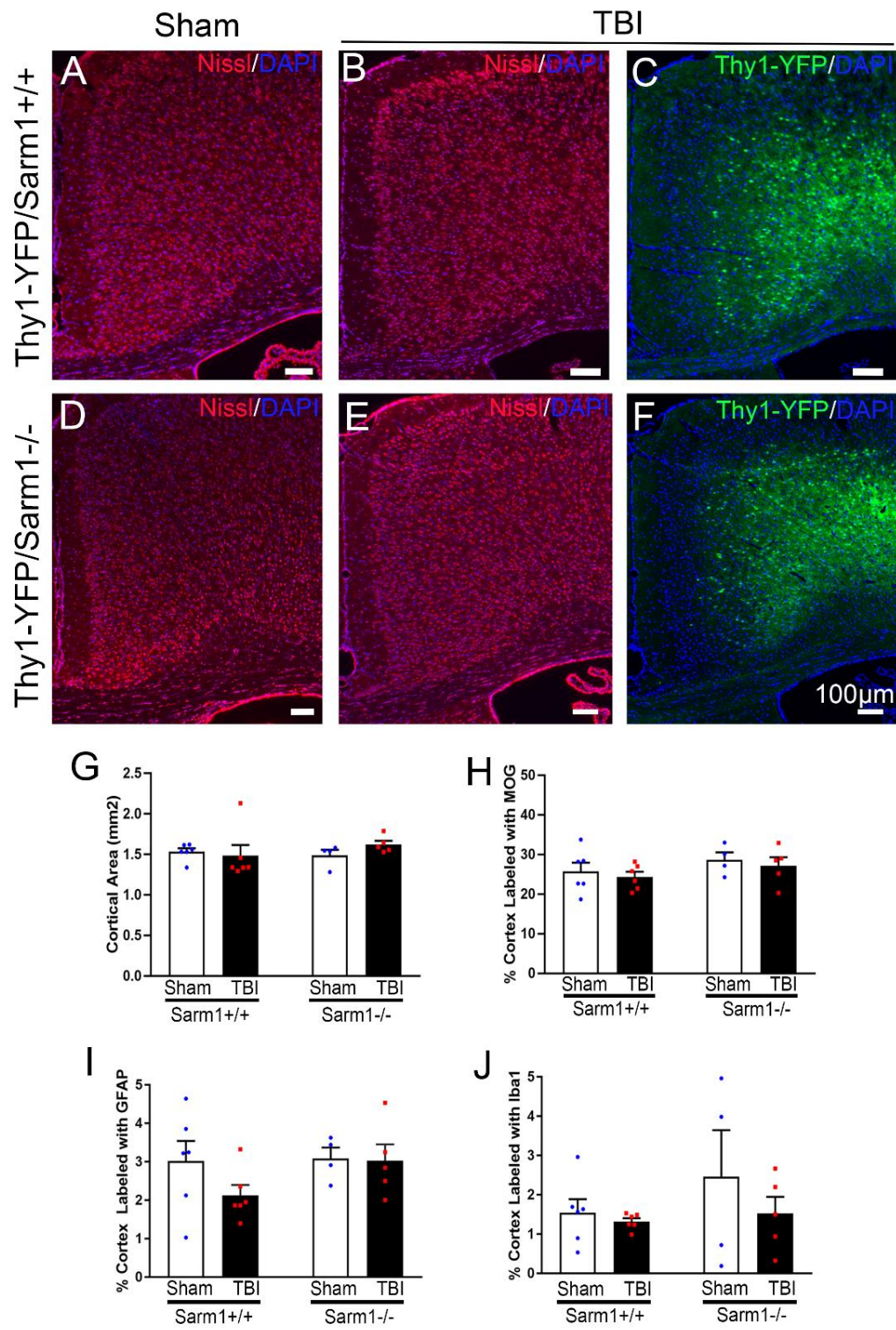


Figure 22. Cortical regions under the impact site in chronic TBI. **A-F**, Representative Nissl and DAPI staining showing cortical neuron cell bodies and nuclei, respectively, in coronal sections from Thy1-YFP/*Sarm1* mice. Cortical areas under the impact site (on the skull at bregma) include premotor/motor cortex areas that project axons through the corpus callosum, as well as cingulate cortex. Both Thy1-YFP/*Sarm1*^{+/+} mice (**A**, **B**, **C**) and Thy1-YFP/*Sarm1*^{-/-} mice (**D**, **E**, **F**) have normal appearing cytoarchitecture of the cerebral cortex at 8 weeks after either sham or TBI procedures. Merged Nissl (red) and DAPI (blue) images are shown without YFP to more clearly show the cytoarchitecture. In addition, for the TBI panels, the matching YFP (green) image of the same field is shown merged with DAPI (blue). **G-J**, TBI did not induce significant cortical atrophy (**G**), myelin loss (**H**), astrogliosis (**I**), or microglial activation (**J**) in either Thy1-YFP/*Sarm1*^{+/+} or Thy1-YFP/*Sarm1*^{-/-} mice.

CHAPTER 5

Experimental Traumatic Brain Injury Identifies Distinct Early and Late Phase Axonal Conduction Deficits of White Matter Pathophysiology, and Reveals Intervening Recovery

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Statement of Contributions:

Christina Marion contributed to the experimental design of all experiments and performed all experimental procedures, except for the electrophysiological recordings performed by Dr. Nathan Cramer and the molecular analysis of nodes and paranodes performed by Dr. Kryslaine Radomski.

ABSTRACT

Traumatic brain injury (TBI) patients often exhibit slowed information processing speed that can underlie diverse symptoms. Processing speed depends on neural circuit function at synapses, in the soma, and along axons. Long axons in white matter (WM) tracts are particularly vulnerable to TBI. We hypothesized that disrupted axon-myelin interactions that slow or block action potential conduction in WM tracts may contribute to slowed processing speed after TBI. Concussive TBI in male/female mice was used to produce traumatic axonal injury in the corpus callosum (CC), similar to WM pathology in human TBI cases. Compound action potential velocity was slowed along myelinated axons at 3 days post-TBI with partial recovery by 2 weeks, suggesting early demyelination followed by remyelination. Ultrastructurally, dispersed demyelinated axons and disorganized myelin attachment to axons at paranodes were apparent within CC regions exhibiting traumatic axonal injury. Action potential conduction is exquisitely sensitive to paranode abnormalities. Molecular identification of paranodes and nodes of Ranvier detected asymmetrical paranode pairs and abnormal heminodes after TBI. Fluorescent labeling of oligodendrocyte progenitors in *NG2CreERTM;mTmG* mice showed increased synthesis of new membranes extended along axons to paranodes, indicating remyelination after TBI. At later times post-TBI, an overall loss of conducting axons was observed at 6 weeks followed by CC atrophy at 8 weeks. These studies identify a progression of both myelinated axon conduction deficits and axon-myelin pathology in the corpus callosum, implicating white matter injury in impaired information processing at early and late phases post-TBI. Furthermore, the intervening recovery reveals a potential therapeutic window.

Significance Statement

Traumatic brain injury (TBI) is a major global health concern. Across the spectrum of TBI severities, impaired information processing can contribute to diverse functional deficits that underlie persistent symptoms. We used experimental TBI to exploit technical advantages in mice while modeling traumatic axonal injury in white matter tracts, which is a key pathological feature of human TBI. A combination of approaches revealed slowed and failed signal conduction along with damage to the structure and molecular composition of myelinated axons in the white matter after TBI. An early regenerative response was not sustained yet reveals a potential time window for intervention. These insights into white matter abnormalities underlying axon conduction deficits can inform strategies to improve treatment options for TBI patients.

INTRODUCTION

Traumatic brain injury (TBI) is a major global health concern (102). TBI ranging from mild to moderate and severe can result in persistent symptoms (113). Speed of information processing is often slowed in patients across the spectrum of TBI severities and can contribute to slowed reaction times, poor attention and learning, impaired executive function, and ineffective emotional adjustment (42; 43; 103; 128; 165; 191).

The pathophysiology underlying information processing speed deficits after TBI is unknown. In severe TBI patients, information processing speed is related to diffuse axonal injury and magnetic resonance imaging (MRI) measures of white matter (WM) integrity (49; 90). Dysfunction involving the neuron cell body, axon, or synapse can impair information processing and desynchronize neural circuits. Of these circuit components, long axons traversing the WM are particularly susceptible to the compression, torsion, and tension forces that cause axonal cytoskeleton breakdown followed by secondary neurodegeneration within WM tracts (85; 160).

Our studies focused on analysis of the corpus callosum (CC), which frequently exhibits abnormalities in TBI patients (29; 85; 153; 157). Importantly, cognitive impairment has been demonstrated in TBI patients with CC atrophy (77; 126; 193). However, disrupted axon-myelin interactions that contribute to impaired WM function or atrophy are not well understood in TBI patients, or even in animal models. Focal areas of axon and myelin loss can result from TBI that produces vascular damage and hemorrhages, since blood vessels are believed to be more vulnerable to TBI forces than

axons (11; 12; 19). Differentiating irreversible focal loss of axons and myelin from potentially reversible myelin pathology along intact axons is important for understanding the progression of WM injury after TBI.

In the CC, traumatic axonal injury affects both unmyelinated and myelinated fibers (23). Unmyelinated axons are highly vulnerable to damage from TBI (146; 147). Myelinated axons also incur damage from TBI, which involves the axons and/or their myelin sheaths (116; 171). Myelin sheaths enwrap axons to enable rapid impulse firing and a 10-fold increase of action potential conduction velocity (76). Myelin is also essential for supporting axonal metabolic functions; losing myelin metabolic support can lead to axonal degeneration (93). Studies in diseases of myelin have established that myelin loss, i.e. demyelination, slows action potential conduction and exposes the axon to potential further injury (189). Furthermore, signal conduction velocity is particularly sensitive to disruption of myelin attachment to axons at paranodal regions, which flank each node of Ranvier between myelin sheaths (13; 38; 177). Prior studies in TBI models have not examined action potential conduction velocity relative to axon-myelin pathology in WM tracts.

This study examined axon and myelin structural and molecular pathology relative to action potential conduction to determine the contribution of WM injury to processing speed deficits after TBI. A mouse single impact TBI was used to exploit technical advantages while modeling the WM traumatic axonal injury of human TBI (116; 171; 197). These combined approaches reveal WM structural and molecular pathology at

paranodes corresponds with slowed action potential conduction velocity early after TBI. At late time points post-TBI, action potentials fail to conduct across the CC in more axons and the CC degeneration progresses to show overt atrophy. Furthermore, between these distinct early and late stages, we demonstrate partial recovery of action potential conduction and evidence of remyelination.

METHODS

Mice and concussive TBI model

All mice were treated in accordance with guidelines of the Uniformed Services University of the Health Sciences and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were socially housed in 27cm x 16.5cm x 12.5 cm cages (2-5 mice per cage) with enrichment objects and maintained on a standard 12 hour cycle of daytime light (6:00-18:00). All procedures took place during the daytime light cycle. The following mouse strains were obtained from The Jackson Laboratory: C57BL/6J (RRID:IMSR JAX: 000664), *ROSA^{mT/mG}* (RRID:IMSR JAX:007676; B6.129(Cg)-*Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo/J}*), *NG2CreERTM* (RRID:IMSR JAX:008538; B6.Cg-Tg(*Cspg4-cre*/Esr1*)BAkik/J), Thy1-YFP-16 (RRID:IMSR JAX:003709; B6.Cg-Tg(Thy1-YFP)16Jrs/J). C57BL/6 mice were purchased as cohorts and acclimated for at least three days prior to use in experiments. All other mice were bred as in house colonies to generate the experimental mice. The overall number of mice of each strain was C57BL/6J (n = 54), *NG2CreERTM*; *ROSA^{mT/mG}* (n = 45; referred to as *NG2CreERTM*; *mTmG*), Thy1-YFP-16 (n = 20), see details in Experimental Design and Statistical Analysis. TBI procedure followed the protocols as previously described (116;

197). Briefly, mice received TBI or sham procedures at 8-10 weeks of age. Under isoflurane anesthesia, the scalp was incised along the midline to expose the skull and a 3-mm-diameter tip was used to impact the skull at bregma (velocity set at 4.0 m/sec; depth of 1.5 mm; dwell time of 100 ms). Sham animals were treated exactly the same for all procedures but did not receive the TBI impact. Depressed skull fracture is a pre-determined exclusion criteria that was the basis for omitting one mouse. Investigators were blinded to animal group allocation until after data analysis. This model results in axon pathology in the CC and external capsule, which is most extensive at coronal levels aligned with the impact site, and in axons in the overlying cingulum and medial cortex (170; 197).

Whole brain tissue clearing

On day 3 after concussive TBI in *Thy1-YFP-16* mice, axon damage under the impact site was visualized through three dimensions (3D) using whole-brain clearing based on the CLARITY process (27; 183). *Thy1-YFP-16* mice have robust yellow fluorescent protein (YFP) expression in motor and sensory neurons that extends through the axons of the corpus callosum and sensitively demonstrate varicosities indicative of axon damage (74; 194). Two male *Thy1-YFP-16* mice (yoked littermates run as sham and TBI) were deeply anesthetized and transcardially perfused with 20 mL of ice cold 0.1M phosphate buffer followed by ice cold hydrogel monomer (HM) solution (4% acrylamide, 0.05% bisacrylamide, 4% PFA, 0.25% VA044 in distilled water), immediately excised and post-fixed for 48 hours in 20 mL of HM solution at 4°C. The slightly opened sample tube was then placed in a desiccation chamber filled with nitrogen, placed under vacuum for 10 minutes, and again filled with nitrogen before the

tube was quickly closed. The sample was then transferred to a 37°C water bath for 4 hours until hydrogel was set, and the brain was removed from the excess hydrogel. The brain was washed 2x with 0.2 M boric acid buffer over 24 hours, then transferred into a clearing solution (4% SDS in 0.2 M boric acid) in an electrophoretic tissue clearing chamber to clear for approximately one month until completely transparent. The pH of the clearing solution and presence of fluorescent labeling were checked weekly during the clearing stage. The brain was washed for 1 day in boric acid buffer then transferred to refractive index matching solution (RIMS; 88% w/v histodenz (Millipore Sigma; D2158), 0.1% Tween-20 (BioRad; 1706531), 0.01% sodium azide (Thermo Fisher Scientific; S2271), 0.02 M PB) for 48 hours before imaging. The brain was mounted with RIMS in a customized chamber made from Wellco dishes (Pelco; 14032E120), BluTack putty, and Kwik-Sil (World Precision Instruments; KWIK-SIL) and imaged with a Zeiss Clr Plan-Neofluar objective (20x/1.0 NA , 1.45 nD, 0.03 correction collar with 5.6 mm working distance) on a Zeiss LSM 7MP two-photon confocal microscope. The yoked sham littermate underwent procedures in parallel to confirm that the clearing process did not alter axonal structure or cause swelling unrelated to the injury. The optical image stacks were stitched together using Zeiss Zen software (ZEN Digital Imaging for Light Microscopy, RRID:SCR_013672) and 3D reconstructions were generated using arivis software (Vision4D Modular Software, arivis AG, Unterschleissheim, Germany).

Electrophysiological recording

A total of 28 male C57BL/6J mice were anesthetized with isoflurane, brains dissected and transferred to a Leica VT1200 vibratome cutting chamber containing chilled sucrose ACSF (in mM: sucrose 206, KCl 2, CaCl₂ 1, NaH₂PO₄ 1.25, MgSO₄ 2,

MgCl-6H₂O 2, NaHCO₃ 26, D-glucose 10, bubbled with a mixture of 95% O₂/5% CO₂). Coronal sections, 400 µm in thickness, were taken from the region near bregma and the lateral ventricles and transferred to normal ACSF (in mM: NaCl 126, KCl 3, CaCl₂ 2, NaH₂PO₄ 1.25, MgSO₄ 2, NaHCO₃ 26, D-glucose 10, bubbled with a mixture of 95% O₂/5% CO₂) at 36°C for 20 minutes then to room temperature for one hour. Recordings were performed in an immersion chamber in flowing room temperature normal ACSF continuously bubbled with 95/5% O₂/CO₂. Conduction velocities were measured by moving the borosilicate glass recording electrode filled with ACSF along the length of the corpus callosum or anterior commissure and capturing the evoked compound action potential (CAP) using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA). The slope of a straight line through the plot of CAP latency versus the inter-electrode distance yields conduction velocity.

Electron microscopy

Prepared grids from 26 male C57BL/6J mice previously quantified for traumatic axonal injury and demyelination were re-assessed for nodal and paranodal pathologies (116).

Tissue preparation for immunohistochemistry

Mice were deeply anesthetized and transcardially perfused with 0.1M phosphate buffer followed by 4% paraformaldehyde, then post-fixed overnight in 4% paraformaldehyde. Dissected brains were cryoprotected in 30% sucrose at 4°C overnight, embedded in OCT compound (Sakura Finetek, Torrance, CA), and sectioned coronally at 14 µm thickness on a Leica CM 1900 UV cryostat (Leica Biosystems, Buffalo Grove, IL).

Confocal microscopy and image processing for analysis of node-paranode complexes

A total of 18 *Thy1-YFP-16* male mice were used for analysis of nodal and paranodal regions. Tissues were prepared for immunohistochemistry, as detailed above, using *Thy1-YFP-16* tissues with primary mouse anti-Caspr antibody (1:500, UC Davis/NIH NeuroMab Facility 75-001, RRID:AB_2083496) and rabbit anti-Nav1.6 antibody (1:500, Alomone Labs; ASC-009, RRID:AB_2040202). Tissues were then incubated with appropriate Alexa dye-conjugated secondary antibodies (1:300, Jackson ImmunoResearch Labs; 715-586-151, RRID:AB_2340858 and 711-606-152, RRID:AB_2340625) and counterstained with DAPI (Sigma Aldrich; D9542) before mounting with Vectashield (Vector Laboratories; H-1400, RRID:AB_2336787). Images of Thy1-YFP with Caspr and Nav1.6 immunolabeling were acquired on a Zeiss 700 laser scanning confocal microscope with individual laser lines sequentially collected for each channel (pinhole size set to 1 Airy unit) using a Plan-Apochromat 63x/1.4 oil objective at 1.5x zoom and 12-bit color depths. Images were acquired with a voxel size of 0.09 μm x 0.09 μm x 0.45 μm in generating an optical image stack of 67.7 μm x 67.7 μm x 5 μm using a 0.45 μm z-interval separation between image planes. The 3D reconstructions of the confocal image stacks were created using arivis Vision4D software (Vision4D Modular Software) to improve signal-to-noise ratio and sharpen immunoreactivity boundaries using Curvature Flow Filter anisotropic denoising algorithm (iterations = 20, time step = 0.125). Reconstructions were then captured as a screenshot to generate two dimensional images which were cropped using Adobe Photoshop CS6 (Adobe Systems, RRID:SCR_014198) and analyzed using NIH ImageJ software (ImageJ,

RRID:SCR_003070). Two images per animal were processed from equivalent regions of the medial corpus callosum of each hemisphere.

Quantification of paranode-node complexes

Paranodal domain organization was assessed in ImageJ by manually drawing a line over the length of each domain: left and right paranodes (Caspr) and the intervening nodal gap delimited by Na_v1.6 immunoreactivity between Caspr domains. The following measurements were tabulated: length of the ‘triplet’ set summed together (Caspr-nodal gap-Caspr), nodal gap length (between Caspr pair), average paranode length (Caspr domains), average length difference within paranode pairs (paranodal asymmetry), and total number of heminodes per image Z-stack. The asymmetry index for each paranodal pair was calculated as the difference between the longer and the shorter paranode divided by the sum of the length of the two paired paranodes multiplied by 100 (156).

Detection of oligodendrocyte lineage cells and myelin in *NG2CreERTM;mTmG* myelin reporter mice

ROSA^{mT/mG} (RRID:IMSR_JAX:007676) were crossed to *NG2CreERTM* (RRID:IMSR_JAX:008538) mice, providing 45 *NG2CreERTM;mTmG* mice that were used in experiments. Mice received 20 mg/mL of tamoxifen (Millipore Sigma; T5648) via oral gavage at 48 and 72 hours post-TBI or sham procedure (117). Tamoxifen induced *Cre* recombination to remove a stop signal and induce a switch from constitutive tdTomato expression in all membranes to green fluorescent protein labeling of membranes in NG2-expressing cells. Cycling cells were labeled with 5-ethynyl-2'-deoxyuridine (EdU) (Thermo Fisher Scientific; Waltham, MA; E10187) in the cohorts aged to 7 days or 2 and 6 weeks. The 7 day cohort mice were given two i.p. injections of

EdU (100 mg/kg) at 2 hours apart on post-procedure days 3 - 7 (10 total injections) and were perfused 2 hours after the last injection. The 2 and 6 week cohorts were given two i.p. injections of EdU on the four days prior to surgery (8 total injections), with the last dose given approximately 22 hours prior to the procedure. EdU was detected in tissue sections using a Click-iT ® Plus EdU kit in the far red channel (Thermo Fisher Scientific; C10637) and counterstained with DAPI.

Slide mounted sections from the 2 week cohort were immunostained with primary monoclonal mouse anti-adenomatous polyposis coli (APC/CC1, 1:100, Millipore Sigma; OP80, RRID:AB_2057371) and incubated with Cy3 conjugated secondary antibody (1:100, Thermo Fisher Scientific; PA1-29773, RRID:AB_10983245). Sections from the 8 week cohort were immunostained with primary polyclonal rabbit anti-oligodendrocyte transcription factor 2 (Olig2, 1:100, Millipore Sigma; AB9610, RRID:AB_570666) then incubated with Alexa dye-conjugated secondary antibody (1:100, Jackson ImmunoResearch Labs; PA1-29773, RRID:AB_10983245). DAPI counterstain (Sigma Aldrich; D9542) was applied before mounting to detect cell nuclei, which aided in differentiating red immunofluorescence of the nuclear localization of Olig2 and the cell body localization of CC1 as distinct from membrane tdTomato (mT) fluorescence remaining in non-recombined cells within the tissue.

Slide mounted sections from the 4 and 8 week cohorts were immunostained with primary monoclonal mouse anti-myelin oligodendrocyte glycoprotein (MOG, 1:20; (10)). Tissues were then incubated with Alexa dye-conjugated secondary antibody (1:300, Jackson ImmunoResearch Labs; 715-605-151, RRID:AB_2340863) and counterstained with DAPI (Sigma Aldrich; D9542) before mounting with Vectashield

(Vector Laboratories; H-1000, RRID:AB_2336789). MOG immunofluorescence was detected using secondary antibodies and filters for the far-red channel, which did not collect detectable signal from the red tdTomato (mT) fluorescence.

Myelin and corpus callosum morphology quantification in myelin reporter mice

Images were acquired on an Olympus IX70 fluorescence microscope using a SPOT RT3 camera (Diagnostic Instruments, Sterling Heights, MI). Myelination was estimated based on pixel intensity values to determine the immunolabeled pixels above background levels within the corpus callosum using Metamorph (MetaMorph Microscopy Automation and Image Analysis Software, RRID:SCR_002368). Myelin fluorescence thresholding to estimate the myelinated area was performed as previously detailed (9). The corpus callosum region-of-interest extended from the midline laterally to under the peak of the cingulum at coronal levels between +0.5 and -0.5 mm relative to bregma. The corpus callosum width was calculated as the average of measurements taken at the midline, approximately 200 μ m lateral to the midline, under the peak of the cingulum, and over the lateral ventricle using MOG staining or Thy1-YFP-16 YFP fluorescence (116). Images of newly synthesized GFP-labeled membranes (mG) images were acquired using filters for green fluorescent protein fluorescence. Images were also acquired a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY) to illustrate morphological details.

Experimental design and statistical analysis

All data collection and analysis was performed by investigators blinded to the sham/TBI condition. All regions-of-interest used coronal levels under the impact site at

bregma. Separate cohorts of mice were required for each of the following experimental procedures.

Large Scale Imaging: Two Male Thy-YFP-16 littermates were sacrificed 3 days after TBI or sham procedures for qualitative analysis. Two confocal image data sets were acquired from the cleared brain of the TBI mouse to allow for more detailed analysis of the region-of-interest. A large scale data set was imaged and a digitally stitched to generate a 12x16 panel image stack roughly 2.64 mm across medial to lateral dimension, 1.3 mm deep superior to inferior, and extending 0.518 mm in the caudal to rostral direction. The second data set was acquired as a single image stack through the CC and cingulum. Both data sets were reconstructed into 3D volumes in arivis to show the scope of injury, and 2-dimensional representative images were generated for figure panels.

Electrophysiology: Male C57BL/6J mice were sacrificed after a sham or TBI procedure with the number of mice per time point and condition as follows: 3 days (n = 4 sham, n = 4 TBI), 2 weeks (n = 5 sham, n = 5 TBI), 6 weeks (n = 5 sham, n = 5 TBI). Surgeries and ex-vivo slice analysis were performed in yoked pairs, so that one sham and one TBI animal were injured and assessed together on each experimental day. Slice recordings were performed by a researcher blinded to the condition of the animals. For each mouse, data was collected from a single 400 μ m thick coronal slice included the affected region of the CC and the anterior commissure, a white matter region unaffected in this model that served as an internal control. Where indicated, each TBI time point was compared to sham groups, which were averaged across time points.

Electron microscopy: Electron microscopy grids prepared from male C57BL/6J mice were assessed for all examples of paranode regions along the axon within the CC. For

qualitative analysis at least two grids of coronal tissue sections were assessed per mouse, with multiple sections imaged per grid. The number of mice evaluated for each time point and condition was as follows: 3 days (n = 3 sham, n = 4 TBI), 1 week (n = 5 TBI), 2 weeks (n = 5 TBI), 6 weeks (n = 4 sham, n = 5 TBI). Representative images of the observations were selected for each time point.

Paranode complexes: Male Thy1-YFP-16 mice underwent either TBI or sham surgery and were perfused for analysis at 3 days (n = 6 sham, n = 6 TBI) or 6 weeks (n = 3 sham, n = 3 TBI) post-procedure. Quantification included two confocal images per mouse from equivalent CC regions in both hemispheres of the same coronal section. From each image Z-stack, 100 paranode-node-paranode triplet sets were measured. All heminodes were counted per Z-stack. As axonal YFP signal is a sensitive indicator of axonal damage, the node-paranode measurements and heminode counts were initially performed without the YFP visible to maintain blinding to the condition. Marked heminodes were later confirmed to have YFP present on both sides of the Nav1.6 labeled node but flanked on only one side by a single Caspr-labeled paranode after careful 3-dimensional viewing in arivis Vision 4D to include image rotation, fly-through and varying channel visibility levels.

Fluorescent reporter labeling of cells and myelin membranes. Cohorts of

NG2CreERTM;mTmG littermates received TBI or sham procedures and were sacrificed for tissue analysis at 7 days (sham n = 3 male; TBI n = 3 male), 2 weeks (sham n = 3 male, n = 2 female; TBI n = 3 male, n = 2 female), 4 weeks (sham n = 4 male; TBI n = 3 male, n = 2 female), 6 weeks (sham n = 4 male, n = 1 female; TBI n = 3 male, n = 2 female), or 8 weeks (sham n = 2 male, n = 3 female; TBI n = 3 male, n = 2 female). Four

or more images were analyzed per mouse and included images collected from at least two tissue sections per mouse. The CC region-of-interest extended from the midline laterally to under the peak of the cingulum at coronal levels between +0.5 and -0.5 mm relative to bregma, which includes the anterior commissure inferior to the lateral ventricles.

NG2mG fate-labeled cells were counted using both the mG and membrane tdTomato labeling to exclude any NG2mG labeled cells along blood vessels to avoid potential pericytes.

Statistical Analysis: GraphPad Prism 7.0 software (RRID:SCR_002798) was used to graph and analyze all data. The sample size was pre-determined from prior experiments as an n of 5-6 mice per condition for quantification of corpus callosum myelination and atrophy or defined after a pre-determined interim power analysis was performed with three mice per condition. The specific sample size and statistical tests used for each set of data is noted in each figure legend. Unpaired Student's *t*-test was used to compare TBI and sham for values analyzed at a single time point. For comparison of the frequency of multiple length distributions, measurements were logarithmically transformed using the $Y=\text{Log}(Y)$ formula and the resulting Gaussian distribution was graphed to demonstrate a log-normal distribution. The mean log lengths for each animal were then averaged and compared between conditions using the unpaired Student's *t*-test. Two-way ANOVA was used for comparisons of TBI and matched sham conditions at multiple time points or between cell types with post-hoc analysis using Sidak's test to determine p values with consideration of multiple comparisons. Two-way ANOVA with Dunnett's post-hoc test was used for comparison of multiple TBI post-injury time points to an averaged sham condition. Cohen's *d* effect size was determined by calculating the mean difference

between the sham and TBI groups, then dividing the result by the pooled standard deviation. For simplicity, statistical details are only provided for significant comparisons.

RESULTS

Large scale imaging of corpus callosum axon damage after TBI.

YFP labeling in axons can reveal swellings from an early reversible stage of damage through the terminal stage when enlarged swellings disconnect to form axonal end bulbs (71; 74). We have previously shown that axon labeling with YFP in Thy1-YFP-16 mice detects traumatic axonal injury in the CC after concussive TBI (197). Therefore, optical imaging with brain clearing was used for large scale imaging to illustrate YFP swellings indicative of axon damage in the CC under the impact site at 3 days post-injury (Fig. 23). This large-scale imaging illustrates the distribution of axon damage relative to the region-of-interest in the CC to be used for electrophysiological recordings (Fig. 23A). Damaged axons extend across the width of the CC as well as rostrocaudally, and axonal varicosities are particularly localized over the ventricles (Fig. 23A, B). The TBI also involves axons of the cingulum, where the lower density of YFP labeled axons allows longitudinal visualization of damaged axons with extensive swellings as well as adjacent large normal appearing axons (Fig. 23C). Smaller YFP labeled axons without swellings are not apparent due to the imaging resolution of the long focal length objective required for imaging the cleared brain specimen, and exposures compatible with the intense YFP accumulation in axon swellings. In the sham condition (data not shown), YFP-labeled axons exhibited relatively minor variation in

diameter with only occasional swellings, similar to cleared images of naïve and sham Thy1-YFP-H mice (27; 202).

White matter conduction deficits after TBI.

To test the effect of concussive TBI on axonal function in the CC, the compound action potential (CAP) velocity and amplitude was evaluated in both sham and TBI mice (Fig. 24). The general placement of the recording and stimulating electrodes in ex vivo slices captured signal from axons traversing the CC, including regions exhibiting axon damage (Fig. 23A). The CAP has two waveform components; N1 is comprised of fast conducting myelinated axons and N2 includes slower conducting axons, which are generally the non-myelinated axons in healthy adults. Representative waveforms indicate both slowing of the N1 component and loss of N1 amplitude at 3 days after TBI (Fig. 24A). Analysis of multiple times post-TBI shows that the N1 conduction velocity is slowed significantly at 3 days, followed by recovery (Fig. 24B). In contrast, N2 conduction velocity is not significantly different from sham across each post-TBI time points (Fig. 24C). N1 amplitude is significantly reduced at each time point in TBI mice as compared to sham mice (Fig. 24D). N2 amplitude increases at 3 days (Fig. 24E), indicating that demyelinated axons may have slowed from N1 to fall within the N2 wave. Combining both N1 and N2 amplitudes shows overall axon loss and/or functional drop out is significant only at 6 weeks post-TBI (Fig. 24F). The anterior commissure, which does not exhibit axon damage in this TBI model, served as a technical control within the same slices. Anterior commissure N1 and N2 conduction velocities were not significantly different from sham mice at any time point (Fig. 24G-H). Together, these results demonstrate slowed conduction velocity early after TBI followed by drop out of

conducting axons during late phase TBI. In addition, the N1 amplitude increases significantly from 3 days to 2 weeks post-TBI (Fig. 24D), indicating functional recovery within a time frame expected for myelin repair.

TBI-induced axon degeneration, demyelination, and node-paranode disruption.

Rapid action potential conduction is particularly sensitive to disrupted axon-myelin interactions. We employed high resolution electron microscopy to identify a full range of axon and myelin pathology, with a specific focus on nodes of Ranvier and adjacent paranode domains, where myelin attaches to axons. Experimental concussive TBI exhibited dispersed axonal degeneration (Fig. 25), consistent with the pattern of traumatic axonal injury observed in human TBI postmortem specimens (85). Sagittal sections through the CC illustrated healthy axons surrounded by myelin in sham mice (Fig. 25A), while TBI caused distinct axonal and myelin pathologies at early (Fig. 3B) and late post-injury time points (Fig. 25C). TBI resulted in both degenerating axons and demyelinated axons, i.e., intact axon without myelin (Fig. 25B,C), see also.(116) Coronal CC sections in sham mice have healthy axons with organized myelin loops contacting the axon at paranodal regions flanking the node of Ranvier (Fig. 25D). In TBI mice, intact axons demonstrated appropriate axon cytoskeleton and myelin preservation (Fig. 25E) while adjacent damaged axons showed cytoskeletal breakdown and abnormal paranodal myelin loops (Fig. 25F). Early and late phases post-TBI exhibited abnormal paranodes with detached, disorganized, or elongated myelin loops associated with damaged axons (Fig. 25F-J).

Abnormal molecular organization of paranodes after TBI.

To further examine these ultrastructural findings of axon-myelin pathology, we used molecular markers to quantify node and paranode domains in Thy1-YFP-16 mice. Three-dimensional reconstructions were used to examine the YFP-labeled axons combined with immunolabeling for Caspr in paranodes and Na_v1.6 voltage gated sodium channels in nodes of Ranvier (Fig. 26A). Enlarged images of these high-resolution reconstructions enable distinct identification of nodal complexes with symmetrical or asymmetrical paranode pairs as well as heminodes, which have only one Caspr domain adjacent to the Na_v1.6 immunolabeled node (Fig. 4B). TBI did not significantly alter the mean nodal gap length (Fig. 26C,D). The individual paranodal lengths were also not statistically different between sham and TBI animals (sham = 2.008 ± 0.045 , TBI = 1.836 ± 0.065 ; unpaired *t*-test, *p* = 0.0539; mean $\pm$ SEM). However, TBI shortened the overall length of the paranode-node-paranode complex (Fig. 26E,F) and caused asymmetry between paranode pairs (Fig. 26G). Importantly, TBI significantly increased the frequency of abnormal heminodes relative to sham mice at 3 days (Fig. 26H) and at 6 weeks (Fig. 26I). The CC width was not significantly reduced at 6 weeks post-TBI (Fig. 26J). Therefore, CC atrophy did not account for this increased heminode density between TBI and sham mice. Heminodes were not associated with large axon swellings along damaged axons in the TBI mice (Fig. 26A).

TBI does not alter oligodendrocyte progenitor density or proliferation in *NG2CreERTM;mTmG* myelin reporter mice.

In this TBI model, we previously showed that NG2 immunolabeled cells were increased in the dorsolateral extension of subventricular zone at 2 and 6 weeks, as

compared to sham mice (117). Within the CC, NG2 cell density was maintained as well, indicating that genetic fate-labeling of NG2 cells may be feasible for detecting newly synthesized myelin after TBI. Transgenic *NG2CreERTM;mTmG* myelin reporter mice were used to examine potential changes in immature oligodendrocyte lineage cells and myelin membrane synthesis in the CC after TBI. In these myelin reporter mice, tamoxifen induces recombination to initiate green fluorescent protein labeling of newly synthesized membranes in NG2-expressing cells, i.e., NG2mG (201). After TBI or sham procedures, tamoxifen was given on days 2-3 to fate label NG2 cells and EdU was given on days 3-7 to label cycling cells (Fig. 27A). NG2mG fate-labeled cells exhibiting the morphology of oligodendrocyte progenitors were identified at 7 days (Fig. 27B). NG2mG labeled cells located on blood vessels (not shown) may be pericytes and so were excluded from these counts. NG2mG cells continued to proliferate in the CC (Fig. 27C). These NG2 and EdU double positive cycling oligodendrocyte progenitors comprised 33.65% of the EdU labeled cells in sham mice and 25.12% after TBI (Fig. 27D). However, there was no significant difference in the number of NG2mG labeled cells, EdU labeled cells, or NG2 and EdU double positive cells between TBI and sham mice at 7 days (Fig. 27D). In addition, NG2mG fate-labeled cells that had undergone further oligodendrocyte lineage differentiation were observed at 7 days (Fig. 27E). At 4 weeks, NG2mG labeling was also present in membranes extending along axons (Fig. 27F). At 8 weeks, NG2mG labeled membranes extended throughout the CC in a pattern consistent with myelin formation (Fig. 27G). The majority of NG2mG fate-labeled cell bodies are co-labeled with the oligodendrocyte lineage marker Olig2, which was not different between TBI and sham mice (Fig. 27H,I).

NG2-mG fate-labeled cycling progenitors and terminal differentiation are not altered by TBI.

To specifically follow endogenous cycling oligodendrocyte progenitors in *NG2CreERTM;mTmG* mice, EdU was administered daily for four days prior to the TBI or sham procedures and then tamoxifen was given on days 2-3 post-procedure to fate label NG2 cells (Fig 28A). Approximately half of NG2mG fate-labeled cells underwent terminal differentiation into mature oligodendrocytes by 2 weeks post-TBI/sham based on immunolabeling for CC1 (Fig. 28B,C). The injury condition did not result in a significant difference in the total population of CC1+ oligodendrocytes, NG2mG fate-labeled cells, or the subset labeled with both CC1 and NG2mG (Fig. 28C). Focusing on the cycling progenitors labeled with EdU prior to the sham or TBI procedures, there was no significant difference in the total number of EdU+ cycling progenitors or the proportion EdU+ cells fate-labeled for NG2mG in TBI versus sham mice at 2 weeks (Fig. 28D) or 6 weeks (Fig. 28E). Taken together with the post-procedure EdU+ labeling (Fig. 28D), this data indicates that TBI does not stimulate proliferation to amplify the subset of cycling NG2mG progenitor cells in the CC.

TBI increases myelin membrane remodeling.

Following this progression of NG2mG fate-labeled cells toward mature oligodendrocytes, we further examined the extent of NG2mG labeling of myelin membranes. Immunohistochemistry for myelin oligodendrocyte glycoprotein (MOG) detected myelin and Caspr identified paranodes in *NG2CreERTM;mTmG* mice (Fig. 29). At 4 weeks post-TBI/sham, NG2mG membranes aligned along axons in a pattern matching with MOG in the CC (Fig. 29A,B). NG2mG membranes also extended to

Caspr-labeled paranodes (Fig. 29C,D), which is indicative of myelin formation. In addition to the expected paranode pairs, single Caspr domains were observed, in agreement with our finding of increased heminodes after TBI (Fig. 26). At 8 weeks post-TBI/sham, NG2mG membranes extended throughout much of the CC (Fig. 29E,F). Quantification of NG2mG and MOG labeling in the CC shows significant new membrane formation at 4 weeks after TBI (Fig. 29G). However, this increase in NG2mG is transient and does not persist at 8 weeks. In sham mice, NG2mG and MOG show continued myelin formation in the adult CC (Fig. 29E-G). This result agrees with an increase in the proportion of myelinated axons and number of myelin lamellae in the mouse CC in this time frame (169). Importantly, the CC area and width do not increase between 4 to 8 weeks post-TBI resulting in significant CC atrophy relative to sham (Fig. 29H,I).

DISCUSSION

Deciphering the diverse pathophysiological mechanisms in play after head injury is important for improving non-invasive biomarkers for TBI diagnosis and prognosis and for developing effective treatments. Experimental TBI in animals enables modeling of specific features of human TBI to examine pathophysiological mechanisms with specialized techniques across early through late post-injury time points. Our studies model traumatic axonal injury in WM tracts, which is a major pathological feature commonly observed in TBI postmortem cases (160). The results reported here are the first to show slowed conduction velocity across a WM tract with traumatic axonal injury from TBI. We show that myelinated axons initially exhibit slowed conduction, which recovers over a two week period after TBI. This pattern is consistent with disrupted axon-

myelin interactions, i.e., demyelination and/or myelin detachment from axons, followed by remyelination. Indeed, this interpretation is strengthened by ultrastructural and molecular pathological evidence of demyelination and disrupted paranode organization. In addition, our studies with *NG2CreERTM;mTmG* myelin reporter mice provide clear evidence of increased myelin membrane synthesis, which is consistent with remyelination occurring after TBI. However, with longer survival post-TBI, our electrophysiological and morphological data show that this WM injury progresses to overall loss of functional axons and CC atrophy. These results demonstrate dynamic structural and functional changes that may contribute to impaired information processing resulting from WM injury in TBI patients.

Neural circuits for sensorimotor, cognitive, and emotional domains depend on myelination to precisely coordinate action potential conduction to synchronize timing and ensure fidelity of transmission (5). Within neural circuits, our studies focused on mechanisms that may impair action potential conduction in injured WM tracts. We focused specifically on myelinated axons since the electrical advantages of myelin enable rapid signal conduction, and transmission along long axons is a major component within neural circuits (5; 22). We show degenerating axons dispersed among intact axons in the CC (Figs. 23, 25), as expected for traumatic axonal injury after TBI (160). These same CC regions also contain demyelinated axons, i.e. relatively large axons missing myelin sheaths (Fig. 25) (116; 171). Among the myelinated axons within white matter, electron microscopy is required to identify this pattern of demyelinated axons, which is not detected well by immunohistochemistry for myelin proteins (171; 197). Similarly,

disruption of myelin attachments to axons at paranodal junctions can be widespread without significant overall myelin loss (178).

Myelin sheath attachment to axons at paranodes flanking each node of Ranvier is critical for rapid action potential transmission via saltatory conduction (140). Prior work in TBI models has shown axonal damage at nodes of Ranvier within 24 hours after either experimental stretch injury in guinea pig optic nerve, fluid percussion injury in rats, or lateral head acceleration in non-human primates (107; 110; 145). Our studies demonstrate ultrastructural pathology of nodes at multiple longer survival times along with abnormalities in paranode regions that flank nodes (Fig. 25). We identify increased heminodes at 3 days post-TBI (Fig. 26) based on Caspr localization, which molecularly defines paranode regions (140). Axonal membrane proteins, such as Caspr and Na_v1.6 at paranodes and nodes connect within the axons to cytoskeletal proteins, including alpha-II spectrin and Ankryin-G, respectively. These cytoskeletal proteins can protect axons from mechanical injury and undergo proteolysis after axon damage from TBI (81; 145). Therefore, damage to nodes and disruption of paranodes are important features of TBI WM injury, in addition to cytoskeletal breakdown and impaired fast axonal transport that produces swellings along the length of myelinated axons (70; 167).

Axonal paranodes have complex molecular interactions in order to attach myelin and segregate nodal sodium (Na_v) channels away from juxtaparanodal potassium (K_v) channels for rapid saltatory conduction and membrane potential repolarization (6; 140). In healthy adult CNS, myelin plasticity adjacent to nodes is a predicted mechanism to

precisely time signaling within the CNS (8). Both demyelination and myelin detachment at paranodes can slow signal conduction along axons while more extensive myelin or axon damage will typically block signal propagation, resulting in failure of the action potential to reach the synapse (7; 55; 75). Fluid percussion TBI studies in rats have reported reduced CAP amplitude in the first week post-TBI (14; 146), but did not examine conduction velocity or the progression of WM conduction deficits out to the late phase of CC atrophy as in the current study. We show that TBI causes early conduction slowing that progress to conduction failure and/or loss of functional axons (Fig. 24), which agrees with the impaired saltatory conduction observed with demyelination and paranode disruption in other neurological diseases (73; 175). Interestingly, computational modeling of TBI also predicts a role for paranode disruption in slowed and/or failed axonal depolarization (187). After TBI, we found that paranodes were abnormally shortened or missing, which resulted in an increased frequency of heminodes (Fig. 26). This alteration of paranode molecular elements may be an early indicator of myelin damage (175). Alternatively, asymmetrical paranodes and heminodes may occur where new myelin sheaths are forming, such as during developmental myelination (20; 55), or where remyelination is incomplete as reported in multiple sclerosis lesions (33).

Transgenic mice facilitated detection of new myelin formation or remodeling after TBI. *NG2CreERTM;mTmG* myelin reporter mice given tamoxifen after TBI, or sham procedure, heritably labeled immature oligodendrocyte lineage cells that differentiated into oligodendrocytes and progressively extended membranes along axons (Figs. 27,v28). Caspr immunolabeling co-localized where these fluorescently labeled membranes ended

along axons, indicating myelin attachment at paranodes (Fig. 29C). This result is consistent with formation of new myelin sheaths and/or new membrane synthesis to remodel existing myelin sheaths. Initial studies using retroviral infection to express *NG2* promoter driven *Cre recombinase* in *mTmG* mice showed that new fluorescently labeled myelin was formed between 2-4 weeks after traumatic spinal cord injury.(141) This time interval for remyelination is in agreement with our findings in TBI for partial recovery of myelinated axon conduction (Fig. 24D) and the transient increase of new membrane formation in our *NG2CreERTM;mTmG* mice (Fig. 29G). Remyelination in the first weeks after TBI is also supported by our ultrastructural finding of thin myelin relative to axon diameter in the CC (116). However, our findings also demonstrate CC atrophy and loss of functional axons at longer survival post-TBI (Fig. 24D,F; 29H,I). Furthermore, oligodendrocyte lineage cell density (Fig. 27) is maintained during CC atrophy (Fig. 29), indicating a corresponding reduction of axons and the oligodendrocyte population at 8 weeks post-TBI. Therefore, interventions that target axon-myelin pathology in the first weeks after TBI may help maintain this remyelination and partial functional recovery to prevent progressive WM degeneration.

These results identify important pathophysiological mechanisms of WM injury after TBI that correspond with symptoms experienced by TBI patients. Further study is warranted using approaches that can extend and address limitations of the current work. Examination of additional molecular components across time post-injury is needed to evaluate loss or redistribution of ion channels in the axolemma to more specifically interpret our electrophysiological data. To more directly interrogate the mechanism(s)

involved, interventions to modify these pathological features should be tested. In addition, the role of neuroinflammation in these identified functional and structural changes should now be explored. Quantitative *in vivo* electrophysiological approaches are needed to corroborate our *ex vivo* recordings of CC axons. Clinically applicable techniques to detect these pathophysiological changes in TBI patients could be extremely helpful for diagnosis and prognosis. In this TBI model, *in vivo* diffusion tensor magnetic resonance imaging detected reduced CC WM integrity (197). Advances in diffusion tensor imaging may improve capabilities for quantifying atrophy and loss of axons within an overall volume of WM atrophy (15). Similarly, demyelination and detached paranodal junctions can be detected by diffusion tensor imaging in simplified models (178; 194).

In conclusion, this study reveals structural, functional and molecular pathology of axon-myelin interactions in WM after experimental TBI. Furthermore, we demonstrate a progression of WM injury that initially recovers yet advances to later phase degeneration with CC atrophy. These pathophysiological mechanisms may be responsible for impaired information processing experienced by patients at acute through late phases after TBI. Repetitive TBI may also damage axon-myelin interactions based on myelinated axonopathy observed in humans and slowed conduction velocity reported in mice (176). Importantly, these results inform diverse therapeutic strategies to maintain axon function and promote myelin plasticity and indicate a potential treatment window in the first weeks prior to the progression of WM degeneration.

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FIGURES

Figure 23

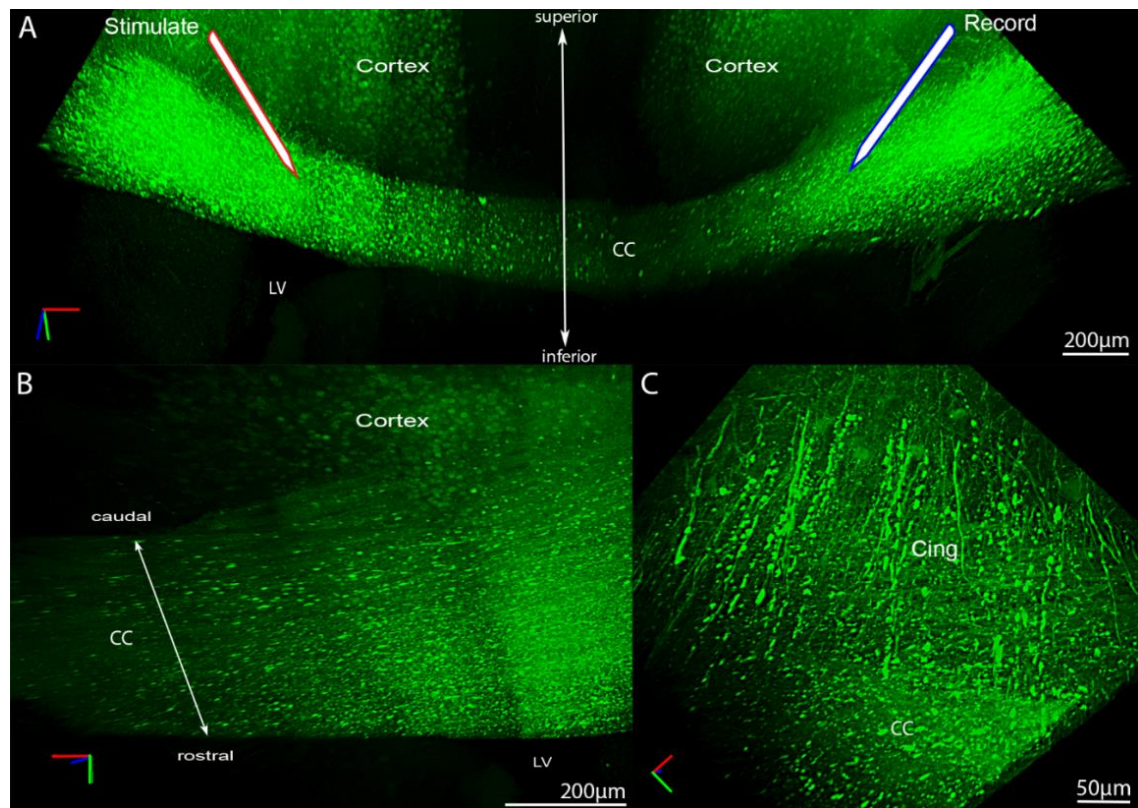


Figure 23. Concussive TBI causes axonal damage in the corpus callosum and cingulum under the impact site illustrated using CLARITY. **A**, Confocal imaging from an optically cleared brain of a Thy1-YFP-16 mouse at 3 days post-TBI shows axon damage in the corpus callosum (CC) as detected by YFP-labeled axonal swellings. This YFP labeling illustrates the distribution of axon damage in the CC between the positions of the stimulating and recording electrodes used for electrophysiological analysis of axonal conduction properties (see Fig. 24). **B**, YFP-labeled axonal swellings are particularly dense over the ventricle and extend rostrocaudally throughout the CC under the site of impact. Scale bar 200 µm. **C**, Higher magnification of the cingulum (cing) also shows damage in axons with YFP swellings as well as adjacent thinner normal appearing axons without swellings. A video of this field is available as Extended Data to better visualize the YFP labeling in an example 3-dimensional reconstructed image stack.

Figure 24

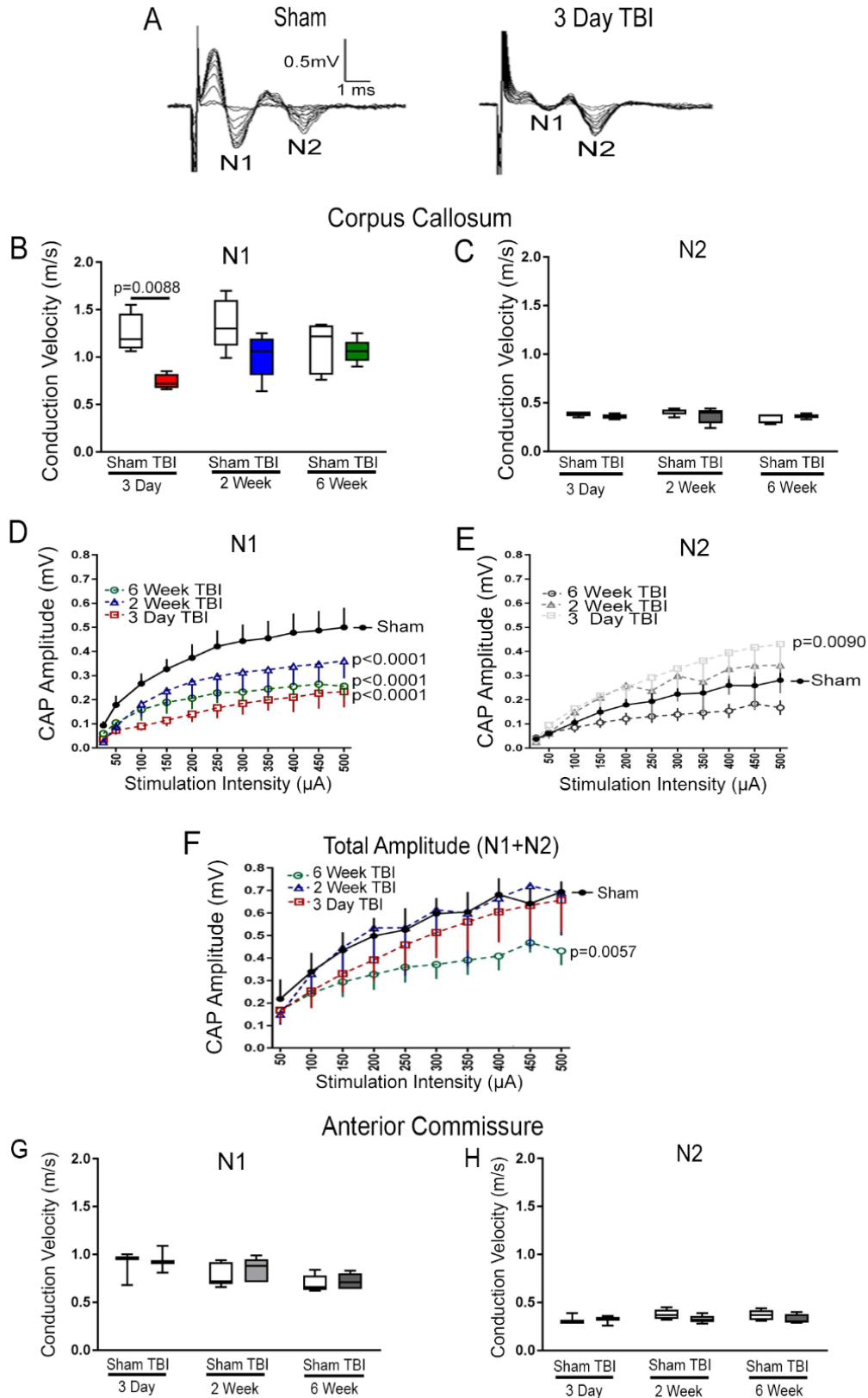


Figure 24. TBI causes slowed conduction velocity followed by axon drop out. *A*, Representative CAP waveforms recorded from axons within the CC at the level of the midline crossing of the anterior commissure. The fastest wave is the N1 component, which is comprised of myelinated axons. TBI impairs conduction in the N1 fast myelinated axons. The second (N2) wave is comprised of non-myelinated axons in sham mice but may also include demyelinated axons after TBI. *B*, TBI slows N1 conduction velocity at 3 days. *C*, N2 conduction is not slowed by TBI at any time point examined. *D*, N1 amplitude is reduced at all post-TBI time points relative to the averaged sham values. Among the TBI mice, the N1 amplitude significantly increases between 3 days and 2 weeks ($p = 0.0003$). *E*, N2 amplitude is increased to above sham levels at 3 days, which may reflect the abnormal contribution of demyelinated axons with conduction velocities that slow to within the timing of the N2 wave. *F*, Combining N1 and N2 amplitudes shows overall viable axon conduction across the CC and indicates that axon loss and/or conduction block is significant only at 6 weeks post-TBI. *G-H*, The anterior commissure is a white matter tract within the same slices served as a technical control for *ex vivo* recording within each brain slice. The anterior commissure is more ventrally located and does not exhibit axon damage in this TBI model. Conduction velocity was not altered in anterior commissure axons in the N1 (*G*) or N2 (*H*) component after TBI, relative to sham mice. Mouse sample sizes were 3 days ($n = 4$ sham, $n = 4$ TBI), 2 weeks ($n = 5$ sham, $n = 5$ TBI), 6 weeks ($n = 5$ sham, $n = 5$ TBI). Velocities were compared by two-way ANOVA followed by Sidak's multiple comparison test. Panel B: Interaction $F(2,22) = 2.7165$, $p = 0.0882$; Time $F(2,22) = 1.7535$, $p = 0.1965$; Injury $F(1,22) = 13.106$, $p = 0.0015$ with posthoc for 3 day $p = 0.0088$ and effect size = 3.15. Amplitudes compared by two-way ANOVA with Dunnett's multiple comparison to sham. Panel D: Interaction $F(30,138) = 0.9489$, $p = 0.9994$; Intensity $F(10,138) = 10.44$, $p < 0.0001$; Injury $F(3,138) = 23.95$, $p < 0.0001$ with posthoc for 3 day $p = 0.0001$ and effect size = 1.96, 2 week $p = 0.0001$ and effect size = 0.91, and 6 week $p = 0.0001$ and effect size = 2.01. Panel E: Interaction $F(30,136) = 0.3408$, $p = 0.9995$; Intensity $F(10,136) = 6.541$, $p < 0.0001$; Injury $F(3,136) = 10.15$, $p < 0.0001$ with posthoc for 3 day $p = 0.009$ and effect size = 1.15. Panel F: Interaction $F(27,125) = 0.1516$, $p > 0.9999$; Intensity $F(9,125) = 5.661$, $p < 0.0001$; Injury $F(3,125) = 5.31$, $p = 0.0018$ with posthoc for 6 week $p = 0.0057$ and effect size = 2.49. Error bars show 10-90% interval.

Figure 25

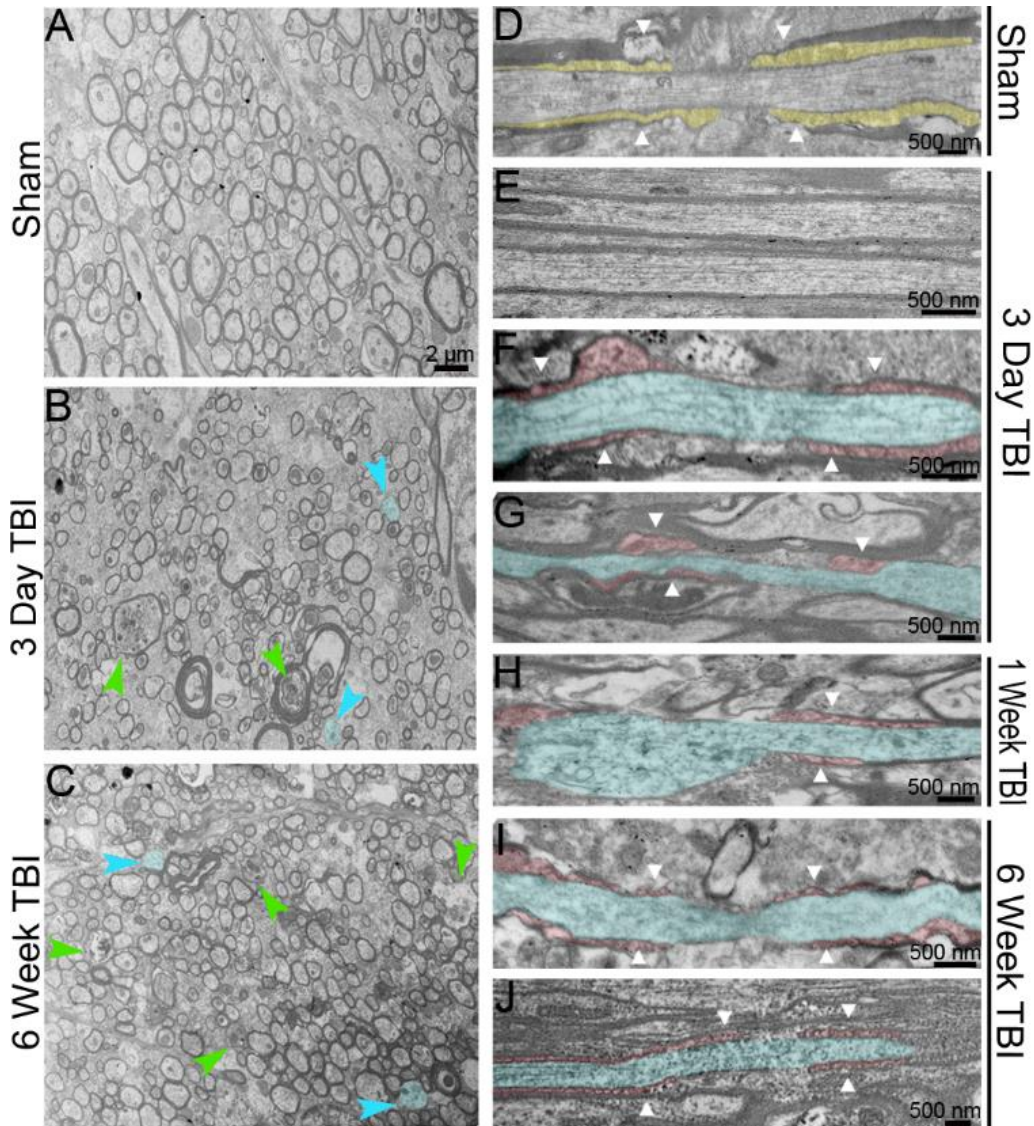


Figure 25. Electron microscopy demonstrates that TBI white matter pathology, with characteristic traumatic axonal injury, involves dispersed demyelinated axons and disrupted paranode structure. A, Sagittal sections through the CC show sham axons, many of which are myelinated. **B-C,** Dispersed degenerating axons (green arrowheads) and demyelinated axons (blue arrowheads and fill) are evident early (B) and late (C) post-TBI. Scale bars 2 μ m. **D,** Coronal section through the CC to illustrate organized myelin loop attachments forming paranodes (yellow fill; white arrowheads) in sham mice. Scale bar 500 nm. **E-G,** Within the CC of injured mice, intact axons with normal myelin (E) are found adjacent to damaged axons (F,G blue) with cytoskeletal breakdown and non-uniform diameter, along with abnormal paranodes (red) and myelin loss (F,G). **H-J,** Damaged axons (blue) with abnormal paranodes (red) continue to be evident later post-TBI. Scale bars 500 nm.

Figure 26

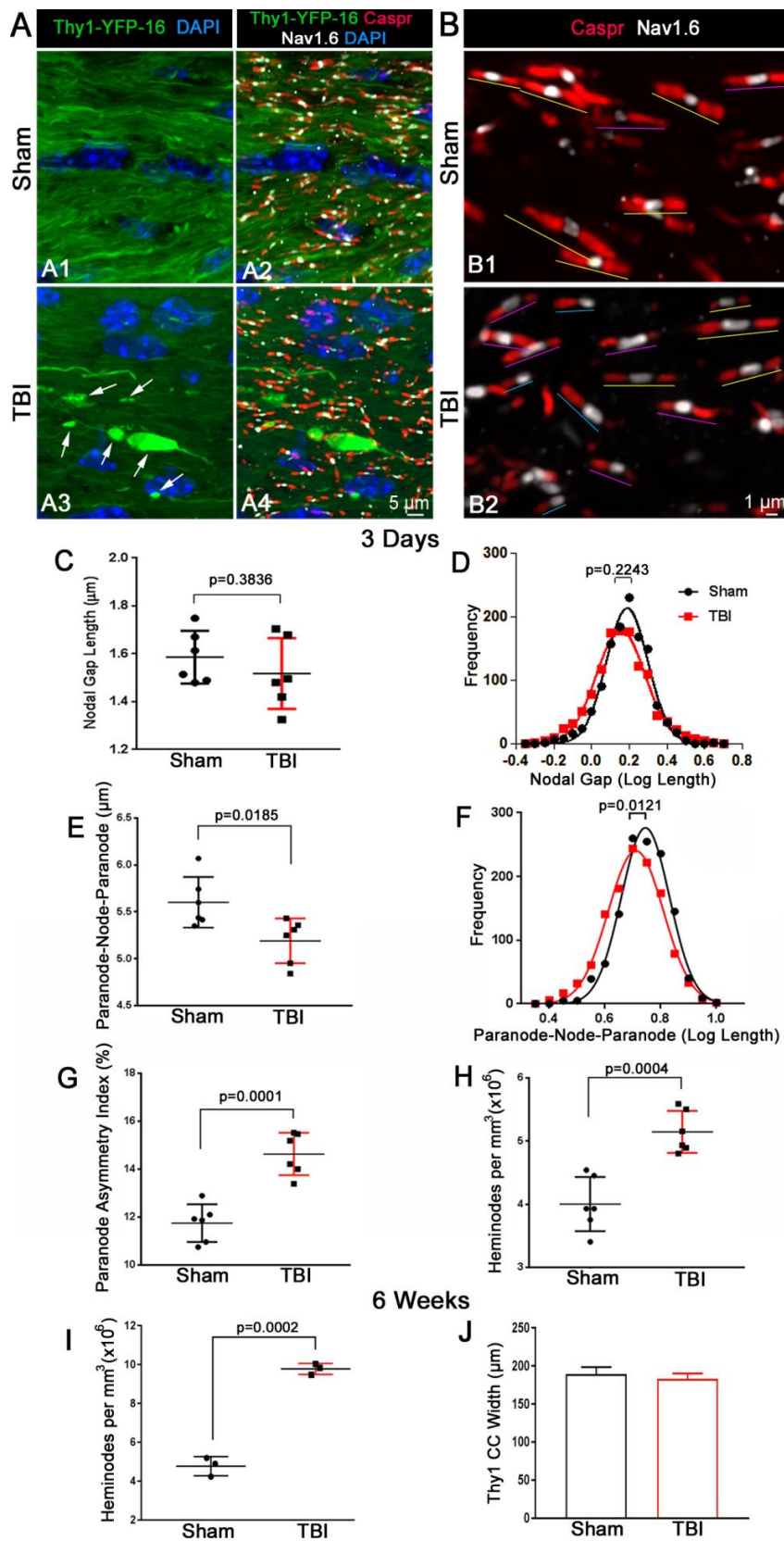


Figure 26. TBI increases paranode asymmetry and formation of heminodes. A, Representative confocal 3-dimensional reconstructions of Thy1-YFP labeled axons in the corpus callosum of sham (A1-A2) and TBI (A3-A4) mice at 3 days post-procedure. Thy1-YFP, which accumulates in swellings (arrows) along damaged axons in TBI mice, aided in the analysis of paranodal organization with Nav1.6 immunolabeling of nodes of Ranvier (white) and Caspr staining of the flanking paranodes (red). Cell nuclei stained with DAPI (blue). Scale bar 5 μ m. **B,** Higher resolution confocal reconstructions show that paranodal complexes in sham animals (B1) mostly appear as normal symmetrical units (yellow lines). Following TBI (B2), disorganized paranodes are evident as asymmetrical paranodes (purple lines) and heminodes (blue lines). Scale bar 1 μ m. **C-H,** Quantification of node and paranode parameters at 3 days after sham or TBI procedures. The mean length of the nodal gap between paired Caspr immunolabeled paranodes remains comparable between sham and TBI animals at 3 days post-procedure (C). Log transformation plot shows a Gaussian distribution of nodal gap length measurements with no significant differences in mean gap lengths between the two groups (D). TBI decreases the overall length of the paranode-nodal gap-paranode regions (E). Log transformation plot shows a Gaussian distribution of paranode-nodal gap-paranode ‘triplet’ length measurements with significant shortening in TBI mice (F). TBI increases paranodal asymmetry, i.e., shortening of one Caspr-positive paranodal domain in a given paranodal pair (G). TBI also increases the frequency of heminodes, i.e., Caspr domains flanking Nav1.6 nodes on only one side resulting in unpaired paranodes (H). **I-J,** At 6 weeks post-procedure the frequency of heminodes is further increased in TBI mice (I), while the overall width of the CC is not significantly different (J). P-values were determined by unpaired Student’s *t*-test. Mouse sample sizes were 3 days (n = 6 sham, n = 6 TBI) and 6 weeks (n = 3 sham, n = 3 TBI); Panel E: $t(10) = 2.18$, $p = 0.0185$, effect size = 1.62; Panel G: $t(10) = 5.973$, $p = 0.0001$, effect size = 3.45; Panel H: $t(10) = 5.158$, $p = 0.0004$, effect size = 2.98. Panel I: $t(4) = 13.71$, $p = 0.0002$, effect size = 11.19. Panels C,E,G-I: Error bars show 10-90% interval. Panel J: Error bars show SEM.

Figure 27

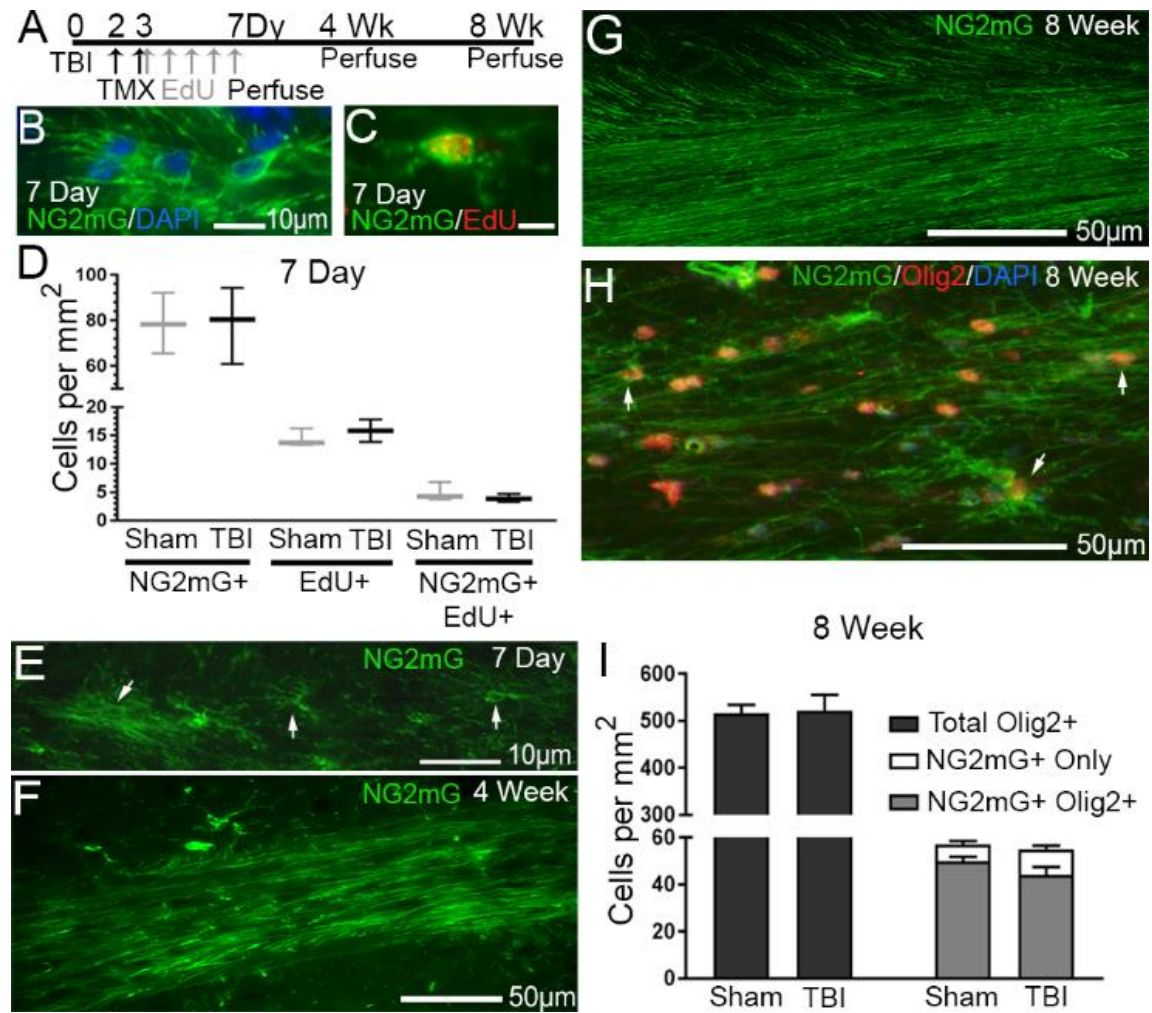


Figure 27. Fluorescent labeling of new membrane synthesis in oligodendrocyte lineage cells using NG2CreERTM;mTmG myelin reporter mice. **A**, NG2CreERTM;mTmG mice were given tamoxifen on days 2-3 after TBI or sham procedures in order to induce expression of membrane-localized green fluorescent protein driven from the NG2 promoter (NG2mG). The thymidine analogue EdU was given daily between 3 and 7 days post-TBI/sham to label cycling cells during DNA synthesis. **B-E**, At 7 days after TBI/sham, NG2mG labels cells with a progenitor morphology (**B**), including EdU-labeled cycling cells (**C**). The density of NG2 cells and/or cycling cells in the CC is not significantly different in TBI mice compared to sham (**D**). **E-G**, NG2mG also labels cells with more elaborate processes that are characteristic of later stage oligodendrocyte lineage cells (**E**). With longer survival time, NG2mG labeled cells extend membranes along axons (**F**) that continue to increase within the CC in a myelinating pattern (**G**). All images show representative examples from TBI mice. **H-I**, The oligodendrocyte lineage marker Olig2 labeled nuclei within the majority of NG2mG cells (**H**, **I**). At 8 weeks post-TBI/sham, the injury condition did not significantly alter the cell populations expressing single or double labeling for Olig2 and/or NG2mG. Mouse sample sizes were 7 days (n = 3 TBI mice, n = 3 sham mice); 4 Weeks (n = 5 TBI, n = 4 sham); 8 Weeks (n = 5 TBI mice, n = 5 sham mice). Two-way repeated measures ANOVA showed no significant effect of injury for a given cell type on posthoc analysis with Sidak's adjustment for multiple comparisons. Panel D: Interaction F(2,8) = 0.03029, p = 0.9703; Cell type F(2,8) = 135, p < 0.0001; Injury F(1,4) = 0.0002, p = 0.9891). Error bars show 10-90% interval. Panel I: Interaction F(2,27) = 0.06392, p = 0.9382; Cell type F(2,27) = 0.05687, p < 0.0001; Injury F(1,27) = 0.00617, p = 0.9380). Error bars show SEM.

Figure 28

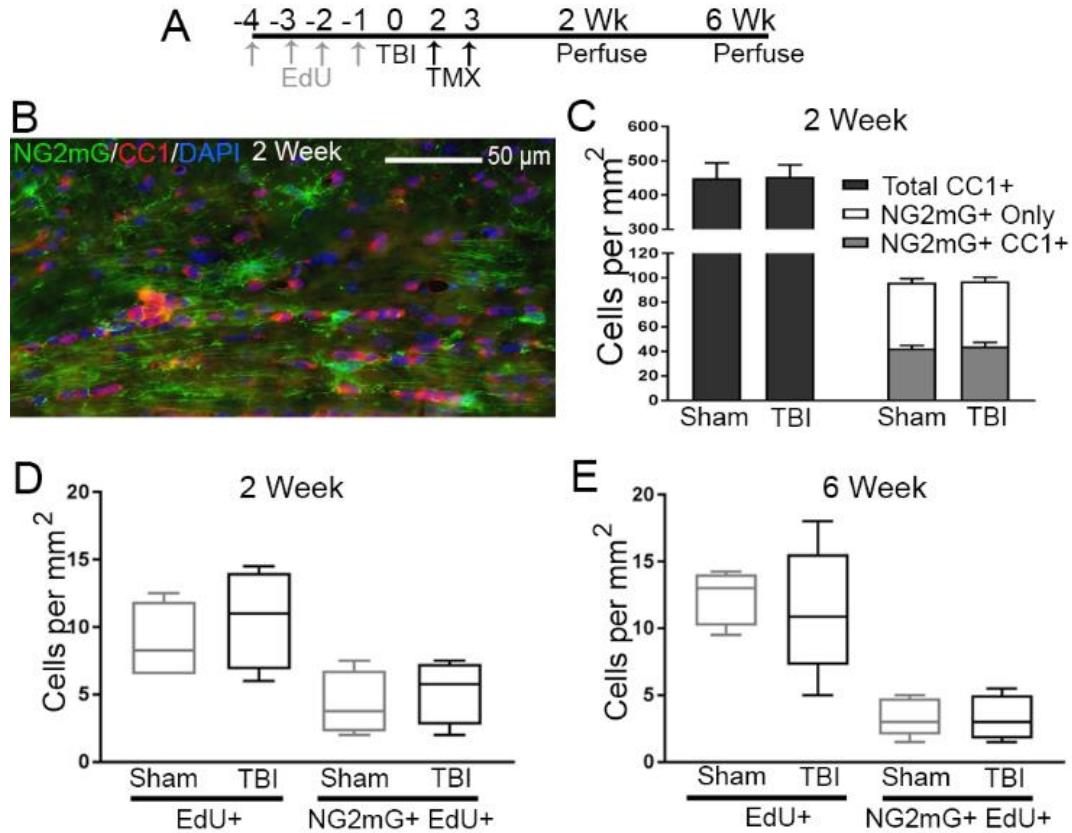


Figure 28. TBI does not alter the response of endogenous cycling cells or differentiation into mature oligodendrocytes in NG2CreERTM;mTmG mice. **A**, NG2CreERTM;mTmG mice were given thymidine analogue EdU daily for four days prior to TBI or sham procedure, followed by tamoxifen on days 2-3 post-TBI/sham to induce NG2mG expression. **B-C**, At 2 weeks post-TBI/sham, NG2mG is expressed in immature oligodendrocyte lineage cells and in cells that express CC1, a mature oligodendrocyte marker (B). The injury condition did not significantly alter the CC cell populations expressing single or double labeling for CC1 and/or NG2mG (C). **D-E**, The most immature oligodendrocyte lineage cells are endogenous cycling cells that incorporate EdU prior to TBI/sham procedures. TBI did not alter the population of EdU labeled cells in the CC, either with or without NG2mG labeling, at 2 weeks (D) or 6 weeks (E) post-TBI/sham. Mouse sample sizes were 2 weeks (n = 5 TBI, n = 5 sham); 6 Weeks (n = 5 TBI, n = 5 sham). Two-way repeated measures ANOVA showed no significant effect of injury for a given cell type on posthoc analysis with Sidak's adjustment for multiple comparisons. Panel C: Interaction $F(2,24) = 0.005887$, $p = 0.9941$; Cell type $F(2,24) = 202.8$, $p < 0.0001$; Injury $F(1,24) = 0.009432$, $p = 0.9234$. Error bars show SEM. Panel D: Interaction $F(1,12) = 0.06658$, $p = 0.8007$; Cell type $F(1,12) = 11.84$, $p = 0.0049$; Injury $F(1,12) = 0.8952$, $p = 0.3627$. Error bars show 10-90% interval. Panel E: Interaction $F(1,16) = 0.1492$, $p = 0.7403$; Cell type $F(1,16) = 45.24$, $p < 0.0001$; Injury $F(1,16) = 0.1649$, $p = 0.6900$. Error bars show 10-90% interval.

Figure 29

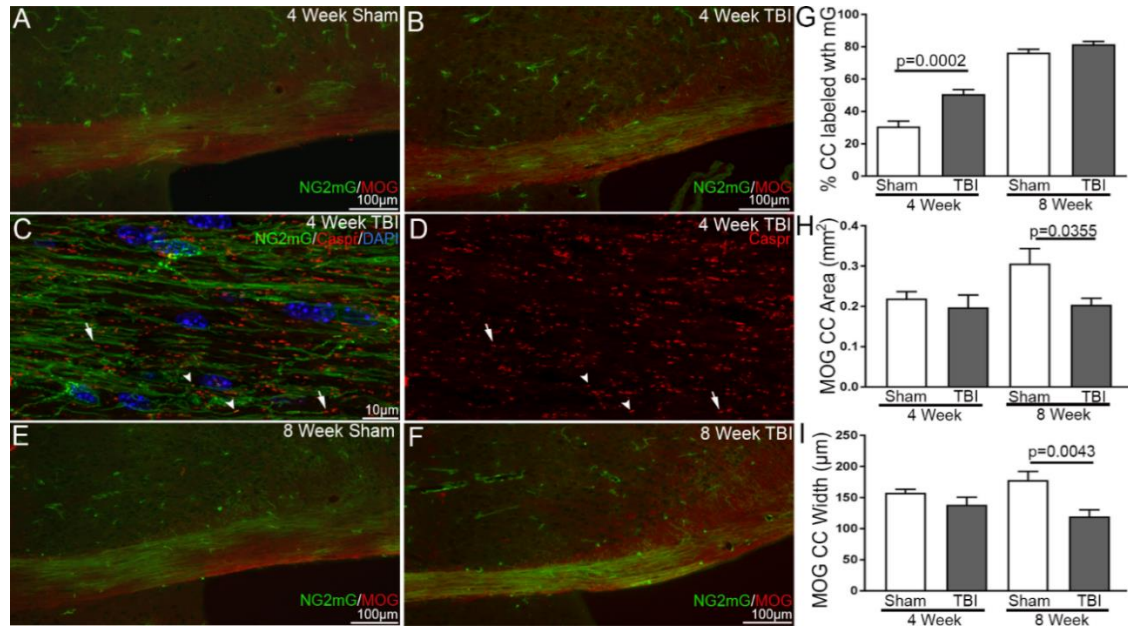


Figure 29. *NG2CreERTM;mTmG* myelin membrane remodeling and CC atrophy after TBI. **A-B**, Sham and TBI mice at 4 weeks elaborate NG2mG labeled membranes in the CC area that is immunolabeled for myelin oligodendrocyte glycoprotein (MOG). **C-D**, Myelin formation by NG2mG labeled cells is indicated by membrane extension to paranodes, identified by Caspr immunolabeling. Caspr labels paranode pairs (arrows) as expected for flanking the node of Ranvier. Individual Caspr regions (arrowheads) reveal abnormal paranode organization after TBI. **E-F**, By 8 weeks after TBI or sham procedures, NG2mG membranes are more widespread within the CC. **G**, Quantification of NG2mG and MOG shows that TBI increases NG2mG membrane formation in the CC at 4 weeks post-injury. **H-I**, However, at 8 weeks, TBI results in significant CC atrophy. MOG measurements of the area (H) and width (I) of the CC are reduced in TBI mice, which do not exhibit the normal continued increase with age that is observed in sham mice. Mouse sample sizes were 4 weeks (n = 5 TBI, n = 4 sham); 8 weeks (n = 5 TBI, n = 5 sham). Two-way ANOVA and posthoc analysis with Sidak's test for multiple comparisons. Panel G: F(1,15) = 8.695, p = 0.0100; Time F(1,15) = 229.5, p < 0.0001; Injury F(1,15) = 25.07, p = 0.0002 with posthoc for 4 weeks p = 0.0002 and effect size = 3.04. Panel H: Interaction F(1,15) = 2.05, p = 0.1727; Time F(1,15) = 2.781, p < 0.1161; Injury F(1,15) = 4.908, p = 0.0426 with posthoc for 8 weeks p = 0.0355 and effect size = 1.89. Panel I: Interaction F(1,15) = 2.887, p = 0.1100; Time F(1,15) = 0.004485, p = 0.9476; Injury F(1,15) = 11.42, p = 0.0041 with posthoc for 8 weeks p = 0.0043 and effect size = 2.14. Error bars show SEM.

CHAPTER 6: DISCUSSION

The primary goals of these studies were to show that myelin changes play an important role in both the deficits and mechanisms of recovery after TBI. Our work has established that in addition to axon damage, distinct myelin pathologies occurred in the white matter after a single relatively mild concussive TBI. Ultrahigh resolution structural assessment, the gold standard for distinguishing different types of axon damage, revealed that demyelination and excessive myelin figures are also TBI-induced pathologies (116). These pathologies are difficult to detect through other means, even in experimental models, and preparing human brain tissue for similar electron microscopy assessment is nearly impossible. Visualizing axon damage on a large scale, however, was possible using transgenic Thy1-YFP mice. After TBI, these mice expressed yellow fluorescent protein that filled axonal swellings across the region-of-interest (197). After a single TBI, these swellings are found throughout the CC (197). This is particularly evident when optically cleared Thy1-YFP brains were three dimensionally reconstructed, as we have presented in this work. We have also now assessed myelin changes across the CC by using a transgenic mouse model to track myelin formation after TBI. This was particularly important in conjunction with ex vivo electrophysiological assessment to identify deficits to myelinated axon conduction across the CC that correspond to our structural findings.

Demyelination is dependent on axon degeneration after TBI

One major goal of these studies was to explore the extent to which myelin pathologies persist in TBI if axon degeneration can be prevented. We used an

experimental model of TBI where *Sarm1* was genetically deleted in mice to prevent execution of the conserved axon death pathway. Electron microscopy in these mice revealed high-resolution ultra-structural detail of types of degenerating axons and myelin pathologies. *Sarm1* reduced the proportion of degenerating axons with vesicle buildup indicative of fast axonal transport disruption and axons with compacted cytoskeleton. It is important to note that axon pathology was not completely resolved with genetic deletion of *Sarm1*. Though significantly reduced compared to wild-type levels, degenerating axons were still present to some extent after TBI despite *Sarm1* deletion. These axons with evidence of degeneration are potentially a subset of axons focally damaged by the mechanical forces of the concussive impact (23).

Additionally, *Sarm1* deletion did not prevent swollen mitochondria within the axon. Mitochondrial swelling is recognized as an early sign of Wallerian degeneration (92; 185; 190). Others have shown that mitochondrial dysfunction may contribute to initiating SARM1-dependent axon degeneration (135; 172). Once activated, *Sarm1* appears to inhibit mitochondrial respiration and promote oxidative stress in turn (120). However, while the conserved axon death pathway likely depends on SARM1, it is only minimally influenced by mitochondrial dysfunction (98; 172). Along with the present results, this suggests that *Sarm1* is downstream of mitochondrial abnormalities in the axon cell death program. These findings also underscore the benefits of ultrastructural analysis for classifying axonal degeneration, as it can show subtle differences in pathology that cannot be detected through other means. The fact that mitochondrial swelling and some degenerating axons are still present when *Sarm1* is genetically deleted also indicates that the forces of TBI are still influencing axons. Ultimately, even if axon

degeneration is reduced by *Sarm1* deletion, the preserved axons are not completely normal as compared to sham axons. This is a key point, as it suggests normal axonal function is still disrupted to some extent.

With axonal degeneration reduced as expected in *Sarm1* mice, the next goal was to determine if this reduction in axon pathology influenced the myelin pathology that also occurs after TBI. Myelin pathology related to TBI has hereto been unexplored with a *Sarm1* genetic deletion. In fact, there is little research on how *Sarm1* deletion may alter myelin in general, though *Sarm1* expression in neurons is approximately three times that of OPCs and tenfold higher than oligodendrocyte expression of *Sarm1* (199). Importantly, *Sarm1* deletion prevented TBI-induced demyelination. This suggests a relationship between axon degeneration and demyelination. When considering SARM1 as a possible target for therapeutic intervention this is an especially encouraging finding. Ideally, intervention along the Wallerian degeneration pathway would not only reduce axon degeneration but additionally be protective for demyelination, which has functional consequences on its own.

Interestingly, *Sarm1* deletion did not prevent all myelin pathologies caused by TBI. After TBI, both wild type and mice with *Sarm1* genetic deletion showed a significant increase in excessive myelin figures. Excessive myelin figures are very long myelin sheaths extending away from a degenerating or intact axon that have folded over on themselves. These myelin figures occur very rarely in sham animals but are a distinct pathology after TBI (116). Though the overall proportion of excessive myelin figures observed after TBI did not change significantly with genetic deletion of *Sarm1*, the figures observed were smaller than those in wild-type mice. The occurrence of these

excessive myelin figures appears not to depend directly on axon degeneration. However, the fact that the figures are smaller in *Sarm1* deleted mice suggests that they are influenced by axonal signaling. Though we do not fully understand the source of these excessive myelin figures, they are of concern for causing additional issues. It has been shown that excessive myelin can slow conduction (46; 136; 138). They may also influence diffusion along axons in the CC, contributing to findings of white matter abnormalities in DTI. Furthermore, it is unknown if these figures resolve or degrade, but if they do generate myelin debris it would likely contribute to microglial activation and persistent neuroinflammation (30; 53).

We do not yet understand how these excessive myelin figures form, but their prevalence in TBI versus other white matter disorders does provide some clues. In much the same way mitochondrial abnormalities persist despite *Sarm1* deletion, it is possible that excessive myelin is a product of early disruptions to normal axon-myelin signaling caused by TBI. Abnormal axon signaling may cause oligodendrocytes to produce this excessive myelin rather than a proper myelin internode. Indeed, similar myelin structures have been observed when scaffolding proteins involved in maintaining axon-myelin interactions are mutated (46; 136). This may be particularly true in the context of TAI, where some axons are damaged while others remain intact. A single oligodendrocyte can myelinate multiple axons, so aberrant signal from one damaged axon could alter normal oligodendrocyte support to the other intact axons it myelinates. Our findings suggest that even minor alterations to the normal interactions between axons and their supporting myelin sheaths have consequences, particularly in the context of TBI.

TBI-induced demyelination corresponds to conduction velocity deficits

Considering the importance of axon-myelin signaling, and the likelihood that TBI disrupts normal interactions between axons and myelin, we wanted to determine if any related structural changes could be observed. Indeed, high-resolution ultrastructural assessment along axons revealed abnormal myelin attachments at the paranode region after TBI. As compared to sham and intact axons where myelin loops evenly contact the axon on either side of the node, after TBI these myelin loop connections were uneven, appeared to be loose, or were missing all together. Using immunohistochemistry to assess the paranode-node-paranode units within a larger scope of the CC after TBI, Dr. Kryslaine Radomski found several alterations. TBI caused an increase in heminodes, defined as a single paranode without a full unit. Of the full paranode-node-paranode units that persisted, there was an increase in asymmetry where one paranode would be longer than the corresponding side. Though these alterations may seem minor, any regions where the myelin internode has been lost or myelin is not properly interacting with the axon could alter action potential propagation (7).

These structural abnormalities at the paranode-node interface, where axon-myelin interactions are most important, are particularly stark given our work has also shown increased demyelination after TBI. To determine if these TBI-induced myelin changes do indeed correspond to slowed axon conduction, we collaborated with Drs. Zygmunt Galdzicki and Nathan Cramer to explore the compound action potential signal across the CC. At three days post-TBI, the time point where the most demyelination is observed, compound action potential conduction velocity was significantly slowed as compared to shams. Importantly, this reduction in conduction velocity was specific to the signal

normally produced by myelinated axons, rather than that of smaller unmyelinated axons. This suggests that myelinated axons are specifically impacted by TBI in this region of the brain, and the electrophysiological deficits correspond to the previously observed demyelinated axons and paranode abnormalities, and possibly excessive myelin figures as well.

Moving out to two and six weeks post-TBI, conduction velocity was restored to roughly sham levels. This was not surprising, given most patients do tend to recover well after mild to moderate TBIs. However, when we assessed the amplitude of the compound action potential, which generally indicates the proportion of myelinated axons contributing to the signal across the CC, we found that it was significantly reduced from sham at all time points after TBI. It was likely that the signals carried by myelinated axons were slowing so much after TBI, likely due to demyelination, that they were becoming part of the signal from unmyelinated fibers. We therefore combined the amplitude from both myelinated and unmyelinated fibers and found that at six weeks after TBI there was a significant overall amplitude loss. This loss of amplitude suggested that roughly six weeks after TBI damaged axons have passed the point of a delayed action potential and have rather lost signaling ability all together. We speculate that this dropout is caused by axons that have, or are in the process of, becoming fully degenerated.

An intervening recovery period after TBI

An interesting timing pattern emerged between our electrophysiological assessment and the electron microscopy performed at corresponding time points after

TBI. As mentioned above, axon demyelination was most prevalent at three days after injury, which corresponds to decreased conduction velocity and compound action potential amplitude for myelinated axons. However, by two weeks after TBI, the proportion of demyelinated axons observed by EM dropped and the amplitude significantly recovered as compared to the three day time point. This timing was of particular interest because it falls within a time frame in which remyelination could occur (141).

We utilized transgenic mice to assess new myelin formation or remodeling occurring after TBI. NG2CreER;mTmG mice were given tamoxifen after the TBI or sham procedure to heritably label immature oligodendrocyte lineage cells. These cells differentiated into oligodendrocytes and extend membranes along axons in both sham and TBI mice, but the overall amount of myelin formed was increased in TBI mice over shams at four weeks after injury. Importantly, these new sheaths colocalized with Caspr immunolabeling of the paranode, suggesting these newly formed myelin sheaths were functionally relevant. Additionally, myelin measured by EM during this intervening period of roughly two to four weeks after TBI is thinner, which is indicative of remyelination (116).

These findings are encouraging for several reasons. First, this suggests that the process of adult myelin plasticity can be harnessed in the context of TBI to potentially replace lost myelin internodes. A similar process is believed to occur in patients who suffer from relapsing/remitting multiple sclerosis, where myelin lesions can be remyelinated and heal to some extent, particularly early on in the disease progression (26; 36). Additionally, myelination is known to occur from birth into adulthood, even in

humans, and evidence of white matter structural changes after learning a new skill in adulthood suggest a similar mechanism of myelin plasticity is possible in the human brain (155). Furthermore, this two to four week post-injury period in mice corresponds to a very realistic timeline for therapeutic intervention in TBI patients. Patients who have been identified as having issues related to slowed processing or indications of white matter abnormalities through DTI could be excellent candidates for therapies that enhance the remyelination process.

Factors contributing to white matter atrophy after TBI

The evidence of remyelination occurring in the weeks following TBI is encouraging. However, by eight weeks after TBI, the proportion of newly formed myelin membrane in the CC evened out with sham animals. While the overall number of fate-labeled mature oligodendrocytes did not differ between the conditions, the size of the CC was altered by TBI. The area and the width, measured at several points along the length of the CC, was significantly reduced in TBI mice as compared to sham. Despite evidence of axon loss at six weeks, CC atrophy was not yet observed. Again, the overall number of new oligodendrocytes was comparable between sham and TBI mice at eight weeks, suggesting CC axons and their corresponding myelin were lost between six and eight weeks. This late CC atrophy was an important finding because it parallels similar outcomes in patients that have suffered moderate to severe TBIs (126; 157; 193). CC atrophy is detectable through MRI in many of these patients, who tend to have more long-term deficits than those without overt atrophy (31; 69). It should be noted that CC atrophy has been described in other diseases such as Alzheimer's, Parkinson disease, and

multiple sclerosis, and furthermore, also corresponds to cognitive impairments in patients (45; 66; 68; 87).

We returned to the *Sarm1* genetic deletion mice to determine if protecting axon integrity reduced late CC atrophy. Indeed, at eight weeks after TBI, atrophy was attenuated in mice with genetic *Sarm1* deletion as compared to the wild type counterparts. This was an encouraging finding, as it suggests an early intervention to preserve axons had a long-term benefit that may improve patient outcomes. Interestingly, immunohistochemistry for neuroinflammation-related cell types revealed TBI activated these cells in both wild type and *Sarm1* deleted mice. Though neuroinflammation is thought to be a major contributing factor to white matter atrophy, these findings suggest that it may not be the prevailing factor. Additionally, it is unlikely that neuroinflammation is spurred by the process of axon degeneration alone, as it persisted despite a reduction in degenerating axons through *Sarm1* deletion.

TBI causes a complex cascade of issues, particularly in the white matter, so it is not surprising that axon degeneration is not the only contributing factor to neuroinflammation. As discussed previously, excessive myelin, particularly if degraded, may contribute to microglial activation. In our model, however, astrocytes were found to have the greatest changes after TBI in the *Sarm1* deletion mice. It is likely that despite no overt signs of microhemorrhage, the vasculature is still disrupted on a smaller scale. White matter vasculature is even more vulnerable to the forces of concussive TBI than axons (11; 19). We suspect that the axon damage observed when *Sarm1* was genetically deleted was due to the direct forces of the concussive impact, which also likely caused focal damage to the vasculature. Astrocytes foot processes have a known role in

maintaining the neurovascular unit and are functionally altered by TBI (96; 144).

Additionally, we suspect that even minor disruptions to normal axon-myelin signaling contribute to the pathologies and deficits caused by TBI. The same is likely true for axon-glia signals in general, as altered axon function likely also induces changes in astrocytes and microglial. Further exploration is certainly needed to better inform the relationship between axon degeneration and neuroinflammation, and in turn neuroinflammation and white matter atrophy.

Future directions

Additional studies are needed to further these findings and address current research limitations. First, there are inherent limitations to the experimental model. While we aimed to reflect specific aspects of human TBI, full human pathology is far more complex. Here we attempted to replicate aspects of concussive TBI, particularly traumatic axonal injury and reduced white matter integrity without overt tissue damage or microhemorrhage on either neuroimaging or neuropathology. It would be interesting to explore myelin pathology in other experimental TBI models, particularly those that include white matter microhemorrhages recapitulating the microhemorrhages and white matter lesions detectable by MRI in patient populations. It would also be interesting to test the effects of *Sarm1* deletion on pathology that occurs in other experimental TBI models.

Additionally, diffusion tensor MRI is currently used to detect reduced white matter integrity after TBI in patients and *in vivo* within our experimental model (28; 94; 126; 129; 154; 157; 197). Ideally, this technology will advance to the point of being able

to detect demyelination and paranodal abnormalities to help identify patients for whom certain treatments may be beneficial. Diffusion tensor MRI could also improve the ability to quantify axon loss and overall atrophy across white matter, potentially allowing for these processes to be tracked over time in patients. Furthermore, structural MRI volume measurements are an excellent potential biomarker that bridges the gap between experimental TBI and human patients. For both populations, volume measurements allow for longitudinal assessments that inform the efficacy of interventions.

The present studies underscore the importance of axon-myelin interactions after injury. The exact signaling mechanisms through which axons and myelin interact under normal circumstances is not yet fully understood. Furthermore, we have few clues as to how these interactions may be altered by a complex injury process like TBI. There are several areas of potential interest to be explored. It would be interesting to assess the ion channels exposed by demyelination, as well as determine the extent to which damaged axons can respond to demyelination by redistributing sodium channels from the nodal cluster. There is also evidence that axons will cluster ion channels and paranode proteins in proper arrangement prior to remyelination (33). Damaged or stressed axons may not be able to fully undergo this process or become vulnerable to further disruption before remyelination can occur fully (162). Investigating these processes in both wild-type mice and in mice with genetically deleted *Sarm1* may also help inform how axon-myelin interactions are preserved. Others have shown that even when fully transected, axons of *Sarm1* deleted mice can continue to propagate action potentials if stimulated (58). We also suspect that an alteration in normal axon-myelin function contributes to excessive

myelin figures. Ongoing projects by others in the lab seek to better identify the nature and potential consequences of this unusual myelin pathology observed in TBI.

Finally, we hope these findings support the effort to identify potential TBI treatments. Here we assessed a specific intervention, genetic deletion of *Sarm1*, to determine the effects preventing Wallerian degeneration has on the major pathologies observed after TBI. While the genetic deletion shows promise for attenuating axon degeneration, demyelination, and overall white matter atrophy after TBI, it is limited by the fact that *Sarm1* initiation is not expected to occur in this model. Additional work is needed to target *Sarm1* after the initiation phase of the axon degeneration pathway. Indeed other models of primary axonal injury have been successfully modified using gene therapy to target *Sarm1* (59). This is important if drugs developed to target SARM1, or axon degeneration in general, are to be effective therapeutics given after a TBI has occurred. Similarly, therapeutics targeted toward remyelination would also require further study to identify the best timing to promote functional and stable myelin sheath formation after TBI.

Summary

This research identified a time course for white matter pathology and functional deficits caused by TBI. Early assessment revealed axon degeneration, demyelination, and disrupted axon-myelin interactions as well as slowed conduction velocity across the CC. An intervening period of remyelination or myelin plasticity corresponded to partial functional recovery. However, ultimately axon loss and overall CC atrophy became apparent at late stages after TBI. Furthermore, *Sarm1* genetic deletion has implications

for altering some of these pathologies by targeting the conserved axon death pathway. By preventing the initiation of Wallerian degeneration through *Sarm1*, axon degeneration and demyelination were reduced after TBI, and late CC atrophy was attenuated. This preservation of the axon did not prevent all pathologies, however, as TBI still induced mitochondrial changes, excessive myelin figures, and neuroinflammation. Overall these studies highlight the important role for myelin after TBI. Furthermore, the findings underscore the need for a thoughtful and possibly multimodal approach to TBI treatment that considers not only axonal damage, but also myelin pathology and the regenerative capacity of myelin.

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