

Sarm1 as a Therapeutic Target for Chronic White Matter Pathology Following
Experimental Traumatic Brain Injury

by

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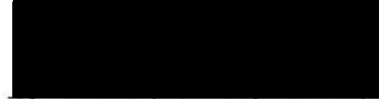
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ABSTRACT

Sarm1 as a Therapeutic Target for Chronic White Matter Pathology Following Experimental Traumatic Brain Injury

Donald V. Bradshaw Jr., Ph.D., 2021

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Traumatic brain injury (TBI) can have long term consequences that include neurodegeneration and persistent symptoms. White matter tracts, such as the corpus callosum (CC), are particularly vulnerable and are associated with functional outcomes and potential neurodegenerative processes. Treatments are needed to protect axons from early traumatic axonal injury (TAI) and mitigate secondary mechanisms that lead to late stage neurodegeneration and atrophy. The *SARM1* (sterile alpha and Toll/interleukin-1 receptor motif containing 1) gene has been identified as a master executioner of the axon degeneration pathway and has become a significant target of interest in the development of therapeutic strategies to prevent axonal degradation. Our previous work demonstrated that mice with genetic deletion of *Sarm1* exhibit significantly less acute axon damage and demyelination following a model of concussive TBI. This project aimed to determine if the constitutive absence of *Sarm1* attenuates chronic white matter pathology and behavioral deficits following experimental TBI. We first evaluated the effect that a military relevant model of repetitive blast exposure had on white matter pathology by

utilizing fluorescent reporter mice that enable visualization of distinct white matter components. Repetitive blast exposure causes acute axon damage accompanied by neuroinflammation without persistence to late-stage myelin disruptions. Next we assessed white matter damage and associated functional deficits in a concussive model of TBI proven to cause significant acute axon and myelin pathology. Magnetic resonance with diffusion tensor imaging revealed chronic CC atrophy, validated by post-imaging neuropathology, and microstructure disruptions that were accompanied by chronic-stage motor learning deficits. The evidence of a chronic phenotype of axon damage after concussive TBI provided a model to evaluate the effect of *Sarm1* genetic deletion on white matter deficits. Electron microscopy revealed that *Sarm1* KO mice had preserved intact axons and reduced damaged or demyelinated axons. Longitudinal MRI analyses demonstrated that CC atrophy after TBI was attenuated in *Sarm1* KO mice. Prevention of CC degeneration was further validated by immunohistochemistry which revealed significant therapeutic effect on CC atrophy, myelin loss, and neuroinflammation in *Sarm1* KO mice after TBI. Functional outcomes showed that preservation of white matter in *Sarm1* KO mice after TBI translated to improvements in motor skill learning and prevention of sleep deficits. Taken together, these findings demonstrate white matter pathology after TBI is a chronic process. Furthermore, SARM1 is a promising therapeutic target for attenuating these chronic axon and myelin pathologies and improving long-term outcomes.

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LIST OF ABBREVIATIONS

ABS:	Advanced blast simulator
AD:	Axial diffusivity
ALS:	Amyotrophic lateral sclerosis
ANOVA:	Analysis of variance
β -APP:	Beta-amyloid precursor protein
CC:	Corpus callosum
DAPI:	4,6-diamidino-2-phenylindole
DoD:	Department of Defense
DTI:	Diffusion tensor imaging
EM:	Electron microscopy
FA:	fractional anisotropy
GFAP:	glial fibrillary acidic protein
GFP:	Green fluorescent protein
IBA1:	Ionized calcium binding adaptor molecule 1
MOG:	Myelin oligodendrocyte glycoprotein
MRI:	Magnetic resonance imaging
NAD ⁺ :	Nicotinamide adenine dinucleotide
NMN:	Nicotinamide mononucleotide
NMNAT2:	Nicotinamide-nucleotide adenylyltransferase 2
OPC:	Oligodendrocyte progenitor cell
rBlast:	Repetitive blast exposure
RD:	Radial diffusivity

Rm-ANOVA: Repeated measures analysis of variance

ROI: Region of interest

rSham: Repetitive sham procedure

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

Sarm1 KO: *Sarm1* knockout mouse

Sarm1 WT: *Sarm1* wildtype mouse

T2(w): T2 weighted image

TAI: Traumatic axonal injury

TBI: Traumatic brain injury

TMX: Tamoxifen

WldS: Wallerian degeneration Slow

YFP: Yellow fluorescent protein

CHAPTER 1: INTRODUCTION

TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is a highly prevalent and concerning global health issue. Greater than 27 million cases occur globally every year and the incidence rate has been steadily climbing over the last 20 years (1). In the United States alone there are more than 2.5 million hospitalizations annually associated with sustaining a TBI (167). Brain injury is of particular concern to the United States Department of Defense (DoD). There have been approximately 430,000 TBI cases amongst military personnel since the year 2000, occurring at a rate that, per capita, is roughly double the civilian population (2; 3). With the United States' involvement in the conflicts in Iraq and Afghanistan unique mechanisms of injury such as blast induced TBI have greatly increased in prevalence. These distinct modes of brain injury combined with the complications of healthcare within a military theater have created a heightened interest in the consequences of TBI on military personnel. The prevalence of TBI and the growing knowledge of the long lasting burden it places on patients and healthcare systems underscores the need for greater research and therapeutic development.

The persistence of symptoms and long-term disabilities associated with a TBI have become a problem of increasing notoriety in recent years. It is estimated that 1% of the U.S. population, > 3 million individuals, suffer from the chronic effects of having sustained a brain injury (189). Longitudinal studies have revealed a variable rate of recovery after TBI wherein some patients have enduring symptoms months to years after their injury. Following treatment for TBI, patients can have short-term improvements on

measures such as the Glasgow Outcome Scale that then progressively decline over time, correlating to increased hospital re-admittance and need for chronic clinical care (30; 32; 134). Additionally, TBI has been correlated to a significantly increased risk of stroke and the development of neurodegenerative and neuropsychiatric disorders (30; 129; 154). These negative long-term outcomes following periods of recovery point to TBI as being an evolving, chronic process.

A more thorough understanding of what contributes to chronic symptoms after TBI is particularly important for the military population. The prevalence of mental health disorders is incredibly high amongst military TBI patients and long-term need for rehabilitative care presents a significant challenge for the military healthcare system (22; 69). Additionally, mechanisms of TBI specific to military populations (e.g. explosive blast) complicate our understanding of the pathological underpinnings that contribute to worsened outcomes. In 2018, the National Defense Authorization Act called for increased surveillance and research around the issue of repetitive blast (rBlast) exposure that military personnel routinely encounter during training exercises. Early studies connected rBlast exposure to worsened neurological outcomes in service personnel (19; 149; 166). However, the pathological profile and long-term effects of this distinctive mechanism of brain injury remain poorly understood.

The epidemiological data, both in civilian and military populations highlights the immense burden TBI places on patients and healthcare systems alike. Of specific concern is the growing body of literature that supports the idea that TBI is a chronic process with a heterogeneous and time dependent profile of symptoms. A more detailed understanding

of the progression of specific TBI pathologies that drive chronic indications will open the door to the development of much needed future therapeutics.

WHITE MATTER PATHOLOGY IN CHRONIC TBI

Damage to the white matter of the brain is a hallmark of TBI and a major driver of negative outcomes after injury (76). Axons are particularly vulnerable to the tension, torsion and compression forces that occur inside the brain. TBI results in traumatic axonal injury (TAI) where damaged axons are found interspersed between healthy axons within white matter tracts (17). Advances in neuroimaging have allowed for the identification and longitudinal tracking of TBI associated white matter disruptions. Magnetic resonance imaging (MRI) studies utilizing diffusion tensor imaging (DTI) have shown that reductions in white matter integrity over time correlated to worsened cognitive performance in TBI patients (23; 24; 87). Studies in military populations have furthered this understanding of the connection between chronic white matter damage and long-term neurological problems in TBI patients. Veterans with a history of TBI showed a correlation between white matter microstructure alterations and cognitive decline (156). Additionally, a history of blast exposure in some military personnel was connected to reductions in white matter integrity detected through neuroimaging (34; 92; 94). These studies demonstrate how damage to major white matter tracts can persist for extended periods of time and that the evolution of those pathologies contributes significantly to the persistence of TBI symptoms in some patients. However, while these studies have proven vital in determining that white matter damage after TBI is a chronic process, they lack the specificity to pinpoint what pathologies are driving post-traumatic degeneration. Some postmortem studies in TBI have found evidence of axonal injury, destruction of myelin

and neuroinflammation, all of which contribute significantly to white matter degeneration (76; 77; 123; 153). In postmortem studies of military personnel hyperphosphorylation of tau, beta-amyloid precursor protein (β -APP) aggregation, myelin disruptions and astroglial scarring have all been identified in tissue from subjects with a history of blast exposure. These findings from human tissue samples are incredibly useful for identifying some pathological features of brain injury but there is an inherent disconnect between findings in vivo and those found postmortem. Preclinical studies should aim to connect results between experiments so that specific pathological features within major white matter tracts can be correlated to changes seen on translational outcomes to better inform findings in human TBI patients.

One of the major white matter tracts in the brain that is particularly important in the context of TBI is the corpus callosum (CC). Evidence in recent years has implicated the CC specifically as being a crucial substrate in terms of both white matter pathology and long-term outcomes after brain injury. In severe TBI, greater than 75% of patients show significant CC atrophy that continues for years after degeneration of other major white matter tracts has plateaued (170). Neuroimaging studies have shown that in moderate to severe TBI patients, DTI measures of CC white matter integrity are significantly altered both acutely as well as months after the TBI event (20; 121; 140). These findings in human cases of TBI emphasize the importance of the CC as a major white matter tract and justify the value of focusing on the CC as a white matter substrate in research for development of therapeutics aimed at attenuating axonal pathologies.

Clinical studies of TBI tend to focus on gross white matter changes as opposed to specific disruptions to the cellular structure of the axons themselves. However, an

understanding of specific white matter pathologies will prove to be crucial in the development of future therapeutics. Myelin, specifically, has become a component of intense research interest following TBI. Myelin ensheathes axons enabling the rapid signal conduction necessary for neuronal communication (62; 110; 115). While significantly harder to distinguish in humans independent of other white matter pathologies, myelin disruptions have been found to accompany axonal injury in post-mortem tissue (123). Neuroimaging studies have shown that MRI-based measures of intracortical myelin are sensitive to a history of TBI and are associated with functional connectivity (54). Preclinical studies have further explained that TBI can cause alterations to myelin integrity or loss of myelin completely even in milder forms of white matter injury (105; 162). An appreciation for the role that myelin plays in chronic TBI will prove vital in effectively addressing unmet therapeutic needs of white matter injury.

There are currently no FDA approved clinical therapeutics for TBI or axon damage. This highlights a significant gap in the field and a need for rigorous preclinical testing of potential therapeutic strategies for attenuating axon and myelin pathology that characterize white matter degeneration, specifically in the context of brain injury.

WALLERIAN DEGENERATION AND SARM1 AS A THERAPEUTIC TARGET

To develop strategies for attenuating white matter pathologies we must first have a thorough understanding of the biological mechanism by which axons degenerate. When an axon is irreversibly damaged it undergoes a highly conserved mechanism of degradation, termed Wallerian degeneration, in which the distal portion of the axon fragments and degenerates but the cell body may remain viable. (28; 60; 61; 185). Importantly, the majority of axon damage is not the result of primary, physical forces

from an injury, such as a TBI. Rather, degeneration is mostly the result of secondary damage cascades that initialize in the days, weeks and months post injury, likely contributing to the persistence of symptoms (18; 99). Until 1989, Wallerian degeneration was thought to be a passive process when the discovery of a spontaneous mutation in a mouse line, dubbed Wallerian degeneration slow (WldS), showed resistance to axonal degeneration that proved to be a genetically regulated process distinct from cell body apoptosis or axonal pruning (70; 91; 152). This finding opened the door to a series of discoveries about the molecular mechanism of Wallerian degeneration in which the process was shown to be completed in two distinct phases: an initiation and an execution phase (28). The initiation phase is driven by an intrinsic enzymatic cascade initiated within the axon rather than from injury-derived signals such as calcium influx at the injury site (40; 51; 144; 178). This new understanding that genetically encoded factors induced the process of axonal degeneration provided optimism that Wallerian degeneration was a mechanism that could be modified for therapeutic benefit.

The recognition that axonal degradation was an active, genetically modulated process led to the discovery of a number of genetic factors involved in the process. A screen in *Drosophila* revealed several mutants that suppress Wallerian degeneration (125). The most promising of these, from a therapeutic development perspective, is the sterile alpha and TIR motif containing 1 (SARM1) gene, which was initially identified as its *Drosophila* ortholog *dSarm* and later found to be highly evolutionarily conserved (89). SARM1, a member of the Myd88 family of toll like receptor adaptor proteins has, through cryo-electron microscopy (cryo-EM), been shown to be comprised of a homomultimeric octamer. Each monomer is made up of an N-terminal autoinhibitory

ARM domain, tandem SAM domains that mediate multimerization, and a C-terminal TIR domain encoding the NADase enzyme(120; 147). Under homeostatic conditions SARM1 is kept in an inactive state by an interaction between the N-terminal ARM domains and the C-terminal TIR domains mediated by the nicotinamide-nucleotide adenylyltransferase 2 (NMNAT2) enzyme (49; 176). When an axon is fatally injured mitochondrial stress causes a loss of NMNAT2 which results in the release of the ARM domains allowing the TIR domains to dimerize (35; 51; 52). This dimerization initiates a molecular cascade that results in a rapid depletion of axonal NAD^+ causing a fatal bioenergetics and metabolic imbalance. (47; 49).

The discovery of *Sarm1* and its mechanism of action represented the first loss-of-function mutation that prevented axonal degeneration in the same manner seen originally in WldS mice. This prompted excitement around SARM1 as a therapeutic target for axonopathies. The therapeutic concept is that if the central function of SARM1 could be blocked or genetically modulated the Wallerian degeneration cascade would not initiate and axons would be spared. A series of studies utilizing a mouse model in which *Sarm1* has been genetically deleted have demonstrated significant attenuation of axon pathology in models of stroke, Amyotrophic Lateral Sclerosis (ALS), diabetic and chemotherapy-induced peripheral neuropathy (44; 45; 80; 171). In the context of TBI, *Sarm1* genetic deletion has been shown to have acute benefits in the form of preventing axon damage, protecting myelin and attenuating some functional deficits (65; 96; 190). This protection of white matter in the early stages after TBI warrants the investigation of the effects of *Sarm1* deletion on chronic stage deficits after injury that drive long-term symptoms and disability in TBI patients.

PURPOSE OF RESEARCH

This project aimed to determine if SARM1 is a viable therapeutic target for attenuating chronic white matter pathology following an experimental model of TBI. Treatments are needed to protect axons from early TAI and mitigate secondary mechanisms that lead to late stage neurodegeneration and atrophy. Therefore these studies serve to provide a preclinical framework for the evaluation of a promising therapeutic strategy to address these unmet clinical needs. The first aim of this project utilized fluorescent reporter mice to identify specific components of axon damage and myelin synthesis following a model of repetitive blast exposure. The second aim characterized chronic white matter degeneration and associated behavioral deficits in a concussive model of TBI that has consistently shown significant TAI and demyelination at more acute time points. The third aim demonstrated the benefit of *Sarm1* deletion by connecting reductions of ultrastructural axon and myelin pathology to benefits observed on translational measures of post-traumatic degeneration such as neuroimaging and functional outcomes.

The chapters of this thesis walk through these specific aims, detailing methodology and results, followed by a thorough discussion of their relevance and impact to the field of science. Chapter two and three focus on the primary data acquired within these specific aims. Chapter two highlights the white matter effects seen using fluorescent reporter mice exposed to repetitive simulated blast. Chapter three focuses on the chronic stage effects of TBI on white matter and the benefits that *Sarm1* deletion exhibited. The final chapter of this dissertation discusses the data from the specific aims in the broader context of how it meaningfully advances the field of white matter degeneration in TBI. This thesis provides strong primary data to support development of

SARM1 focused treatments for axonopathies and lays out an experimental framework that will serve to increase translational value of preclinical models in therapeutic development.

CHAPTER 2:

Repetitive Blast Exposure Produces White Matter Axon Damage Without Subsequent Myelin Remodeling: *In Vivo* Analysis of Brain Injury Using Fluorescent Reporter Mice

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AUTHOR CONTRIBUTION STATEMENTS

Donald V Bradshaw Jr. wrote first draft and edited paper. Designed and conducted experiments and analyzed data. Yeonho Kim and Amanda Fu conducted blast exposures. Christina M. Marion and Kryslaine L. Radomski performed research and edited paper. Joseph McCabe contributed to experimental design and edited paper. Regina C. Armstrong designed and performed research, analyzed data, wrote and edited paper.

ABSTRACT

The potential effects of blast exposure on the brain health of military personnel have raised concerns and led to increased surveillance of blast exposures. Neuroimaging studies have reported white matter abnormalities in brains of service members with a history of blast exposure. However, blast effects on white matter microstructure remain poorly understood. As a novel approach to screen for white matter effects, transgenic mice that express fluorescent reporters to sensitively detect axon damage and myelin remodeling were exposed to simulated repetitive blasts (once/day on five consecutive days). Axons were visualized using *Thy1-YFP-16* reporter mice that express yellow fluorescent protein (YFP) in a broad spectrum of neurons. Swelling along damaged axons forms varicosities that fill with YFP. The frequency and size of axonal varicosities were significantly increased in the corpus callosum (CC) and cingulum at 3 days after the final blast exposure, versus sham procedures. CC immunolabeling for reactive astrocyte and microglial markers was also significantly increased. *NG2CreER;mTmG* mice were given tamoxifen on days 2 and 3 after the final blast to induce fluorescent labeling of newly synthesized myelin membranes, indicating plasticity and/or repair. Myelin synthesis was not altered in the CC over the intervening 4 or 8 weeks after repetitive blast exposure. These experiments show the advantages of transgenic reporter mice for analysis of white matter injury that detects subtle, diffuse axon damage and the dynamic nature of myelin sheaths. These results show that repetitive low-level blast exposures produce infrequent but significant axon damage along with neuroinflammation in white matter.

Key Words: blast exposure; white matter; traumatic brain injury; transgenic reporter mice; axon damage; myelin

INTRODUCTION

Traumatic brain injury (TBI) is a significant public health concern for civilian and military populations. Brain injury associated with an explosive blast raises additional concerns due to the unknown effects of blast exposures and the potential for long-term symptoms (5; 93). High explosives can result in injury from the blast wave along with projectiles impacting the head and/or the person being thrown. TBI associated with high explosive blasts accounted for approximately one third of the nearly 400,000 military TBIs between 2001-2008 (136). The effects of repetitive exposure to occupational blasts are an ongoing concern within the military. The National Defense Authorization Act (2018-2020) called for occupational surveillance to evaluate blast exposure, for example among those engaged in breaching exercises or firing of heavy artillery (150). Early studies indicated a potential correlation between military personnel exposed to repeated low-level blast exposure and worsened long term neurological and cognitive outcomes (19; 166).

The long-term effects of blast exposure on brain health remain poorly understood. Magnetic resonance imaging (MRI) studies using diffusion tensor imaging (DTI) detected changes in major white matter tracts of the brain in some of the deployed soldiers who experienced TBI with blast exposure (34; 92; 94). Studies of blast exposed veterans with and without TBI found that blast exposure decreased DTI fractional anisotropy in several white matter tracts (72). Both breachers and veterans examined more than one year after blast exposure with TBI exhibited DTI changes in white matter (159; 165). Furthermore, veterans with blast-related mild TBI and loss of consciousness had increased white matter abnormalities and cognitive impairments (108).

Post-mortem studies of brains of service members who experienced blast-related TBI have identified multiple neuropathological features. Axon damage, identified as axon swellings with accumulated β -amyloid precursor protein (β -APP), has been observed along with disruptions to the myelin that ensheaths axons (102; 142). Tau, a protein that is bound to microtubules in the cytoskeleton of healthy axons, has been reported to become hyperphosphorylated and form aggregates in neurons in the brains of some veterans who experienced repetitive head injury and blast exposure (53). Additionally, brains from military service members with a history of blast exposure exhibited astroglial scarring at interfaces between tissues of differing densities; this astrogliosis involved the subpial glial plate, penetrating cortical blood vessels, structures lining the ventricles, and white matter-gray matter junctions (148). Together, these neuroimaging and neuropathology studies indicate vulnerability of the brain to blast exposure and the potential for axons and white matter to be critical pathological factors. However, the full range of neuropathological effects of repetitive blast exposure are challenging to determine because potentially subtle pathological features may be sparsely distributed throughout the brain, and may change over time. To overcome these challenges, we evaluated the effects of blast exposure on axon and white matter pathology using two lines of transgenic mice with genetic reporter constructs that enable detection of *in vivo* changes in specific cellular processes.

Thy1-YFP-16 reporter mice express yellow fluorescent protein (YFP) in a broad spectrum of neurons so that individual axons can be visualized in diverse brain regions, including the corpus callosum (CC) (38; 97; 182; 188). We used *Thy1-YFP-16* reporter mice to screen for individual damaged axons across the brain and brainstem. YFP-filled

enlargements form at sites of damage that result in swelling and formation of varicosities along axons. The localization of YFP in axon swellings is a sensitive indicator of axonal injury ranging from small swellings observed early, when damage may still be reversible, through to intensely fluorescent terminal end bulbs of disconnected axons (59; 61). Our previous work in *Thy1-YFP-16* mice demonstrated YFP detection of traumatic axonal injury in white and gray matter regions of the brain at 3 days after single or repetitive concussive TBI (97; 188). Importantly, in comparison with β -APP immunohistochemistry used for standard neuropathology, confocal analysis in *Thy1-YFP-16* mice can visualize YFP in 3-dimensions to identify terminal end bulbs at sites where axon damage has progressed to disconnection (180).

Larger axons in white matter are wrapped by myelin sheaths that increase the efficiency and speed of action potential conduction. Myelin can be damaged by direct insults or as a secondary effect of axon damage. New myelin can be generated during activity-dependent adult plasticity and can form after injury or disease through regeneration and replacement of lost myelin sheaths (9; 104; 109; 124; 145). *NG2CreER;mTmG* transgenic fluorescent reporter mice enable *in vivo* analysis of newly formed myelin sheaths over a specific time interval (97; 133). The *NG2* promoter is expressed in oligodendrocyte progenitor cells (OPCs) that proliferate and differentiate into myelin-forming oligodendrocytes. Tamoxifen (TMX) administration activates *Cre recombinase* that switches membrane fluorescent labeling from red to green in OPCs and their progeny. TMX given to *NG2CreER;mTmG* mice on days 2 and 3 after TBI results in green fluorescent protein (GFP) labeling of newly generated oligodendrocytes and their myelin sheaths (97). We previously showed that a single concussive TBI increased

formation of new myelin in the CC through 4 weeks, yet CC atrophy was evident when survival was extended to 8 weeks after TBI (97). We will now leverage the advantages of the *NG2CreER;mTmG* mice to examine new myelin formation over 4 and 8 weeks after repetitive low-level blast exposure.

The effect of blast waves on the brains of *Thy1-YFP-I6* and *NG2CreER;mTmG* mice was tested using an Advanced Blast Simulator (ABS) to model the blast wave component of low level exposures to explosives or heavy artillery relative to potential effects of repetitive blast exposures during deployment or training. Studies of service members have shown that experiencing three or more TBI events, with or without blast exposure, may increase effects compared to those with fewer or no TBI history (29; 122). In mice, our prior studies of concussive mild repetitive TBI, with impacts once per day on each of five consecutive days, resulted in persistent neuroinflammation in the CC and adjacent cerebral cortex (188). Therefore, the current studies in transgenic reporter lines used repetitive low-level blast exposures given once per day on each of five consecutive days for analysis of axon damage, myelination, and neuroinflammation.

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 as 2-4 mice per cage in 35cm x 16.5cm x 18cm cages that contained enrichment objects and maintaining a 12h daytime light cycle (0600 – 1800) during which all experimental procedures were performed. The

following mouse strains were obtained from the Jackson Laboratory (Bar Harbor, ME): *NG2CreER* (RRID: IMSR_JAX:008538; B6.Cg-Tg(Cspg4-cre/Esr1*)BAkik/J), *ROSA^{mTmG}* (RRID:IMSR_JAX:007676; B6.129(Cg)-Gt(*ROSA*)26Sortm4(*ACTB-tdTomato,-EGFP*)Luo/J), and *Thy1-YFP-16* (RRID:IMSR_JAX:003709 ; B6.Cg-Tg(Thy1-YFP)16Jrs/J). Mouse strains were bred as in-house colonies to generate experimental mice. First generation offspring from *NG2CreER* and *ROSA^{mTmG}* breeders were used as *NG2CreER;mTmG* mice. The total number of mice used was *Thy1-YFP-16* (n = 15; 8 male; 7 female) and *NG2CreER;mTmG* (n = 14; 10 male; 4 female). Male and female mice were combined for analyses after separate analyses showed no sex-based differences. Littermates of *Thy1-YFP-16* mice or *NG2CreER;mTmG* mice were used for quantitative analyses. The number of mice for each condition in each experiment is provided in each figure legend.

Repetitive Blast Procedures

At 8-9 weeks of age, mice were randomly assigned to repetitive sham (rSham) or repetitive blast (rBlast) procedures. The rBlast mice were exposed to five consecutive simulated blast exposures, at 24 hour intervals, delivered in an Advanced Blast Simulator (ABS) (ORA, Inc., Fredericksburg, VA). ABS procedures were performed with mice in the prone position for frontal blast exposure. Each mouse was anesthetized with 3% isoflurane and the head and body was secured to a wooden tongue depressor, wrapped in gauze, placed in mesh to prevent rotational movement, and positioned to face the oncoming blast as previously detailed (73; 175). The rSham mice were anesthetized and followed the same procedures except that the blast was not deployed. There was no significant difference in mean peak incident overpressure (21.7 ± 1.11 psi, ~ 149.62 kPa),

mean duration ($7.25 \pm 0.235\text{ms}$) or mean impulse ($62.9 \pm 0.795\text{ psi-ms}$) across the five blast exposures for any groups. Righting reflex time was significantly prolonged in rBlast mice (mean = 78.55s) compared rSham mice (mean = 45.8s) (Two way ANOVA; $F(1,28) = 206.6$; $p > 0.0001$). Righting reflex time did not change significantly across the five days of rBlast or rSham procedures for either genotype or injury condition.

***Thy1-YFP-16* Mice - Analysis of Axon Damage**

At 3 days following the final rBlast or rSham procedure, *Thy1-YFP-16* mice were perfused with 4% paraformaldehyde and brains cut as 14 μm -thick cryosections (97). For qualitative analysis of the distribution of axon damage, sagittal sections of *Thy1-YFP-16* mice were imaged using a Zeiss Axioscan Z.1 (Carl Zeiss) with a Plan-Apochromat 20x objective to image the entire brain section, from olfactory bulb to brainstem. To examine the 3-dimensional structure of YFP-filled axons, confocal image stacks were acquired on a 700 laser scanning confocal microscope (Carl Zeiss) with a Plan-Apochromat 40x/1.4 oil or 63x/1.4 oil objective and processed using Vision4D Modular Software (Arivis Vision4D, Phoenix, AZ; RRID:SCR_018000).

Quantification of axonal varicosities focused on regions-of-interest (ROIs) in the CC and cingulum in coronal sections at levels between +0.5 and -0.5 mm relative to bregma. The CC ROI extended from the midline bilaterally to the turning point of ventral descent in the external capsule. The cingulum ROI was evident as fibers oriented perpendicular to the underlying CC and distinct from the superior cortical tissue. Images acquired on an Olympus IX-70 fluorescence microscope with a SPOT RT3 camera (Diagnostic Instruments, Arnold, MD) were analyzed using Image J software (National Institutes of Health, Bethesda, MD; RRID: SCR_003070). The ROI area was measured

with a 10x objective. Morphological measurements of YFP-filled swellings used images acquired with a 40x objective. The width of each axonal varicosity was measured in the plane transverse to the length of the axon. The width of CC axons is $< 1.5 \mu\text{m}$ as measured by electron microscopy (105; 160). Pilot analyses in rSham and rBlast mice confirmed that axon diameter varied along normal appearing axons but ranged from 0.5-1.5 μm . Therefore, quantification of axon damage included only those YFP-filled swellings of $> 2.5 \mu\text{m}$ in width with peak fluorescent value (measured as max pixel intensity) of at least 25% greater than adjacent axons, which was above the 95% confidence interval of the YFP fluorescence intensity of adjacent normal appearing axons. Cell bodies were distinguished using 4',6-diamidino-2-phenylindole (DAPI) nuclear staining. Axonal varicosities included YFP-filled swellings along an axon and terminal end bulbs, i.e. swellings at the end of an axon segment. Analysis included 6-8 sections per mouse.

Thy1-YFP-16 mice – Analysis of Neuroinflammation

Mice perfused at 3 days following final rBlast or rSham procedures were further analyzed using immunohistochemistry in coronal sections at levels between +0.5 and -0.5 mm relative to bregma. The CC ROI extended from the midline bilaterally to under the peak of the cingulum (97). Astrocytes were evaluated by immunostaining for glial fibrillary acidic protein (GFAP; polyclonal rabbit anti-GFAP; 1:1000; DAKO, Carpinteria, CA; Agilent Cat# N1506, RRID:AB_10013482). 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). All tissue sections were counterstained with DAPI nuclear stain (Sigma-Aldrich. St.

Louis, MO; D9542). For quantification, images within the CC ROI were acquired with a 10x objective on an Olympus IX-70 microscope using a SPOT RT3 camera. ImageJ software was used to threshold fluorescence levels to quantify the area of immunolabeling above background (9). Images were also acquired with a 40x objective to examine the morphology of IBA1 immunolabeled cells to compare the density of resting or activated cells (105; 162; 188). Analysis included 6-8 sections per mouse.

***NG2CreER;mTmG* Mice - Analysis of Myelin**

Cohorts of *NG2CreER;mTmG* littermates received rBlast or rSham procedures. TMX (10 mg in 20 mg/ml corn oil suspension; Millipore, Sigma-Aldrich; T5648) was administered via oral gavage on day 2 and 3 after the final blast or sham procedure (97; 106). TMX induces *Cre recombinase* mediated deletion of a stop codon switches transcription from constitutive expression of membrane-localized tdTomato (mT) to NG2 driven expression of membrane-localized GFP (mG). Fluorescence was quantified in coronal sections at levels between +0.5 and -0.5 mm relative to bregma. The CC ROI extended from the midline bilaterally to under the peak of the cingulum (97). Images were acquired on an Olympus IX-70 microscope using a SPOT RT3 camera. ImageJ software was used to threshold the signal to above background and quantify the area of membrane fluorescence from the CC ROI, as previously detailed for CC mG labeling and for immunolabeling of myelin (9; 97). Confocal images were acquired with a 40x objective to examine the morphology of membranes labeled with mG and mT. Additional tissue sections were immunostained for total myelin based on myelin oligodendrocyte glycoprotein (MOG, mouse monoclonal, 1:100; EMD Millipore, Burlington, MA; mab5680, RRID:AB_1587278).

Statistical Analysis

Mice were allocated to the rBlast or rSham procedures using the random number generator function in Microsoft Excel. Investigators were blinded to animal injury condition during tissue analysis until after completion of the data analysis. GraphPad Prism 8.0 software (RRID:SCR_002798) was used for statistical analysis and graphing. Bar graphs demonstrate means with standard error of the means as well as symbols for individual mouse values. Two-way ANOVA with repeated measures, for CC and cingulum within same subjects, and Sidak's posthoc multiple comparisons test was used for axonal varicosity comparisons. An unpaired Student's *t*-test was used to compare immunolabeling of astrocytes and microglia between groups. Two-way ANOVA with Sidak's posthoc multiple comparisons test was used at each time point for comparing mG fluorescence and CC width between rSham and rBlast in *NG2CreER;mTmG* mice. Statistical significance was set at values of $p < 0.05$.

RESULTS

Screening in *Thy1-YFP-16* fluorescent reporter mice detects damaged axons following repetitive blast exposure.

The effects of repetitive blast exposures on axons were examined in *Thy1-YFP-16* fluorescent reporter mice that express YFP in diverse neuronal populations but in only a subset of those neurons, which allows visualization of individual axons (Figure 1). Confocal microscopy identified YFP-filled axonal swellings, which can detect axonal injury ranging from early reversible damage through terminal axon disconnection (59; 61). As an initial qualitative assessment, tissues were screened in parasagittal sections of the brain and brainstem at 3 days after the last of five repetitive blast exposures (rBlast;

Figure 1A-E) that were delivered at 24-hour intervals, or after matched procedures (rSham; Figure 1B'-E') without deploying the blast. YFP-filled axonal swellings were observed in rBlast mice in diverse brain regions, including the cerebellum (Figure 1B), pons (Figure 1C), fornix (Figure 1D), and CC (Figure 1E). Overall, axons with YFP-filled swellings were found diffusely distributed in the brain of rBlast mice.

Repetitive blast exposure induces acute axon damage in white matter tracts

Further analysis of blast exposure in *Thy1-YFP-16* mice focused on the CC and overlying cingulum, which had readily detected YFP-filled axonal varicosities in the rBlast mice (Figure 1) and are clinically relevant areas of damage identified in human blast patients (72; 148). These long white matter tracts also run perpendicular to one another, which is of interest relative to screening for broad effects of blast waves across brain regions. YFP-filled axonal varicosities were visualized at high resolution using confocal microscopy of coronal tissue sections through the CC and cingulum at 3 days after the final rBlast or rSham procedure (Figure 2). The rSham mice show YFP signal distributed within axons running longitudinally in the coronal plane or within interspersed crossing fibers (Figure 2A). The rBlast mice exhibited distributed YFP signal in most axons but also rare axons with large accumulations of YFP (Figure 2B). Three-dimensional reconstruction of confocal image stacks identified distinctly large axonal swellings to be terminal end bulbs. YFP accumulations were more frequent and often larger in rBlast mice relative to rSham mice (Figure 2C-E).

Repetitive blast exposure induces neuroinflammation in the corpus callosum

To determine whether a neuroinflammatory response accompanied blast-induced axon pathology, coronal sections were immunolabeled for GFAP as a marker for

astrocytes and IBA1 for microglia/macrophages in *Thy1-YFP-16* mice at 3 days following final rBlast or rSham procedure (Figure 3). The astrocytes and microglia have fine processes in the CC of rSham mice (Figure 3A-B). Following rBlast exposure, reactive astrocytes and activated microglia exhibited shorter, thicker processes with increased immunoreactivity, indicative of a mild innate immune response (Figure 3C-D). Quantitative analysis demonstrated a significant increase of immunoreactivity for GFAP and IBA1 in rBlast mice when compared to rSham (Figure 3E-F). Furthermore, rBlast exposure increased the density of IBA1 immunolabeled cells (Figure 3G-I). These results provide evidence that rBlast exposures produce an acute neuroinflammatory response in the same tissue regions that exhibit axon damage.

Screening new myelin formation in *NG2CreER;mTmG* reporter mice following repetitive blast exposure

In addition to axon damage and neuroinflammation, white matter microstructural effects of blast detected by MRI could also involve myelin membranes that ensheath axons (102; 130; 157). We previously showed that *NG2CreER;mTmG* myelin reporter mice revealed an increase of new myelin synthesis in the CC over a 4-week period after single impact TBI, possibly indicating replacement of damaged myelin sheaths (97). Therefore, *NG2CreER;mTmG* mice were used to quantify new myelin synthesis following rBlast exposure (Figure 4). TMX was administered on days 2 and 3 after the final blast or sham procedure, which corresponds with the time point for analysis of axon damage (Figures 1-3). Fluorescent green mG labeling identified new membranes synthesized after TMX induction. Clusters of green myelin membranes were distributed in the CC at 4 weeks after the final rBlast/rSham procedure (Figure 4A-B) with further myelin formation by 8 weeks (Figure 4C-D). These clusters are as expected with genetic

fate-labeling of NG2 expressing progenitors that generate new oligodendrocytes, which each form myelin sheaths around many axons. Higher magnification shows green myelin aligned along axons (Figure 4E). Green membranes extending along blood vessels are also occasionally observed due to NG2 expression in pericytes (Figure 4F).

Immunolabeling for MOG to detect myelin shows the presence of persisting myelin as well as newly synthesized mG labeled membranes (Figure 4G,H). Quantification of mG membrane labeling in rSham mice shows substantial formation of new myelin membranes continues in adulthood (Figure 4I). This findings is in agreement with studies that show CC myelination continues well beyond 16 weeks of age (160). which is the age match of 8 weeks post-blast. rBlast exposure did not change the extent of new myelin membrane synthesis in the CC after either 4 or 8 weeks (Figure 4I). Furthermore, in our prior study using *NG2CreER;mTmG* mice, single impact TBI resulted in reduced CC width at 8 weeks (97), which was not found after rBlast exposure (Figure 4J). Finally, rBlast exposures does not produce significant loss of CC myelin, as detected by MOG immunolabeling (Figure 4K).

DISCUSSION

The ability to detect subtle and distributed pathological features is vital to determine the effects of blast exposure on the brain. This study utilized two distinct fluorescent reporter mouse lines to assess components of potential axon and myelin white matter injury after rBlast exposures. The results demonstrate that rBlast exposure produces acute axon damage in multiple white matter tracts of the brain, as detected by screening in *Thy1-YFP-16* mice. Acute axon damage was accompanied by an innate

immune response of mildly reactive astrocytes and microglia. However, this white matter pathology did not lead to later effects on myelin synthesis, an indicator of myelin repair and remodeling, which was tracked *in vivo* after blast exposure using *NG2CreER;mTmG* mice.

To model the blast wave component of low level exposures to explosives or heavy artillery, the current experiments used an ABS device to generate controlled high-fidelity simulation of both positive and negative phases of “free-field” blast (11; 66; 137; 146; 175). Studies using single blast exposures in the ABS in adult rats have produced long term behavioral effects.(11) Similar ABS exposures can alter the cerebral vasculature and the blood-brain barrier (66; 137). Blast exposure can also alter cellular pathology including expression of markers for gliosis and myelinated axons (CNPase and neurofilament H), but appears to vary with the degree of head immobilization (146). Indeed, single ABS exposure at 15 psi using a mesh to position the mouse in the prone position and minimize head movement resulted in a low level blast frontal exposure that did not induce detectable microglial activation in the CC (175). The current studies used this same mesh procedure to similarly secure and position the mouse in the ABS. Our prior studies of concussive mild TBI in mice demonstrated persistent neuroinflammation in the CC and adjacent cortex after one impact per day on five consecutive days (188). With repetitive exposure pattern and increasing the ABS exposure to approximately 20 psi, we find a significant but low level neuroinflammatory response in both astrocytes and microglia (Figure 3). This CC neuroinflammation indicates a repetitive blast effect that warrants investigation of axon and myelin pathology.

Thy1-YFP mice facilitated detection of axonal varicosities, including terminal end bulbs, which are significantly increased at 3 days after the final ABS exposure in the CC and cingulum (Figures 1, 2). Previous studies in rodent models of blast exposure found electrophysiological deficits and axon damage within white matter tracts along with behavioral deficits (128; 186). Axon damage after blast exposure appears to be subtle and not readily detected by immunohistochemistry (43; 82; 113). The current YFP approach revealed individual damaged axons in mice even when exposed to a relatively low mean peak incident overpressure of approximately 20 psi (Figure 2).

Our findings of acute neuroinflammation and axon damage in the CC led us to further examine myelination as an indicator of longer term white matter injury after rBlast that is consistent with neuroimaging DTI findings in patients who have been experienced blasts. Myelin has been shown to be disrupted in TBI and myelin repair may have profound implications for recovery of axon function (10). Studies with open field explosive blasts reported CC pathology with increased silver staining of damaged axons and ultrastructural evidence of myelin disruption (157). However, this electron microscopic technique to evaluate myelin can only sample small regions. Additionally, the ability to monitor the dynamic turnover of myelin membranes is important to capture in evaluating potential myelin repair. In the current study, the use of *NG2CreER;mTmG* mice enabled quantification of *in vivo* myelin synthesis during specific intervals after blast exposures. During these periods, rBlast exposure did not produce late phase effects on synthesis of new myelin membranes or induce CC atrophy (Figure 4 G, H). This result is in agreement with an overall mild level of white matter injury based on the limited degree of neuroinflammation and rare axon damage in the CC of rBlast mice. Possibly,

TMX on days 2 and 3 could lessen blast pathology at 8 weeks. However, at 8 weeks after a concussive TBI reduced CC width was found in two separate mouse studies in which one did not involve TMX and the other administered TMX using the current protocol (96; 97).

The advantages of mouse models to leverage the genetic labeling of cellular components are balanced by the limitations of small size and lack of complexity of the mouse brain. Mouse models are most useful as tools to screen for pathology that may be otherwise hard to distinguish. These findings in mice should facilitate further development of techniques applicable to higher mammals and humans, and interpretation of clinically applicable techniques such as white matter microstructure effects identified by MRI DTI. The devices available to model blast exposures increase reproducibility by reducing experimental variables and so do not fully replicate the human experience. In the broadest sense, the blast field overall is limited by the need for further studies to more fully establish the human neuropathology of TBI associated with blast exposure.

CONCLUSIONS

Evaluating effects of low-level repetitive blast exposure is challenging due to potentially subtle and diffuse pathology involving multiple cell types and tissues. *Thy1-YFP-16* mice detected axon damage in white matter tracts across multiple brain regions. *NG2CreER;mTmG* mice detected new myelin synthesis and provided the ability to visualize the neurovascular-pericyte unit. Taken together, this study demonstrates that fluorescent reporter mice are effective tools for evaluating cellular pathology of blast-

induced neurotrauma and that repetitive low-level blast exposures produce infrequent but significant axon damage along with neuroinflammation in white matter.

ACKNOWLEDGEMENTS

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Figure 1.

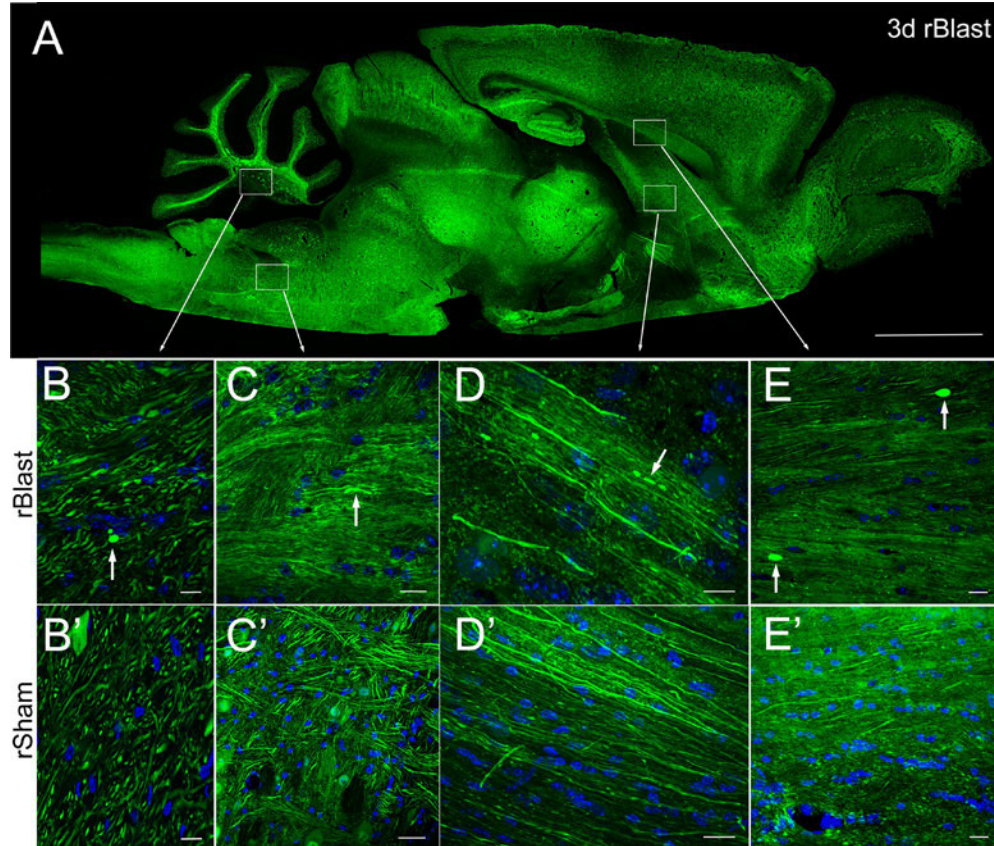


Figure 1. Broad, sensitive screening for axon damage in brain sections after repetitive blast exposure using *Thy1-YFP-16* mice.

A: Sagittal section from a *Thy1-YFP-16* mouse at 3 days after rBlast exposure shows widely distributed neuron cell bodies and processes expressing yellow fluorescent protein (YFP; shown in green). **B-D':** Sagittal high magnification confocal microscope images of axons in white matter tracts in distributed brain regions of rBlast mice (B, C, D) and matching regions in rSham mice (B', C', D'). YFP shown in green. DAPI nuclear stain shown in blue. Example of YFP accumulated in damaged axon to illustrate a terminal end bulb (arrow; transverse view) in the cerebellum of an rBlast mouse (B). Note that other YFP-filled oval and elongated structures are axons shown in angled cuts in both rBlast (B) and rSham (B') mice. The intense YFP of damaged axons is distinct from larger, more diffuse labeling in neuron cell bodies (adjacent to B' label). Examples from rBlast mice of YFP in an axonal thickening (arrow; longitudinal view) in the pons region of the brain stem (C) and in fragmented axons (arrows; longitudinal view) in the column of the fornix (D). **E,E':** Coronal confocal image showing corpus callosum axons in longitudinal view with two examples of intense YFP labeling in large terminal end bulbs in an rBlast section (E arrows), in comparison with an rSham section (E'). Low power image (A) is representative of images from three *Thy1-YFP-16* mice examined in full sagittal sections. Higher power images (B-E') were acquired from sections of these or additional *Thy1-YFP-16* mice examined in coronal sections for quantification in Figure 2. Scale bars = 2 mm (A), 20 μ m (B-E).

Figure 2

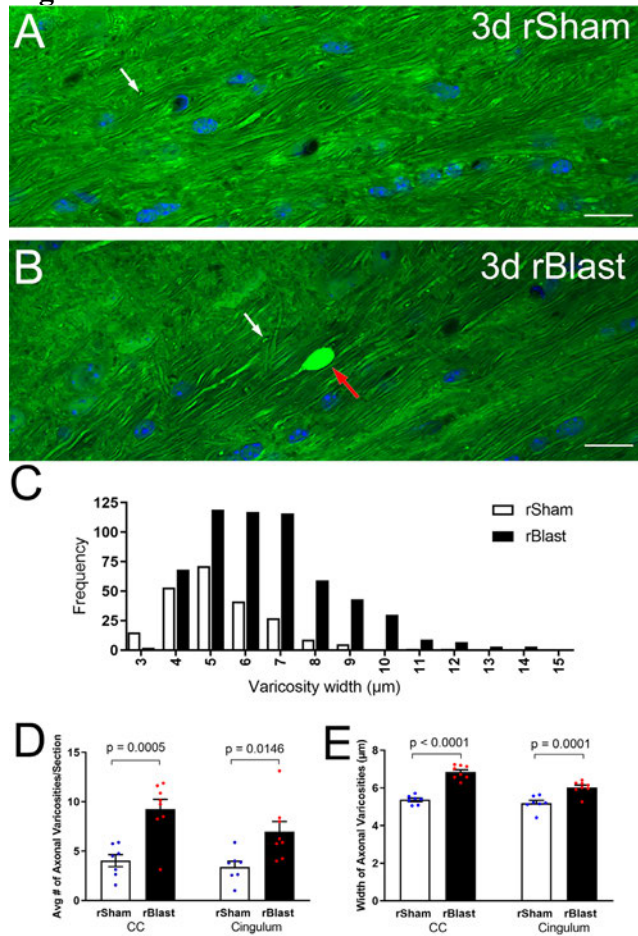


Figure 2. Repetitive blast exposure increases axon damage in the corpus callosum (CC) and adjacent cingulum.

A-B: Confocal images of coronal sections of the CC from *Thy1-YFP-16* mice at 3 days after repetitive sham procedures (rSham; A) or repetitive blast exposure (rBlast; B) showing high magnification of yellow fluorescent protein signal (YFP) within axons and DAPI nuclear stain shown in blue. The rSham mice exhibit relatively uniform YFP signal filling of axons viewed longitudinally (A). There are small variations in diameter along the length of the axons. In addition, YFP signal is visible in axons of crossing fibers cut transversely (A; white arrow). An example in an rBlast mouse of an axon with a large, intensely fluorescent YFP-filled enlargement indicative of axon damage (B; red arrow) that is distinct from crossing fiber axons (B; white arrow). **C:** Analysis of the frequency distribution indicates an increase in both the number and diameter of YFP accumulations in the CC and cingulum of rBlast mice as compared to rSham controls. **D:** The rBlast significantly increases the incidence of damaged axons with abnormal YFP localization (thickenings, varicosities, and terminal end bulbs) in the CC and cingulum, as compared to rSham mice. **E:** YFP accumulations in axonal varicosities are significantly larger in rBlast compared to rSham mice (B). Values are mean $\pm$ s.e.m. Two-way ANOVA with repeated measures, for CC and cingulum within same subjects, and Sidak's posthoc multiple comparisons test. rSham (n = 7) and rBlast (n = 8). Scale bars = 20 μ m (A and B).

Figure 3.

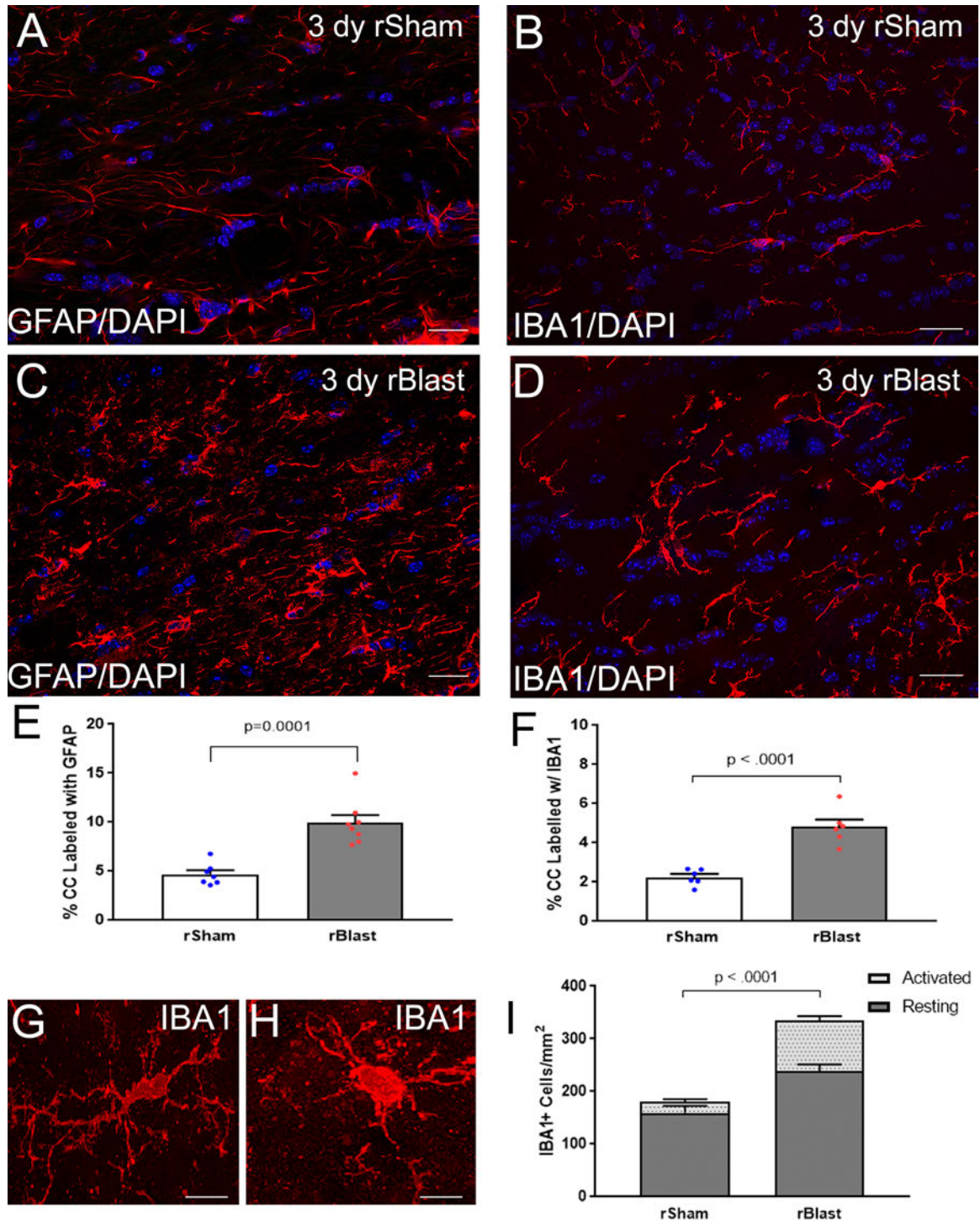


Figure 3. Neuroinflammation is significantly increased in the corpus callosum (CC) after blast exposure.

A-D: Immunohistochemistry to detect GFAP in astrocytes and IBA1 as a marker of microglia/macrophages. Confocal microscope images of coronal CC sections from *Thy1-YFP-16* mice at 3 days after repetitive sham procedures (rSham; A, B) or repetitive blast exposure (rBlast; C, D). Astrocytes (pseudocolored white) and microglia/macrophages (red) have thicker processes with more intense immunoreactivity in rBlast mice as compared to rShams. DAPI nuclear stain shown in blue. **E-F:** The rBlast exposure significantly increased immunoreactivity for GFAP (E) and IBA1 (F). **G-H:** Representative confocal images showing IBA1+ cells as examples of resting state morphology with an elongated cell body and numerous processes (G) or activated morphology with a more rounded and intensely immunolabeled cell body and processes (H). **I:** rBlast exposure significantly increases the number of IBA1+ cells in the CC. Values are mean $\pm$ s.e.m. Student's t-test to compare between groups. GFAP groups (rSham n = 7; rBlast n = 8). IBA1 groups (rSham n = 6; rBlast n = 7). Scale bars = 20 μ m (A-D) and 5 μ m (G, H).

Figure 4.

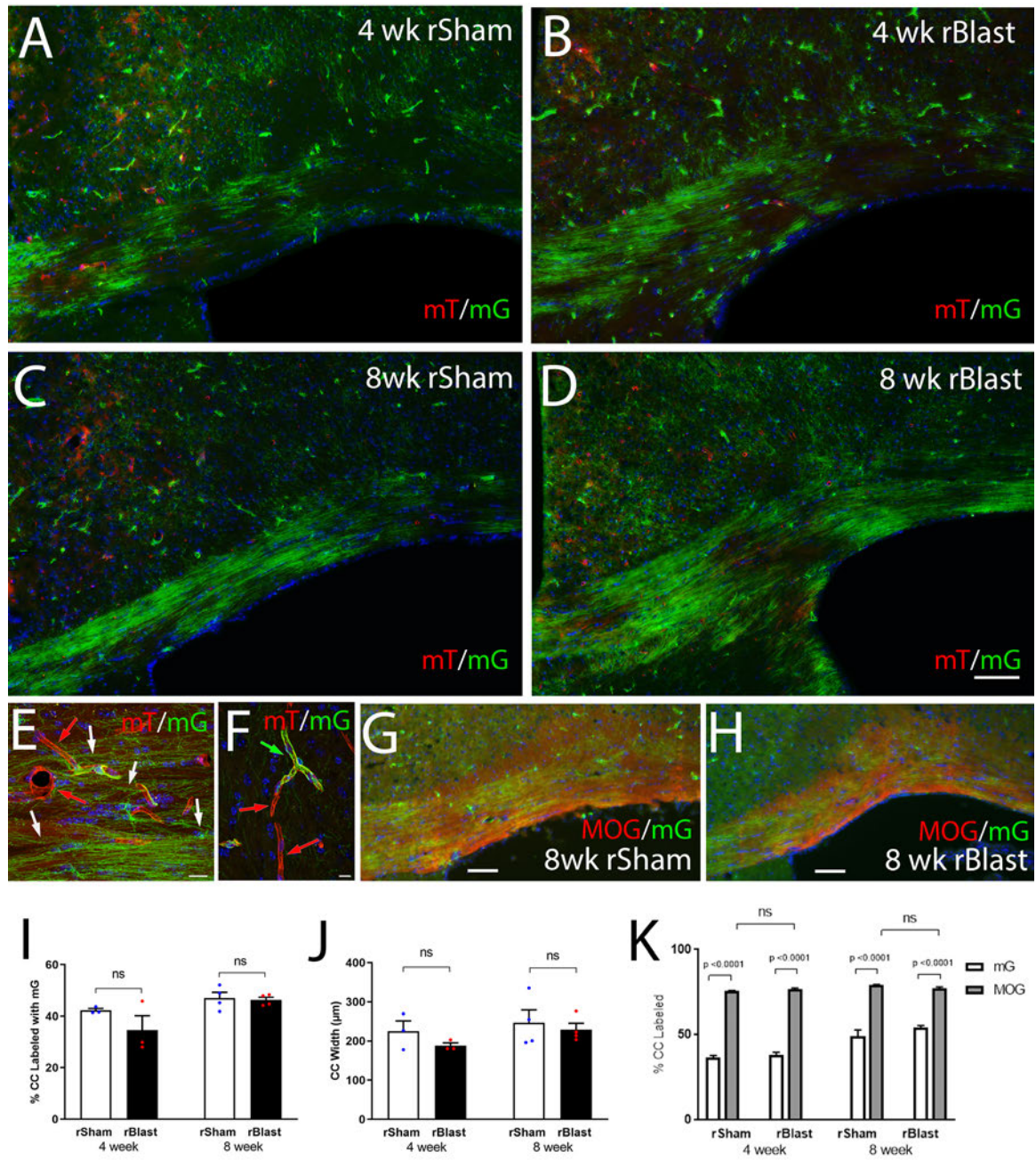


Figure 4. Myelin membrane synthesis in the corpus callosum (CC) after repetitive blast exposure in *NG2CreER;mTmG* reporter mice.

NG2CreER;mTmG mice were given tamoxifen on days 2–3 after repetitive blast (rBlast) or sham (rSham) procedures. Tamoxifen induces recombination to stop expression of membrane-localized td-Tomato (mT) and initiate expression of membrane-localized GFP (mG) driven from the NG2 promoter (NG2mG). NG2 expression drives genetic mG fate-labeling of oligodendrocyte progenitors that is maintained in newly generated oligodendrocytes and their myelin membranes. **A–D**: Representative images of mG (green) labeled membranes in coronal sections through the corpus callosum (CC) at 4 weeks (A, B) or 8 weeks (C, D) after rSham (A, C) or rBlast (B, D). **E–F**: High magnification confocal microscope images within the CC (E) and cortex (F). Constitutive mT (red) labeling is visible in membranes of non-recombined cells, including blood vessels (E, red arrows). NG2 fate-labeled oligodendrocytes extend myelin-like membranes (E, white arrows) along axons. NG2 fate-labeled pericyte (F, green arrow) extending processes around blood vessels (F, red arrows). DAPI nuclear stain shown in blue. **G–H**: Representative images of mG fluorescence (green) in newly synthesized myelin membranes along with immunolabeling for MOG in new and persisting myelin membranes (red). The mG membranes represents only a subset of the total myelinated area of the CC. **I**: rBlast did not significantly alter the proportion of mG labeled myelin membranes synthesized in the CC at either time point. **J**: rBlast exposure did not result in significant CC atrophy at either time point. **K**: Quantification of mG labeling relative to total MOG immunolabeled myelin in the same sections. The myelinated fraction in the CC is significantly greater than the mG new myelin and is not changed by rBlast exposure. Values are mean $\pm$ s.e.m. Two way ANOVA with Sidak's posthoc multiple comparisons test. Four week groups (rSham n = 3; rBlast n = 3). Eight week groups (rSham n = 4; rBlast n = 4). Scale bars = 100 μ m (A–D, shown in D), 20 μ m (E–H).

CHAPTER 3:

Genetic inactivation of SARM1 axon degeneration pathway improves outcome trajectory after experimental traumatic brain injury based on pathological, radiological, and functional measures

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K.L.R., D.V.B, and R.C.A. designed neuropathological analysis of tissue sections

D.V.B performed TBI surgical procedures

D.V.B., X.Z. and D.P.M performed neuropathological procedures

D.V.B. and R.C.A. performed neuropathological analyses

R.C.A, A.K.K, and A.K., B.J.D. and D.V.B. designed longitudinal MRI studies

A.K. and D.V.B. performed MRI studies

A.K.K. performed image processing for MRI studies

D.V.B., A.K.K., A.K. and R.C.A. analyzed MRI data

D.V.B. and G.M.S. performed behavioral studies

D.V.B., R.C.A, and G.M.S. designed and analyzed behavioral studies

R.C.A. secured funding for project

D.V.B. and R.C.A wrote the manuscript

All authors edited and approved the manuscript

ABSTRACT

Traumatic brain injury (TBI) causes chronic symptoms and increased risk of neurodegeneration. Axons in white matter tracts, such as the corpus callosum (CC), are critical components of neural circuits and particularly vulnerable to TBI. Treatments are needed to protect axons from traumatic injury and mitigate post-traumatic neurodegeneration. The *Sarm1* gene is a central driver of axon degeneration through a conserved molecular pathway. *Sarm1*^{-/-} mice with knockout (KO) of the *Sarm1* gene enable genetic proof-of-concept testing of *Sarm1* inactivation as a therapeutic target. We evaluated *Sarm1* deletion effects after TBI using a concussive model that causes traumatic axonal injury and progresses to CC atrophy at 10 weeks, indicating post-traumatic neurodegeneration. *Sarm1* wild-type (WT) mice developed significant CC atrophy that was reduced in *Sarm1* KO mice. Using electron microscopy to quantify individual axons demonstrated that *Sarm1* KO preserved more intact axons and reduced damaged or demyelinated axons. MRI in live mice identified significantly reduced CC volume after TBI in *Sarm1* WT mice that was attenuated in *Sarm1* KO mice. MR diffusion tensor imaging detected reduced fractional anisotropy in both genotypes while axial diffusivity remained higher in *Sarm1* KO mice. Immunohistochemistry revealed significant attenuation of CC atrophy, myelin loss, and neuroinflammation in *Sarm1* KO mice after TBI. Functionally, TBI resulted in late-stage motor learning and sleep deficits that were ameliorated in *Sarm1* KO mice. Based on these findings, *Sarm1* inactivation can protect axons and white matter tracts to improve translational outcomes associated with CC atrophy and post-traumatic neurodegeneration.

Keywords: Chronic traumatic brain injury / SARM1; Sterile alpha and Toll/interleukin-1 receptor containing motif 1 / white matter / corpus callosum atrophy / magnetic resonance imaging

INTRODUCTION

Traumatic brain injury (TBI) results in long term disability in more severe cases and can cause persistent symptoms even in patients who receive a “mild” diagnosis (78; 117; 135). TBI may also lead to post-traumatic neurodegeneration and increase the risk for co-morbid neurodegenerative diseases, such as Alzheimer’s disease (27; 37; 58; 138). In patients with moderate-severe TBI, diffuse axonal injury has been shown to predict the extent of post-traumatic neurodegeneration, based on MRI volumetric and diffusion tensor imaging (DTI) data (56). The strongest relationship was found in central WM tracts, including the corpus callosum (CC). The CC is one of the main structures exhibiting atrophy across patients with complicated mild to severe TBI (27; 58). Furthermore, DTI tractography demonstrated disrupted fiber tract continuity in anterior CC regions after concussions while broad areas of disrupted tracts were found throughout the CC in patients with MRI findings of diffuse axonal injury (74). *Ex vivo* MRI of a patient who died 26 days after TBI demonstrated that white matter tract disruptions detected by DTI correlated with neuropathological identification of axonal injury (119).

Long axons in white matter tracts are particularly vulnerable to damage in all forms of closed head TBI (15; 76; 100). At an early stage of damage, axons can recover; once mechanical or molecular processes fragment the axon, then the distal axon irreversibly degrades by Wallerian degeneration (61; 71; 98; 99; 118; 183). The majority of axon damage is due to secondary mechanisms of damage that can cause axons to initiate Wallerian degeneration for weeks or more after the initial TBI event (18; 98; 99). Irreversible axonal injury leads to disconnection between brain regions and disruption of neural circuits that may contribute to diverse TBI symptoms (63). To improve outcomes

for patients after TBI, research is needed to identify approaches to protect against axon degeneration and, further, to determine whether acute axon protection can reduce post-traumatic neurodegeneration.

Wallerian degeneration is an “active program of axon self-destruction” (147). After injury or mitochondrial dysfunction, the SARM1 (sterile alpha and Toll/interleukin-1 receptor motif-containing 1) protein executes a highly conserved molecular axon death pathway (49; 88; 126; 163). Injury releases SARM1 from an auto-inhibited state that is found in healthy axons (42; 147; 158). Active SARM1 is a glycohydrolase which depletes nicotinamide adenine dinucleotide (NAD⁺) that is critical for energy stores in axons (36; 44; 48). Loss of sufficient NAD⁺ leads to a cascade of axon destruction in which ionic imbalance leads to cytoskeletal and structural breakdown within the axon (46; 48; 164).

Deletion of the *Sarm1* gene provides a genetic “proof-of-concept” to examine the effects of long-term inactivation of this axon death pathway in chronic TBI and to evaluate the relationship of axonal injury to post-traumatic neurodegeneration. Knockout of *Sarm1* in cultured human sensory neurons and in live mice reduced axon damage in trauma and in chemotherapy-induced models of peripheral neuropathy (21; 45; 48; 126). In multiple models of TBI, *Sarm1* knockout (KO) mice exhibited significantly reduced axon damage at acute and late phase time points (65; 96; 101; 190). Long-term studies of TBI in mice can model post-traumatic neurodegeneration that includes atrophy of the CC (86; 97; 112). Initial results from our prior studies provided the first evidence that *Sarm1* deletion may reduce both late stage axon damage and CC atrophy (96).

We now focus on CC atrophy as an important outcome measure of post-traumatic neurodegeneration. We evaluate the effect of *Sarm1* deletion on axon damage, demyelination, and neuroinflammation on the progression of CC atrophy after closed head TBI. We use longitudinal MRI to detect changes in CC volume and white matter integrity in live mice, which provides a highly translational outcome measure of prolonged *Sarm1* inactivation. To demonstrate whether the pathological effects of *Sarm1* deletion have a meaningful impact on complex functions, we assess motor skill learning and sleep behavior. This experimental design provides a rigorous and translationally relevant screen of *Sarm1* genetic inactivation as a potential therapeutic strategy for improving outcome measures after TBI.

MATERIALS AND 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 h cycle of daytime light (6:00–18:00). *Sarm1*^{-/-} knockout (KO) mice, B6.129 × 1-Sarm1^{tm1Aidi}/J (RRID:IMSR_JAX:018069) were originally obtained from The Jackson Laboratory. *Sarm1* KO mice were crossed to C57BL/6J mice and then heterozygotes were bred to generate littermates for experiments. *Sarm1* is highly expressed in the brain; lack of detectable SARM1 protein in *Sarm1* KO mice does not

produce gross or microscopic brain pathology (52; 80). The colony was maintained at Charles River Laboratories (Wilmington, MA) where ear biopsies were genotyped using a 3-primer allele specific PCR assay that targets the mutated *Sarm1* region. Experimental mice were acclimated for 3 days after shipment and prior to the start of experiments. The total number of mice used in experiments was *Sarm1* WT (n = 66; 33 male, 33 female) and *Sarm1* KO (n = 75; 34 male, 41 female) littermates.

TBI and Sham Procedures

The TBI model has been characterized in our previous studies (96; 105). This concussive, closed head injury model results in pathology under the impact site at bregma in the CC and the adjacent cingulum (97; 162). Under 2% isoflurane anesthesia, 8–10 week old mice received a single impact onto the skull at bregma using an ImpactOne stereotaxic impactor (Leica Biosystems, Buffalo Grove, IL) with a 3-mm-diameter tip (velocity set at 4.0 m/s; depth of 1.5 mm; dwell time of 100 ms). Sham mice received the same procedure without the impact. Righting reflex demonstrated a significant injury effect of longer time to righting after TBI, as compared to sham procedures, which did not differ with *Sarm1* genotype or sex (Supplemental Figure S1). The predetermined study design criteria required exclusion of three mice for depressed skull fracture and/or impactor malfunction.

Electron Microscopy

Transmission electron microscopy (EM) was used to analyze axon and myelin subcellular structure, which can reveal a broad range of axon and myelin pathology (97; 105). 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 0.1 M phosphate buffer. After overnight post-fixation, brains were cut into sagittal (40 μ m) sections using a Leica VT-1200 vibrating blade microtome (Leica Biosystems, Buffalo Grove, IL). Parasagittal sections were immersed in 2% osmium tetroxide (OsO₄; Electron Microscopy Sciences; Cat #19100) infiltrated with Spurr epoxy resin (Electron Microscopy Sciences; Cat #14300), flat-embedded and then polymerized at 70 °C for 11 h. Thin sections (~70 nm) were cut on an Ultracut UC6 ultramicrotome (Leica Biosystems). Copper grids containing thin sections were post-stained for 20 min in 2% aqueous uranyl acetate (Electron Microscopy Sciences; Cat #22400) and for 5 min in Reynolds lead citrate (Reynolds, 1963).

Quantification of sagittal CC width and electron microscopy analysis

Cohorts consisted of *Sarm1* WT sham (n = 9; 4 female, 5 male) and TBI (n = 8; 4 female, 4 male) along with *Sarm1* KO sham (n = 7; 3 female, 4 male) and TBI (n = 9; 5 female, 4 male). Mice were sacrificed at 10 weeks after sham or TBI procedure. Resin-embedded 40 μ m sagittal sections approximately 200 μ m lateral to the midline were osmicated to stain myelin and imaged in bright field on an Olympus IX-70 microscope. CC width was measured across the superior to inferior borders in five locations at ~100 μ m intervals across a 0.5 mm rostro-caudal region centered under bregma. This region-of-interest (ROI) consistently exhibits traumatic axonal injury in this TBI model, as evidenced by dispersed damaged axons among adjacent intact axons, and matches the EM analysis performed in our previous work with this *Sarm1* line of mice (96; 97).

Thin sections for EM analysis were then cut from within the CC ROI of the 40 μ m thick sections. The EM grids of sagittal thin sections were reviewed on a JEOL JEM-

1011 transmission electron microscope (JEOL USA Inc., Peabody, MA) and images were acquired using an AMT XR50S-A digital camera (Advanced Microscopy Techniques, Woburn, MA). Images were taken at 5000 $\times$ magnification and 8-10 images per animal were quantified for classification of axon and myelin pathology. For each image, a 17 μm $\times$ 12.5 μm region defined the counting frame, within which >120 axons were quantified. All axons within the counting frame were counted. Axons partially crossing the top and right lines were included while those partially crossing the left and bottom line were excluded. Axons were classified as intact axons, de/unmyelinated axons, axons with abnormal mitochondria, or damaged axons (96; 97; 105). Damaged axons were defined as axons with cytoskeletal disruption or axons with accumulated vesicles and debris. Mitochondria that appeared swollen encompassed > 50% of the area of the axon cross-section and were considered abnormal. De/unmyelinated axons were > 0.3 μm in diameter and lacked detectable compact myelin, but otherwise appeared intact. Axons without myelin and with a diameter < 0.3 μm were excluded as this axon size is typically unmyelinated in the CC of healthy adult mice (160). TBI-induced demyelination was inferred when de/unmyelinated axon counts were significantly greater after TBI, as compared to sham. Myelin outfoldings were identified as myelin extending out from an axon and folding back onto itself to form double layered or redundant myelin (105) but were not significantly induced by TBI at this 10 week time point in mice of either genotype (data not shown). Additional images were taken at 10,000–15,000 $\times$ for illustration of pathological features.

Magnetic resonance imaging (MRI) analysis of CC volume and microstructure

MRI with multi-spin-echo, high resolution T2-weighted (T2-w) and diffusion tensor images (DTI) were acquired to assess the longitudinal effects of *Sarm1* deletion in the CC ROI following TBI. Longitudinal MRI studies were conducted in live mice with repeated scans of the same mouse at baseline (prior to TBI) and at 3 days and 10 weeks post-TBI. Mice were scanned on a 7 tesla Bruker BioSpec 70/20 USR Magnetic Resonance Imaging System with a 660 mT/m, 12 cm diameter gradient coil, 86 mm quadrature TX/RX coil, and Bruker Mouse head 4 channel receive coil array (Bruker BioSpin GmbH, Reinstetten, Germany). Mice were anesthetized in an induction chamber with a mixture of 4% isoflurane in medical air and maintained with 1.5–1.75% isoflurane in medical air delivered by nose cone during the MRI procedures. Respiration rate (range 40–70 BPM, maintained by adjusting isoflurane concentration) and temperature were continuously monitored throughout the experiments. MRI slices were positioned using a sagittal localizer so that coronal slices were oriented perpendicular to the length of the CC axis and each brain was aligned with the midline crossing of the anterior commissure within in the same coronal slice (161; 169; 188).

A whole brain T2 relaxation time map was generated from a two-dimensional rapid acquisition with relaxation enhancement (2D RARE, coronal (64) using the following parameters: TR = 4000 ms, echo time (TE) = 10, 30, 50, 70, 90, 110 ms, echo train length (ETL) = 2, number of averages NA = 4, field of view (FOV) 14 mm × 14 mm, matrix 112 × 112, in-plane resolution 125 μ m × 125 μ m, slice thickness 750 μ m, number of slices NS = 21, no fat suppression, BW 36 kHz, time 14:56 min. Additionally, a high resolution proton density weighted (PD-w) 3D MRI was acquired to measure CC volumetrics using the following parameters: 3D RARE, TR = 2500 ms, TE = 30 ms, ETL

= 8, number of averages NA = 1, FOV 18 mm × 14 mm × 11 mm, matrix 144 × 112 × 88, 125 μ m isotropic resolution, no fat suppression, BW 36kHz, time 55:25 min. DTI data was acquired using the following parameters: 2D 2-shot echo planar imaging (EPI), TR = 3000 ms, TE = 27, NA = 2, 4 B0 and 30 non-collinear diffusion directions, b = 600, 1200 s/mm², δ = 5 ms, Δ = 12 ms, FOV 14 mm × 14 mm, in-plane resolution 175 μ m × 175 μ m, matrix 80 × 80, slice thickness 750 μ m, number of slices NS = 21, fat suppression, BW 300kHz, time 12:48 min.

T2-w images were converted to the NIFTI file format using a custom script in Matlab. A bias field correction was performed on the 30 ms echo time image using the N4BiasFieldCorrection command in the Advanced Normalization Toolkit (ANTs) (12). The computed bias field was then applied to the other echo times (10, 50, 70, 90, 110 ms). Estimates of T2-decay (T2) and amplitude (S₀) images (maps) were obtained using a nonlinear fit to the equation $S_i(TE) = S_0 \cdot \exp(-TE_i/T2)$, where S_i is the signal intensity for echo time TE_i. The T2-map and amplitude values were fed into a deep learning algorithm to create a brain mask (139; 161; 188). Brain masks were manually corrected as needed. The ROI in the CC was drawn manually using VivoQuant software (inviCRO, Boston, MA). The CC ROI was defined as extending from the midline bilaterally to the point of ventral curvature in the external capsule (Supplemental Figure S2), as in our previous studies (161; 188).

DTI were processed using the TORTOISE v3.2.0 software package (114; 131). Motion correction, eddy current correction, and EPI distortion correction were performed using the DIFFPREP function. Tensors were computed using a nonlinear tensor fit with RESTORE, and fractional anisotropy (FA), trace (TR), axial diffusivity (AD), and radial

diffusivity (RD) were computed from the diffusion tensor images. An ROI was drawn manually in the CC within the slice under the impact site at bregma (Supplemental Figure S2) and average CC values of FA, TR, AD, and RD were computed. TR did not show an effect of injury or genotype (data not shown).

To calculate volume change in the CC the 125 μm isotropic 3D T2-weighted image slices were converted to NIFTI file format using a custom script in Matlab. A bias field correction was performed using the *N4BiasFieldCorrection* command in the Advanced Normalization Toolkit (ANTs) (12). The brain mask from the multi-spin-echo T2 image set was transformed to the 3D T2-w imaging using rigid registration and linear interpolation and thresholded at 0.5 to create a new binary mask. A template was created from the baseline scans using the *antsMultivariateTemplateConstruction2.sh* script (13). Each image for each time point was then registered to the template using the nonlinear registration algorithm in ANTs (12). Registration parameters were selected based on the approach described by Anderson et al. (7). Voxel-wise maps of volume change were created using the *CreateJacobianDeterminateImage* function in the ANTs toolkit. A ROI was drawn manually in the CC on each of seven 125 μm coronal image slices (Supplemental Figure S2) that encompassed the CC over the lateral ventricle and under the site of injury within the +0.5 and -0.5 mm window relative to bregma. The average value of volume change was then computed. The statistical parametric mapping (SPM) v12 software was used to perform a voxel-wise analysis of the volume change images.

Immunohistochemistry

At 10 weeks following TBI or sham procedure, *Sarm1* mice were perfused with 4% paraformaldehyde and brains cut as 14 μm -thick coronal cryosections for

immunohistochemistry. Myelin was detected by immunolabelling for myelin oligodendrocyte glycoprotein (MOG; polyclonal mouse anti-MOG; 1:100; Millipore, Burlington, MA; Cat# MAB5680, RRID: AB_1587278). Astrocytes were evaluated by immunostaining for glial fibrillary acidic protein (GFAP; monoclonal mouse anti-GFAP; 1:500; Millipore, Burlington, MA; Cat# MAB3402, RRID:AB_94844). Microglia/macrophages were identified using polyclonal rabbit antibody against ionized calcium binding adaptor molecule 1 (IBA1; 1:500; Wako, Richmond, VA; Cat# 019-19741, RRID:AB_839504). All tissue sections were counterstained with DAPI nuclear stain (Sigma-Aldrich, St. Louis, MO; Cat# D9542).

Immunohistochemical analysis of coronal CC width, myelination, and neuroinflammation

Immunohistochemical analysis used cohorts of *Sarm1* littermates perfused at 10 weeks after the TBI or sham procedure for *Sarm1* WT sham (n = 7; 3 female, 4 male) and TBI (n = 7; 3 female, 4 male) along with *Sarm1* KO sham (n = 6; 2 female, 4 male) and TBI (n = 9; 5 female, 3 male). Images within the CC ROI were acquired with a 10x objective on an Olympus IX-70 microscope using a SPOT RT3 camera. For quantification in coronal images, the CC 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 measured as the average of measurements taken at the midline and bilaterally at ~200 µm lateral to the midline, under the peak of the cingulum, and ~200 µm lateral to the peak of the cingulum using MOG staining (Supplemental Figure S2). ImageJ software was used to threshold fluorescence levels to quantify the area of immunolabeling above background (9; 96). Images were also acquired with a 40x objective to classify the morphology of IBA1 immunolabeled

cells as resting or activated cells (105; 162; 188). Quantification included 4–6 sections per mouse.

Motor skill learning task

The Miss-step wheel motor assay was performed using a protocol previously described (161). The Miss-step wheel motor assay has been shown to engage the CC and be sensitive to changes in myelination (67; 103; 107). From 8 to 10 weeks post TBI, mice were singly housed in home cages with a Miss-step running wheel equipped with an optical sensor to detect wheel revolutions (Mouse Miss-step Activity Wheel system, Cat#80821, Lafayette Instruments, Lafayette, IN). The Miss-step running wheels have 16 rungs missing from a standard wheel so that the remaining 22 rungs are distributed in an irregular interval pattern (67). Mouse whiskers were clipped so that avoiding miss-steps was dependent on learning to follow the rung located on the prior step by bringing the hind paw forward to grasp the rung used by the forepaw (103). Activity Wheel Monitor software (Lafayette Instruments) counted wheel revolutions at 6 min intervals during the “lights on” phase and 1 min intervals during the “lights off” phase. Results were exported to a Microsoft Excel file every 24 hrs.

Sleep/wake pattern

Sleep pattern data was collected using a non-invasive automated scoring system (Signal Solutions LLC, Lexington, KY). Mice were single housed during the 8th week following TBI or sham procedures for 72 hrs and maintained on a standard 12 h cycle of daytime light (6:00–18:00) with continuous data collection. A cage floor matt with piezoelectric sensors recorded 4 second epochs and used the 2-4 Hz breathing rhythm of mice to classify intervals of 30 seconds or more as asleep or awake (95). This

piezoelectric sensor system compares well with sleep data collected by visual observation and with electrophysiological discrimination of sleep/wake intervals, yet avoids the surgical procedures of electrophysiological techniques that could confound other assessments of TBI (111; 184).

Statistical Analysis

Sample sizes were estimated for > 80% power based on prior data in *Sarm1* and in C57BL/6 mice (96; 188) (Supplemental Figures S2 and S3). Mice were randomized to TBI/sham using the RAND function in Microsoft Excel. Investigators involved in data collection and analysis were blinded to genotype and injury condition until after the conclusion of the study. GraphPad Prism 8.0 software (RRID: SCR_002798) was used for statistical analysis and graphing. Bar graphs show means with standard error of the mean and symbols for individual mouse values. Two-way ANOVA was used to determine statistically significant differences when comparing by genotype and injury between groups. Repeated measures two-way ANOVA (RM ANOVA) was used to assess differences between genotypes over time in the longitudinal MRI analysis and within behavioral assessments. Corrections for multiple comparisons were done using Sidak's test. An alpha-level of p value < 0.05 was considered statistically significant.

RESULTS

***Sarm1* deletion protects against CC atrophy and axon-myelin pathology in chronic TBI**

The potential for *Sarm1* inactivation to provide long term axon protection and/or to mitigate post-traumatic neurodegeneration was first evaluated using transmission EM (Figure 5). Before thin sectioning for EM, sagittal brain slices were osmium-stained to

label myelin and examined with bright field microscopy to measure the CC width, which is a clinically relevant measure of white matter pathology in TBI and neurological diseases (39). Both *Sarm1* WT and *Sarm1* KO mice developed significant CC atrophy by 10 weeks after TBI, yet the extent of atrophy was significantly less in the *Sarm1* KO mice (Figure 5A). For in-depth analysis of the underlying pathology associated with changes in CC size, the tissue slices were then thin sectioned and imaged by transmission EM at subcellular resolution to examine individual myelinated axons within the same region of the CC. This quantification revealed a significant reduction of intact axons due to TBI in the *Sarm1* WT mice that was rescued by *Sarm1* deletion (Figure 5B).

Myelinated axons exhibit subcellular features that can distinguish intact axons from indicators of axon damage including swollen mitochondria, compacted cytoskeletal structure, accumulation of vesicles or debris, or loss of ensheathing myelin (Figure 5C-F). After the sham procedure, *Sarm1* WT mice illustrate the healthy adult high density of myelinated axons in this anterior region of the CC (Figure 5G). As previously characterized in this concussive TBI model (96; 105), TBI results in damaged axons that are dispersed among adjacent intact axons and may also exhibit demyelination in *Sarm1* WT mice (Figure 5H). *Sarm1* KO mice exhibit normal appearing myelinated axons after the sham procedure and dispersed damaged axons after TBI (Figure 5I, J). Importantly, while both *Sarm1* WT and *Sarm1* KO mice exhibited axon damage after TBI, *Sarm1* deletion significantly reduced the frequency of damaged axons (Figure 5K). *Sarm1* deletion also normalized the frequency of TBI-induced mitochondrial pathology and demyelination (Figures 5L, M).

Longitudinal *in vivo* MRI detects CC atrophy after TBI and attenuation by *Sarm1* deletion

Longitudinal MRI studies were conducted to advance the translational impact while further evaluating the effects of *Sarm1* deletion on white matter integrity and CC atrophy. Each mouse was scanned prior to injury (i.e., baseline), and with follow up scans at acute and chronic stages after TBI. While DTI measures of white matter integrity have long been effectively used for analysis of adult mouse CC (155; 161), volume measurements have only recently been validated for quantification of atrophy and hypertrophy of the relatively small structures of adult mouse brains (7; 14); Therefore, an initial cross-sectional *in vivo* study was conducted in C57BL/6 mice to optimize the MRI outcome measures. DTI analysis at 10 weeks after sham or TBI demonstrated significant changes after TBI in CC integrity while high resolution T2-w volumetrics detected significant CC atrophy that was validated by post-imaging neuropathology (Supplemental Figure S2).

Longitudinal MRI studies were then conducted to compare *Sarm1* WT and *Sarm1* KO mice. Volumetric analysis showed significant atrophy of the CC between baseline and 10 weeks in *Sarm1* WT mice (Figure 6A-C) that was attenuated in *Sarm1* KO (Figure 6D-F). Across time points, DTI detected reduced white matter integrity after TBI based on reduced fractional anisotropy (FA) in both *Sarm1* WT mice (Figure 6G, I) and *Sarm1* KO mice (Figure 6H, I). Progressive decrease in FA was driven by decreased axial diffusivity (AD) at 3 days post-TBI (Figure 6J) and subsequent elevation of radial diffusivity (RD) at 10 weeks post-TBI (Figure 6K). *Sarm1* genotype resulted in a significant effect of higher AD values in *Sarm1* KO mice (Figure 6J).

Immunohistochemistry shows reduced neuroinflammation in addition to with reduced CC atrophy and myelin loss after TBI in mice with *Sarm1* deletion.

Microglia and astrocyte responses have been linked to chronic white matter damage and atrophy (77; 84; 85; 90; 105; 162). Therefore, immunohistochemistry was used to evaluate CC atrophy and myelination (Figure 7A-D) and examine CC neuroinflammation in adjacent sections (Figure 8). CC width was measured in coronal sections with myelin immunolabeled for MOG and cytoarchitecture was evaluated using DAPI to stain nuclei (Figures 7E and Supplemental Figure S2). CC width was reduced at 10 weeks after TBI in comparison with sham mice (Figure 7E). Quantification of the pixel area of MOG immunoreactivity within the CC showed loss of myelin (Figure 7F), in agreement with the distributed demyelination observed by EM (Figure 5M). *Sarm1* KO mice had significantly less CC atrophy and less myelin loss after TBI as compared to *Sarm1* WT mice (Figure 7E, F).

Neuroinflammation was estimated by immunolabeling for reactive astrocytes and microglia using GFAP and IBA1, respectively (Figure 8). TBI resulted in more intense immunoreactivity of individual cells and an increase of overall immunolabeling for both GFAP and IBA1 (Figure 8A-G). Immunolabeling within the CC after TBI was reduced in *Sarm1* KO mice for GFAP (Figure 8H) but not for IBA1 (Figure 8I). Further analysis to count the IBA1 immunolabeled cells within the CC revealed that *Sarm1* deletion reduced microglia with either a resting state morphology (Figure 8J) or an activated morphology (Figure 8K) after TBI. Taken together, these results show that the reduction in CC atrophy and myelin loss seen in *Sarm1* KO mice after TBI was accompanied by a reduced neuroinflammatory response.

***Sarm1* deletion improves functional outcome measures in chronic stage TBI**

Behavioral assessments targeting CC axons were selected to test whether the beneficial effects of *Sarm1* deletion on TBI pathology translated to improved functional outcome measures. This TBI model did not cause overt symptoms at any time out through 10 weeks post-TBI. Therefore, an initial set of experiments in C57BL/6J mice evaluated two assays associated with CC axon-myelin pathology to determine whether either assay revealed deficits during this late phase of TBI. Miss-step wheel running is a motor skill task that engages CC axons and is sensitive to myelination status (67; 103; 161). C57BL/6 mice showed a deficit in learning to run on the Miss-step wheels after TBI as compared to sham mice (Supplemental Figure S3). C57BL/6 mice with CC pathology from experimental demyelination or repetitive mild exhibit social interaction deficits TBI (107; 188). However, with the current single impact concussive model of TBI, C57BL/6 mice did not show social interaction deficits (Supplemental Figure S4). Based on the results of these assays, the Miss-step wheel task was selected for functional assessment of TBI deficits in *Sarm1* WT versus *Sarm1* KO mice.

Motor learning and performance on the Miss-step wheels was assessed in *Sarm1* WT and *Sarm1* KO mice from 8-10 weeks post-TBI. *Sarm1* KO mice ran at a faster average velocity compared to *Sarm1* WT mice during the learning phase of the assay although this improvement did not reach statistical significance ($p = 0.0821$) (Figure 9A). Further analysis of running parameters showed that *Sarm1* KO mice ran on the wheels more times during the learning phase compared to *Sarm1* WT mice (Figure 9B). Additionally, the cumulative distance traveled by *Sarm1* KO mice during the learning phase is further for *Sarm1* KO mice than for *Sarm1* WT mice (Figure 9C).

Sleep behavior was selected in place of social interaction as a highly translational assessment for post-traumatic neurodegeneration (Figure 10). Sleep disorders are common in patients with chronic TBI and sleep patterns may contribute to neurodegeneration, particularly white matter degeneration (6; 116; 143). Sleep data was analyzed for *Sarm1* WT and *Sarm1* KO mice during two light/dark cycles. In *Sarm1* WT mice, TBI causes an overall difference from shams in the percent of time spent sleeping (Figure 10A). This difference was not found in *Sarm1* KO mice (Figure 10B). More specifically, *Sarm1* WT mice slept less during the lighted period that is the sleep phase for nocturnal mice (Figure 10C) without a change during the awake phase (Figure 10D). *Sarm1* KO mice did not exhibit a sleep deficit after TBI, as compared to shams (Figure 10C, D).

DISCUSSION

The combined results from this study support a direct role of axonal injury in post-traumatic neurodegeneration and provide key pre-clinical evidence for TBI as a clinical indication for treatments to inhibit SARM1. Genetic inactivation of the gene for SARM1, which mediates a conserved axon degeneration pathway, had a beneficial effect on chronic white matter injury after TBI. Analysis of CC atrophy in longitudinal and cross-sectional MRI studies was an effective outcome measure in mice that is a clinically useful measure of post-traumatic neurodegeneration. Complementary neuropathological techniques validated CC atrophy measures and identified beneficial effects from deletion of the *Sarm1* gene on chronic stage axon damage, demyelination, and neuroinflammation. Importantly, *Sarm1* deletion preserved healthy axons and prevented the progression of

CC atrophy after TBI. *Sarm1* deletion also ameliorated functional deficits which, together with the pathological and radiological benefits, indicate improved outcome trajectory after TBI.

Insights afforded by the subcellular detail of EM (Figure 5) address important gaps in the interpretation of long-term effects of *Sarm1* deletion in experimental TBI. In the TBI model used in the current studies, EM data from the acute time point of 3 days (96) and chronic phase of 10 weeks post-TBI (Figure 5) are matched by scan time points in our longitudinal MRI of live mice (Figure 6). Prior studies in *Sarm1* KO mice have shown significant axon protection in the acute (2 hours - 3 days) or chronic (2-6 months) phase after closed head TBI based on β -amyloid precursor protein (β -APP) immunohistochemistry (65; 101) or using *Thy1-YFP* fluorescence to visualize axonal varicosities (96; 190). The observation of subcellular details of axon and myelin ultrastructure afforded by EM informs a fuller interpretation of myelinated axon health.

EM enables quantification of abnormal swollen mitochondria that are an early feature of axonal injury linked to Wallerian degeneration (174; 181) and have important implications for the SARM1 pathway (71; 88). Mitochondrial dysfunction challenges axon energy metabolism and creates a vulnerable axon state (71; 118). Mitochondrial stress, trauma or other insults lead to loss of NMNAT2, an NAD-synthesizing enzyme, in axons that depletes NAD⁺ and raises the level of its precursor nicotinamide mononucleotide (NMN) (41; 88). Low NAD⁺ and high NMN levels regulate release of auto-inhibition of SARM1 NADase activity, which further depletes NAD⁺ and axon energy stores (16; 42; 75; 147; 158). Axons with swollen mitochondria were significantly increased at 3 days after TBI in *Sarm1* WT and *Sarm1* KO mice (96). Compared to this

acute data, axons with swollen mitochondria were less frequent at 10 weeks but still significantly increased after TBI in *Sarm1* WT mice (Figure 5), which indicates the potential for continued activation of SARM1.

EM identifies damaged axons based on breakdown and compaction of the cytoskeleton and/or accumulated vesicles due to impaired axonal transport (Figure 5). The frequency of damaged axons and the effect of *Sarm1* deletion is very similar at the 10 week time point (Figure 5) as compared to 3 days post-TBI (96). The slow clearance of degenerating axons in the CNS may play a part in this result (173). Alternatively, surviving axons may succumb to insults in a late phase after TBI and *Sarm1* deletion may have an ongoing benefit across acute and chronic time points. For example, axons continue to initiate Wallerian degeneration long after stretch injury that models TBI forces (99). Clinically, persistent neuroinflammation in white matter is associated with axon damage in the CC in human postmortem cases several years after TBI (76).

Finally, EM is the gold standard to quantify demyelination, i.e. loss of the myelin sheath around otherwise healthy axons. TBI induced demyelination in *Sarm1* WT mice that was not found in *Sarm1* KO mice at 10 weeks post-TBI or sham procedures (Figure 5). *Sarm1* is expressed mainly in neurons but a low level of expression in oligodendrocyte lineage cells could have a role in the lack of TBI-induced demyelination in *Sarm1* KO mice (81). However, the effect of *Sarm1* deletion may not be an autonomous effect in myelinating oligodendrocytes as zebrafish studies have shown a glioprotective effect of *Sarm1* deletion is dependent on axon protection (168). Loss of myelin along otherwise healthy axons can slow information processing speed and desynchronize neural circuits; myelin also protects from insults and provides trophic

support to axons (10). Therefore, demyelination that is resolved by *Sarm1* deletion may have ongoing benefit at 10 weeks post-TBI that can impact axon health and neural circuit function.

The current study is the first to use translational measures of MRI with CC atrophy to evaluate the effects of *Sarm1* deletion after TBI. Based on histological measures of CC width, our prior studies in this TBI model did not detect significant CC atrophy at the 3 day, 2 week, or 6 week time points (96; 105). In contrast, significant CC atrophy was evident by histological measures at the longer time point of 8 weeks post-TBI (96; 97) and now at 10 weeks post-TBI (Figures 5A, 7E and Supplemental Figure S2). At this 10 week time point, these histological measures validate the changes in CC volume in live mice determined by quantitative MRI (Figure 6C and Supplemental Figure S2). Significantly, the histological and MRI measures corroborate attenuation of CC atrophy in *Sarm1* KO mice as compared to *Sarm1* WT mice (Figures 5A, 6F, 7E). These longitudinal MRI studies also analyzed DTI FA as a measure of CC microstructure (179; 184). Reduced FA values indicated an injury effect at 3 days and at 10 weeks after TBI, as compared to baseline (Figure 6I-K and Supplemental Figure S2). A main effect of *Sarm1* genotype was only observed for the AD parameter (Figure 6J) that is most often associated with axonal anisotropy within white matter voxels. Interpreting reduced AD as an indicator of axon pathology and increased RD as an indicator of myelin pathology can be useful when each pathology predominates (155; 183). The presence of simultaneous pathology of axon damage and demyelination is more difficult to detect and interpret using DTI, and is further complicated in the presence of significant edema or neuroinflammation (179; 183). Histological measures confirm the presence of

simultaneous axon damage, demyelination, and neuroinflammation at 10 weeks after TBI along with significant reduction of each pathology with *Sarm1* deletion (Figures 5, 7, 8), which is not fully appreciated using the DTI parameters (Figure 6I-K).

Pathological and structural benefits of *Sarm1* deletion were complemented by behavioral studies that show amelioration of functional deficits (Figures 9, 10). Our results extend beyond previous reports in *Sarm1* KO mice to now examine complex behaviors during the chronic phase post-TBI. Studies in a weight drop model of TBI used the neurological severity score battery of simple tasks from 2 hours through 4 weeks post-TBI and found reduced deficits in *Sarm1* KO versus *Sarm1* WT mice only during the first week (65). Studies of repetitive TBI showed normalization toward sham responses in *Sarm1* KO for motor performance and memory deficits during the first week and context fear discrimination at 4 weeks (101). With assessments conducted beyond 8 weeks post-TBI, *Sarm1* deletion reduced deficits during motor learning (Figure 9) and normalized the time spent sleeping (Figure 10). The Miss-step wheel running system (Figure 9) and the piezoelectric sleep system (Figure 10) use automated multi-day continuous data collection of spontaneous complex behaviors, which may be advantageous for analysis of subtle deficits after TBI.

Limitations of the experimental design should be considered in the interpretation of the results. The *Sarm1* KO mice have been backcrossed to the C57BL/6 strain but may harbor genes associated with the embryonic stem cell origin in the 129 background strain (172). However, the findings regarding axon degeneration in this line of *Sarm1* KO mice have been confirmed in additional *Sarm1* KO mice generated using CRISPR technology (172). In addition, all experiments in the current study used *Sarm1* WT and *Sarm1* KO

littermates to minimize the variability due to genetic background. The assessment of sleep disorders after TBI used a non-invasive screening approach to avoid surgical procedures and electrode placement through skull burr holes that would be required for electrophysiology. The sleep behavior in the *Sarm1* KO mice appears normalized to the sham sleep pattern but electrophysiological recordings would be needed for a more in-depth comparison of sleep architecture. Finally, the neuropathological and radiological analyses focused on white matter, and specifically the CC. Further studies would be of interest to better understand the relationship of axon damage and the progression of white matter pathology relative to broader neural circuits and gray matter pathology, including analysis of synapse loss that is associated with chronic neuroinflammation after TBI (4).

CONCLUSIONS

These results demonstrate that genetic inactivation of *Sarm1* improves the outcome trajectory after TBI based on pathological, radiological, and functional measures. These studies advance strategies to develop TBI treatments for axon damage by demonstrating a genetic proof-of-concept of the long-term benefit of *Sarm1* deletion. The therapeutic potential of SARM1 inhibitors has drawn intense interest and a small molecule inhibitor of SARM1 has already been developed that recapitulates *in vitro* aspects of the *Sarm1* KO phenotype (71; 89; 151). The current studies also highlight CC atrophy as an important outcome measure of white matter degeneration after TBI in mice that may have translational relevance as a biomarker for clinical studies (55). White matter degeneration may be a tractable therapeutic target for TBI and chronic traumatic

encephalopathy, with potential application to other neurodegenerative diseases including Alzheimer's disease and multiple sclerosis (8; 26; 33; 138).

ACKNOWLEDGMENTS

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Figure 5

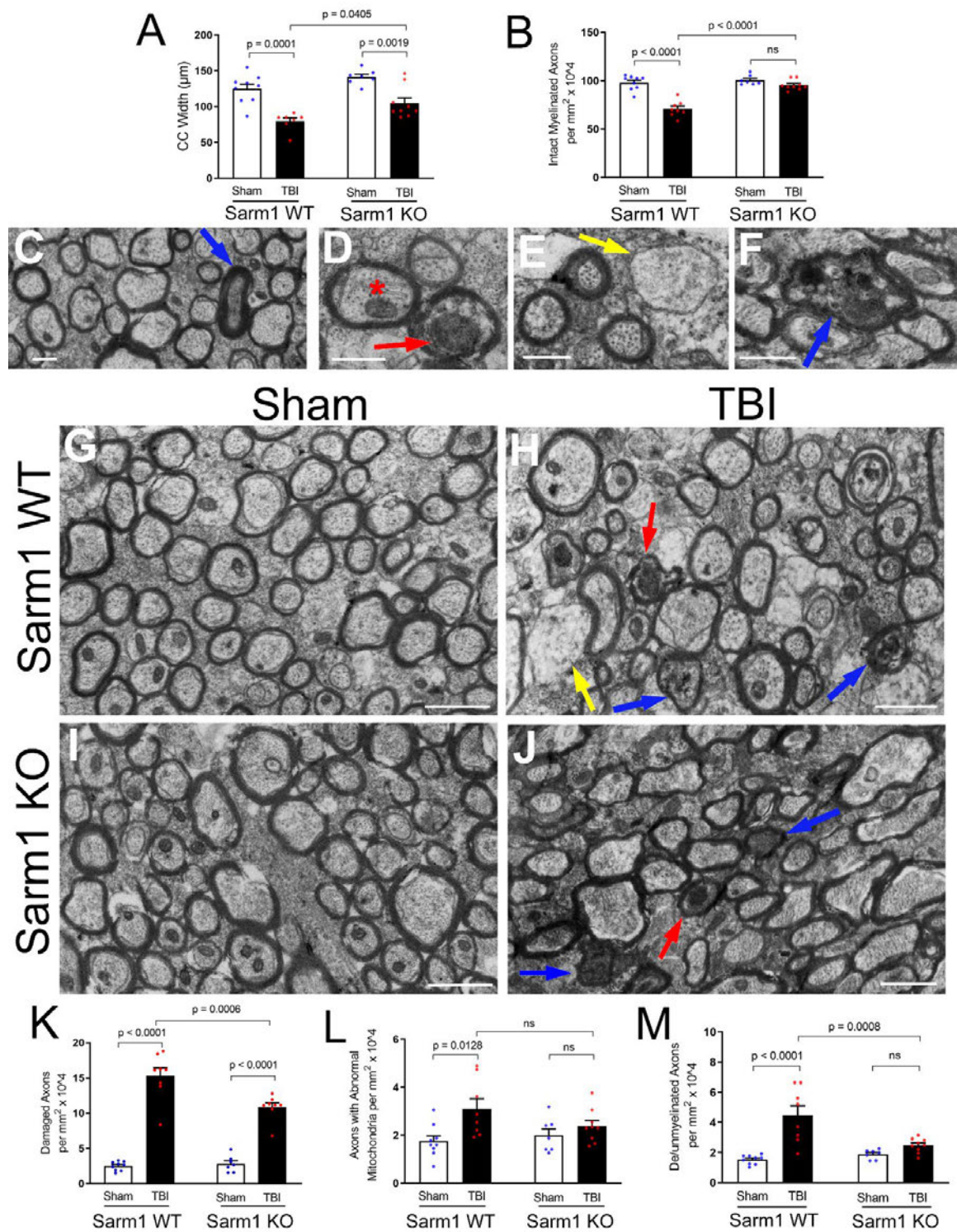


Figure 5. *Sarm1* deletion reduces corpus callosum atrophy and axon pathology at 10 weeks post-TBI.

A: *Sarm1* deletion attenuates CC atrophy that develops after TBI. **B:** *Sarm1* deletion preserves intact, myelinated axons after TBI. **C-F:** Representative images of pathological features from high resolution electron microscopy in CC sagittal sections. **C:** Example from a *Sarm1* WT sham mouse to show the cytoskeleton, mitochondria and surrounding myelin sheath of intact axons in contrast with a rare damaged axon with a densely compacted cytoskeleton (blue arrow). **D:** *Sarm1* WT TBI mouse example of an axon with an abnormally large mitochondrion (red arrow) and an axon with a typical mitochondrion (red asterisk). Mitochondria were considered abnormal when swollen to > 50% of the axon area. **E:** Demyelinated axon (yellow arrow) lacking ensheathing myelin but with otherwise intact cytoskeleton in a *Sarm1* WT TBI mouse. **F:** Damaged axon (blue arrow) with vesicle accumulation and cytoskeletal breakdown in a *Sarm1* KO TBI mouse. **G-J:** Representative electron microscopy images from CC sagittal sections for *Sarm1* WT sham (G) and TBI (H) mice in comparison with *Sarm1* KO sham (I) and TBI (J) mice. Arrows identify examples of damaged axons with accumulated vesicles and/or compacted cytoskeleton (blue), abnormal mitochondria (red), or demyelination (yellow). **K-M:** Quantification of axon and myelin pathology at 10 weeks after TBI or sham procedure. *Sarm1* deletion reduces chronic stage axon damage (K), normalizes mitochondria morphology (L), and eliminates TBI-induced demyelinated component of de/unmyelinated axons (M). *Sarm1* WT: n = 9 sham, n = 8 TBI. *Sarm1* KO: n = 7 sham, n = 9 TBI. ns = not significant. C-F, scale bars = 0.5 μ m. G-J, scale bars = 1 μ m.

Figure 6

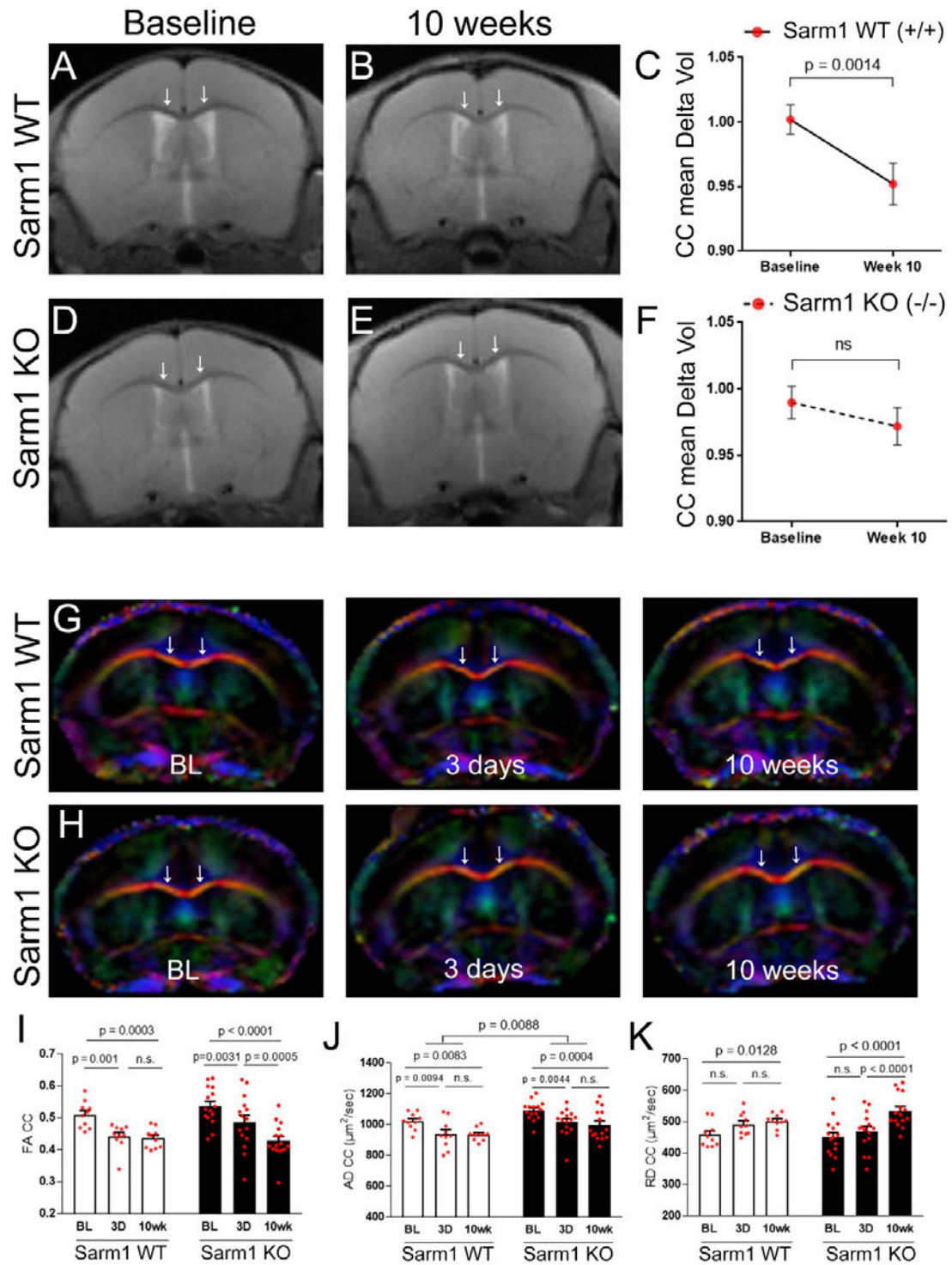


Figure 6. Progression of corpus callosum atrophy after TBI is sufficient for MRI detection in live mice and is attenuated by *Sarm1* deletion.

A-C: TBI induces significant CC atrophy in *Sarm1* WT mice. T2-weighted images showing the coronal view at the level of the impact site at baseline (A, before surgery) and at 10 weeks (B) post-TBI/sham procedures. Quantification of volume change in CC regions under the impact site (C). **D-F:** In *Sarm1* KO mice, CC atrophy is not detected in representative T2-weighted images (D, E) or based on changes in CC volume (F). **G-H:** Direction encoded color images of diffusion tensor imaging (DTI) from a longitudinal MRI series at baseline (BL) and again at 3 days and 10 weeks post-TBI or sham procedures. Colors represent fiber directions as red (medial-lateral), blue (anterior-posterior), and green (superior-inferior). **I-K:** Quantification of DTI measures reveals a chronic progression of CC pathology following TBI. Fractional anisotropy (FA) significantly decreases over time following TBI (I). The acute change is driven by a decrease in axial diffusivity (AD) between baseline and 3 days (J). *Sarm1* KO mice have significantly higher AD values than *Sarm1* WT mice (J). The FA at 10 weeks corresponds with a delayed increase in radial diffusivity (RD) in *Sarm1* KO mice (K). Arrows indicate medial CC regions. *Sarm1* WT: n = 10 TBI; *Sarm1* KO: n = 15 TBI. ns = not significant.

Figure 7

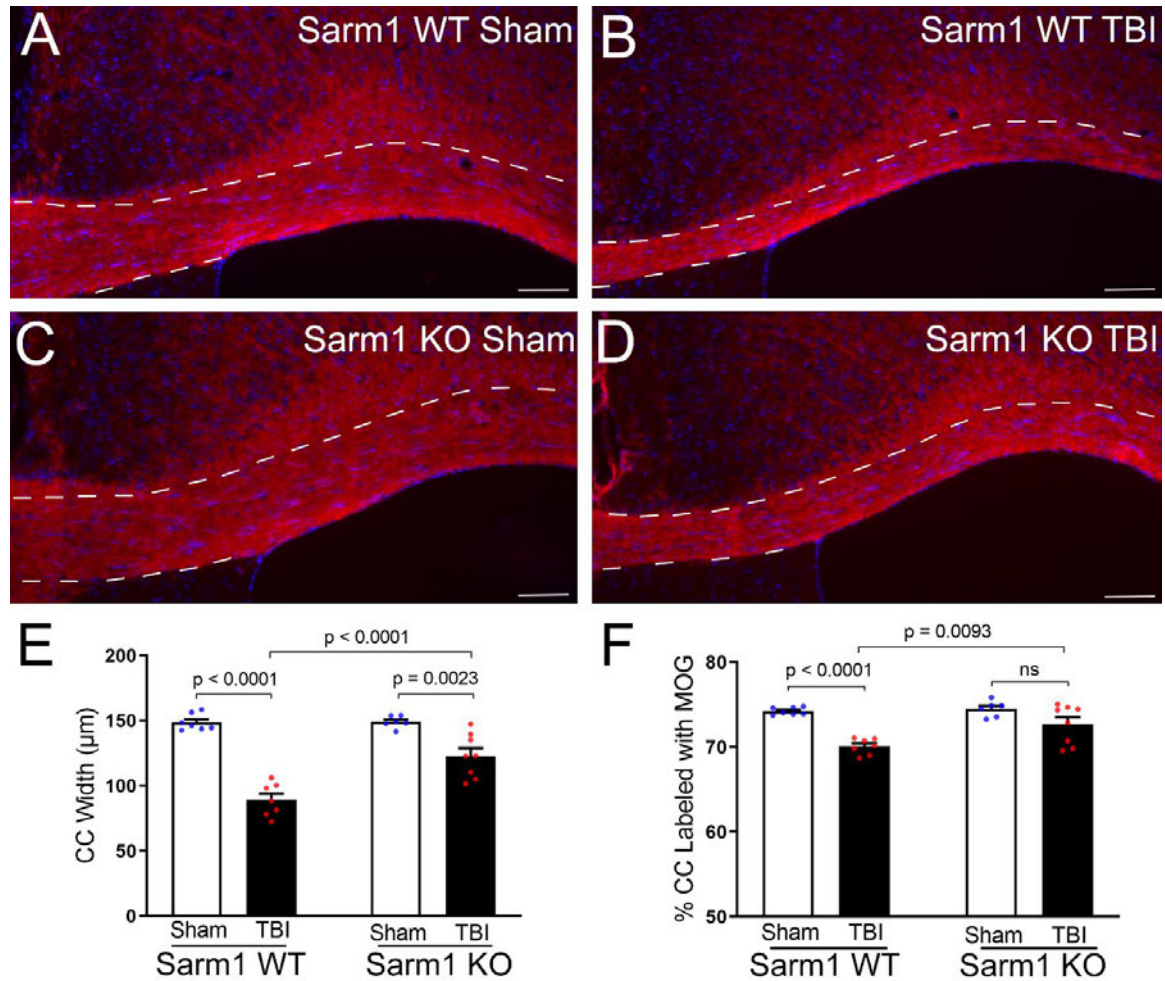


Figure 7. *Sarm1* deletion reduces corpus callosum atrophy and myelin loss at 10 weeks post-TBI.

A-D: Representative images from CC coronal sections of *Sarm1* WT and *Sarm1* KO mice after sham or TBI procedures. Myelin is detected with immunolabeling for MOG (red). DAPI nuclear stain is shown in blue. CC borders are indicated by dashed lines. **E:** *Sarm1* deletion attenuates CC atrophy, which is quantified based on the CC width. **F:** TBI results in significant myelin loss as detected by reduced MOG immunoreactivity. Myelin loss after TBI is significantly reduced in *Sarm1* KO mice compared to *Sarm1* WT mice. *Sarm1* WT: $n = 7$ sham, $n = 7$ TBI. *Sarm1* KO: $n = 6$ sham, $n = 8$ TBI. ns = not significant. A-D, scale bars = 100 μm .

Figure 8

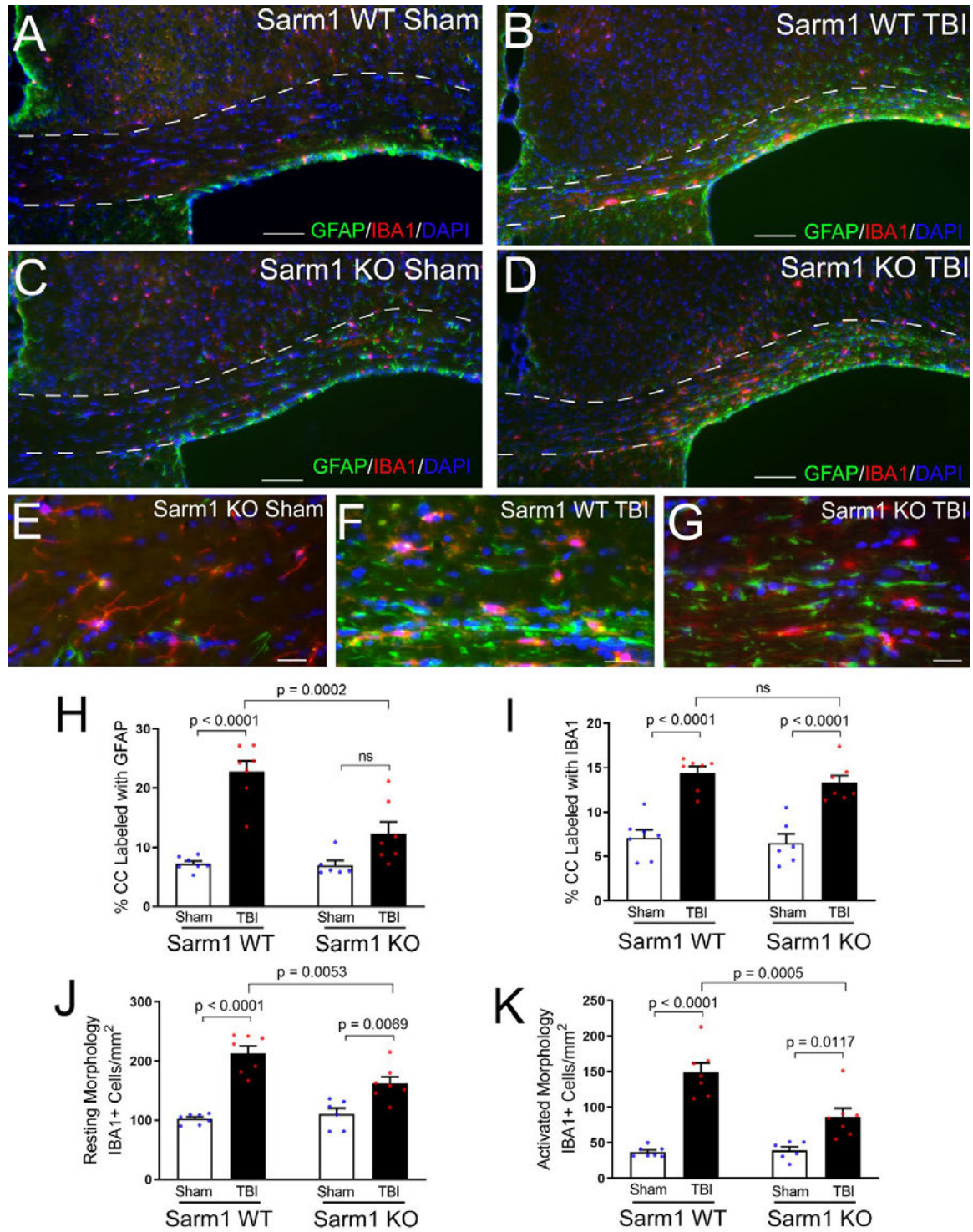


Figure 8. *Sarm1* deletion reduces neuroinflammation at 10 weeks post-TBI.

A-D: Representative images from CC coronal sections of *Sarm1* WT (A, B) and *Sarm1* KO (C, D) mice after sham (A, C) or TBI (B, D) procedures. Neuroinflammation is detected with markers of astrocytes (GFAP, green) and microglia (IBA1, red). DAPI nuclear stain shown in blue. The CC borders are indicated by dashed lines. **E-G:** Higher magnification examples of astrocyte (GFAP) and microglia (IBA1) morphology. In sham mice (E), astrocytes and microglia exhibit homeostatic morphology with thin processes. Following TBI (F, G), reactive astrocytes and microglia have intensely immunolabeled cell bodies and shorter, thicker, processes. **H:** *Sarm1* deletion significantly reduced astrogliosis after TBI, based on GFAP immunolabeling within the CC area. **I:** The microglial response also indicated CC neuroinflammation after TBI, based on IBA1 immunolabeling, but did not detect differences due to *Sarm1* deletion. **J-K:** More detailed counting of IBA1 immunolabeled (+) cells revealed that *Sarm1* deletion significantly reduced the frequency of both resting (J) and activated (K) microglia after TBI. *Sarm1* WT: n = 7 sham, n = 7 TBI. *Sarm1* KO: n = 6 sham, n = 7 TBI. ns = not significant. A-D, scale bars = 100 μ m. E-G, scale bars = 25 μ m.

Figure 9

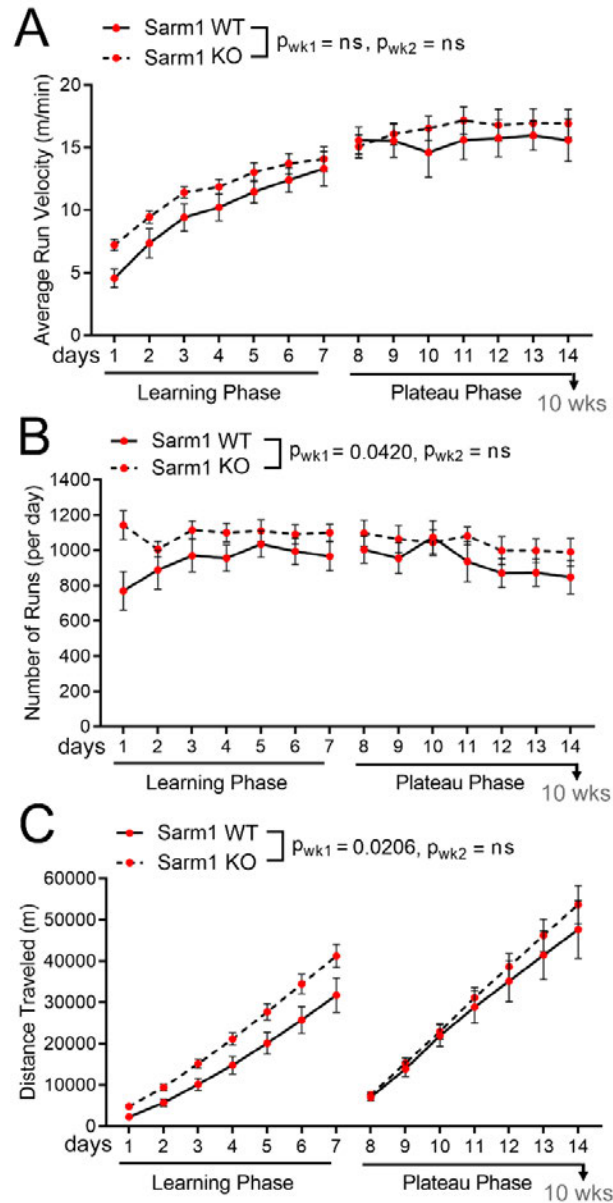


Figure 9. *Sarm1* deletion improves motor learning in chronic stage TBI.

Miss-step wheels have irregularly spaced rungs to assess motor skill learning (week 1) followed by a plateau phase (week 2) that tests bilateral sensorimotor function. **A:** *Sarm1* KO mice more quickly learn to run at a faster average velocity compared to *Sarm1* WT mice, but this improvement does not reach statistical significance ($p = 0.0821$). **B:** *Sarm1* KO mice run on the wheels more frequently than *Sarm1* WT during the learning phase. **C:** Running behavior accumulates to increased total distance traveled during the learning phase in *Sarm1* KO mice compared to *Sarm1* WT mice. There were no statistically significant differences between genotypes in the three measures during the plateau phase (A-C). *Sarm1* WT: $n = 9$ TBI. *Sarm1* KO: $n = 15$ TBI. ns = not significant.

Figure 10

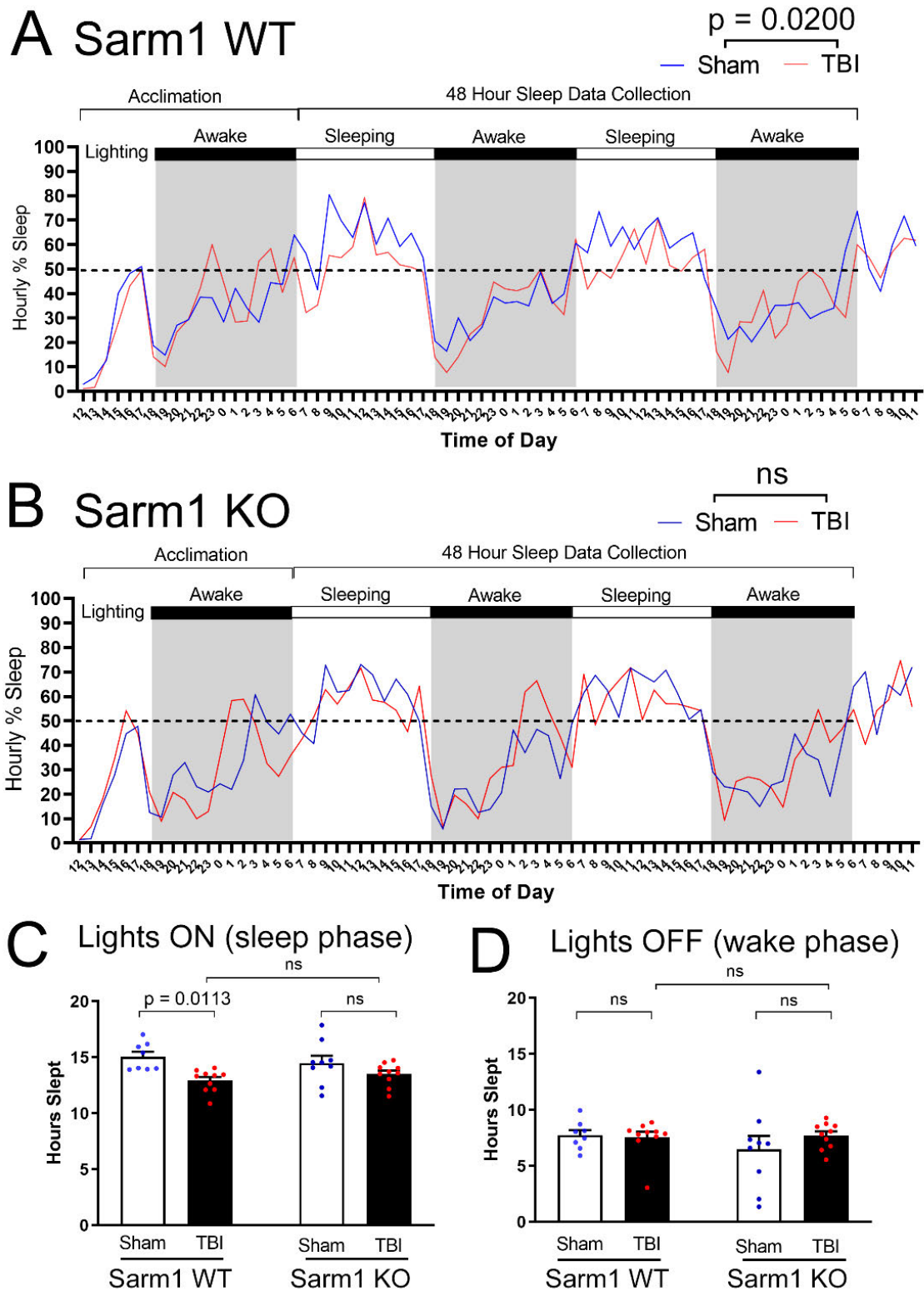
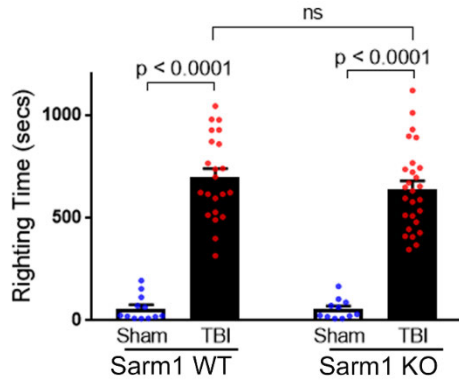


Figure 10. *Sarm1* deletion normalizes time spent sleeping in the chronic stage of TBI.

Mice were single housed in cages equipped with a PiezoSleep Mouse Behavioral Tracking System for 72 hrs during the eighth week after TBI. **A-B:** Mice were acclimated for 18 hrs. Statistical analysis focused on data collected during 48 hrs across two complete cycles of lights on (white bars) and lights off (dark bars and gray background). Recordings continued for a subsequent 6 hrs to show the final wake/sleep transition. In *Sarm1* WT mice (A), the time spent sleeping per hour is significantly different between sham and TBI, with the injured mice appearing to sleep less during the normal sleeping period when lights are on. In *Sarm1* KO mice (B) the sleep pattern was not different between sham and TBI conditions during the 48 hr data collection period. **C:** TBI significantly reduced the time spent sleeping for *Sarm1* WT mice during the normal sleeping period when lights are on. *Sarm1* KO mice did not exhibit sleep loss after TBI. **D:** During the dark period, sleep time was not different based on injury or genotype. *Sarm1* WT: n = 8 sham, n = 10 TBI. *Sarm1* KO: n = 9 sham, n = 10 TBI. ns = not significant.

SUPPLEMENTARY METHODS AND RESULTS

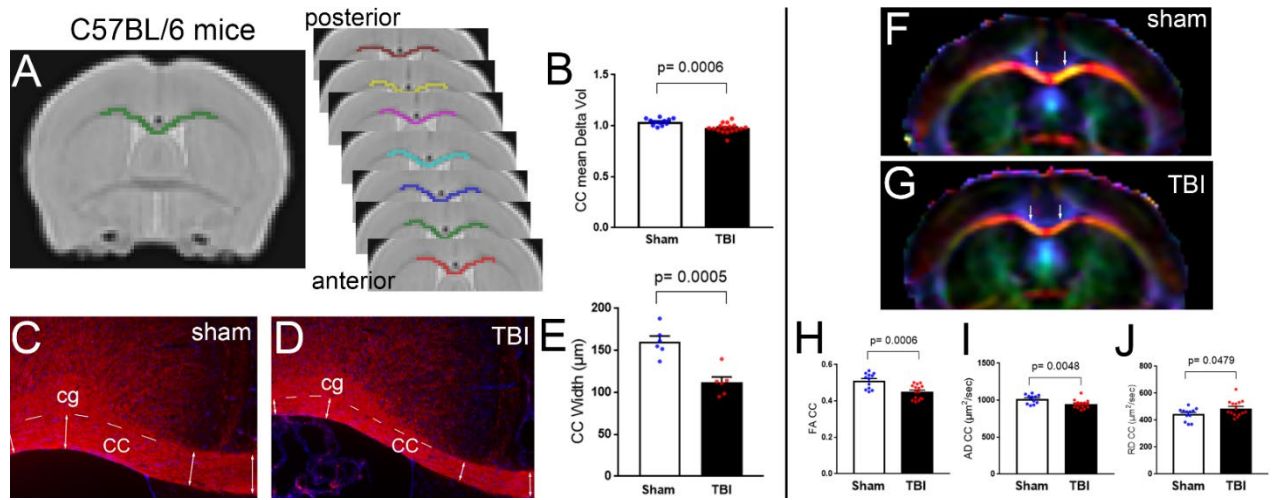
Supplemental Figure S1



Supplemental Figure S1: Righting reflex times after sham or TBI surgical procedure in *Sarm1* WT and *Sarm1* KO mice.

Post-surgical righting reflex times were measured as the time interval from the end of anesthesia until mice returned to the upright position. Righting time is shown for all mice used in experiments. Prolonged righting reflex times are an indicator of TBI severity that serve as a measure of arousal that estimates loss of consciousness after TBI or sham procedures. Righting reflex time did not differ between male and female littermates, so the sexes were combined for analysis by genotype. Righting reflex time was significantly prolonged after TBI as compared to the sham procedure for *Sarm1* WT ($p < 0.0001$) and *Sarm1* KO ($p < 0.001$) mice without a difference based on genotype ($p = 0.8287$) (Two-way ANOVA; $F(1,68) = 183$).

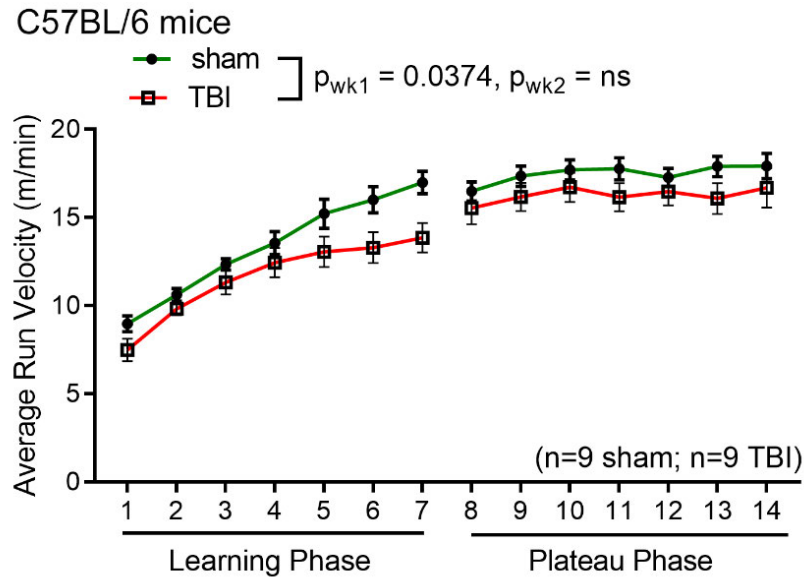
Supplemental Figure S2



Supplemental Figure S2. TBI reduces white matter integrity and produces significant corpus callosum (CC) atrophy in C57BL/6 mice.

A cross-sectional *in vivo* MRI study used male C57BL/6 mice between 9-10 weeks post-TBI/sham. After scanning, mice were perfused for tissue analysis at 10 weeks post-TBI/sham. **A-B:** CC volume changes were calculated as the change in volume compared to a registered template image of all animals in the study. Seven ROIs were manually drawn on coronal images (125 μm thickness) encompassing the rostro-caudal CC over the lateral ventricle and underneath the impact site at bregma (A). TBI significantly reduced the mean CC volume, as compared to the sham procedure (B) **C-E:** Post-imaging neuropathology validated CC atrophy after TBI. Myelinated fibers were immunolabeled for myelin oligodendrocyte glycoprotein (MOG; red) and cellular distribution was detected using nuclear counterstain (DAPI; blue) (C, D). The CC borders (dashed lines) were evident by the pattern of myelinated fibers oriented in the medial-lateral direction in the CC in contrast to the myelinated fibers of the cingulum (Cg) that align in the rostro-caudal (i.e. anterior-posterior) direction. Arrows (C, D) show examples of sites for CC width measurement for quantification (E). **F-J:** MRI diffusion tensor imaging (DTI) illustrates fractional anisotropy signal in the CC (white arrows) and adjacent regions in direction encoded color maps (F, G). Colors represent fiber directions as red (medial-lateral), blue (anterior-posterior), and green (superior-inferior). TBI significantly reduced the CC fractional anisotropy (FA; H) which was driven by reduced axial diffusivity (AD, I) and increased radial diffusivity (RD; J). MRI $n = 12$ per condition; male C57BL/6 mice (RRID:IMSR_JAX:000664), Jackson Laboratories, Bar Harbor, ME). Neuropathology $n = 6$ per condition randomly selected from the MRI cohort. Student's *t*-test.

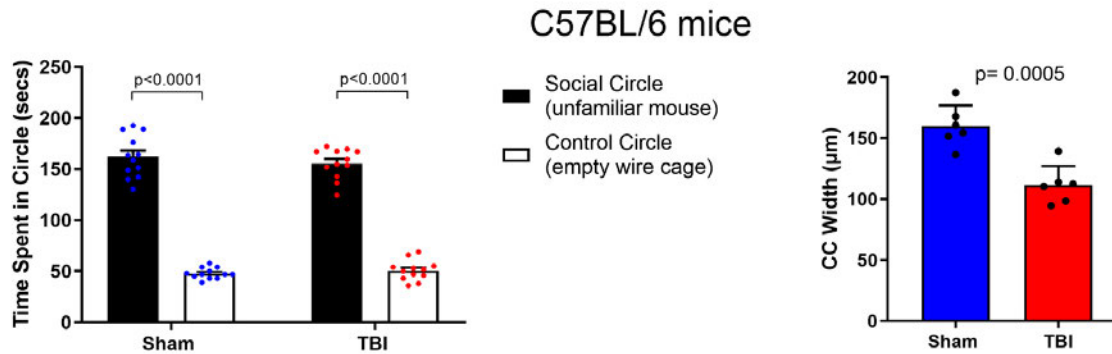
Supplemental Figure S3



Supplemental Figure S3. TBI causes a chronic stage motor learning deficit in C57BL/6 mice.

Miss-step wheels, which have been shown to engage the CC and be sensitive to myelination status, were used to assess chronic stage changes between sham and TBI mice. The wheels have irregularly spaced rungs to assess motor skill learning (week 1) followed by a plateau phase (week 2) that tests bilateral sensorimotor function. TBI mice show a significant decrease in average running velocity compared to sham during the learning phase (week 1). There was no statistically significant difference between TBI and sham groups during the plateau phase of the assay (week 2). Wheels n = 9 per condition; male C57BL/6 mice (RRID:IMSR_JAX:000664), Jackson Laboratories, Bar Harbor, ME). (Two-way REML ANOVA; $F(1,16) = 5.151$).

Supplemental Figure S4



Supplemental Figure S4. TBI does not induce a late-stage social deficit despite CC atrophy in C57BL/6 mice.

In their 8th week post injury mice were tested using a social interaction assay to measure propensity to engage in social behavior. For five minutes mice freely explore a three chamber apparatus with one chamber containing a novel, unfamiliar mouse in a wire cage and another possessing an empty wire cage while their time spent interacting with the cages is measured. Our previous study showed that CC pathology in an experimental model of chronic demyelination induces a social deficit (161). This study looked to determine if a social deficit, that wasn't present at more acute time points after TBI, occurs at a chronic time point (188). Both TBI and sham mice spent a significantly greater amount of time interacting with the cage containing the unfamiliar mouse compared to the empty cage. There was no statistically significant difference in interaction time between sham and TBI groups. At 10 weeks post TBI mice were sacrificed for neuropathological evaluation of CC width. TBI mice exhibit significant late-stage CC atrophy compared to sham. The absence of a social deficit at 8 weeks post TBI despite ongoing, chronic atrophy of the CC resulted in the omission of the social interaction assay from our study evaluating the therapeutic benefit of *Sarm1* genetic deletion. Social Interaction n = 12 per condition; male C57BL/6 mice (RRID:IMSR_JAX:000664), Jackson Laboratories, Bar Harbor, ME). Neuropathology n = 6 per condition randomly selected from the MRI cohort. Social interaction: Two-way ANOVA; $F(1,44) = 0.2116$. Neuropathology: Student's *t*-test.

Chapter 4: DISCUSSION

The main goal of this study was to provide a genetic proof-of-concept that inactivation of the SARM1 mediated axon degeneration pathway attenuates chronic white matter pathology in a relevant model of TBI. Our work demonstrated the advantages of utilizing fluorescent reporter mice to label axon and myelin components in a repetitive blast exposure model where white matter pathologies are diffuse and subtle. In a concussive TBI model, where acute axon and myelin pathologies are robust (97), we identified a chronic phenotype of TBI using a series of translational assessments. The persistent deficits in this model are analogous to those in human patients which supports its use for investigating the therapeutic effects of *Sarm1* deletion. High resolution electron microscopy revealed that *Sarm1* deletion prevents the loss of intact axons and myelin and attenuates cytoskeletal and mitochondrial disruptions seen in *Sarm1* WT mice in the chronic stage of injury. This ultrastructural preservation of white matter likely contributes to reduced CC atrophy and neuroinflammation seen on histopathology in *Sarm1* KO mice. Additionally, neuroimaging measures are able to detect the prevention of CC degeneration in *Sarm1* KO mice. Finally, functional outcomes show that attenuating axon and myelin damage in the CC results in improved motor learning performance and a reduction in sleep deficits. These studies provide valuable data, demonstrating the vital role that white matter plays in TBI outcomes and the value of protecting axons and myelin as a therapeutic strategy.

SUBTLE AND ACUTE AXON DAMAGE AFTER REPETITIVE BLAST EXPOSURE

The first major goal of this study was to evaluate the effects of a model of repetitive blast exposure on white matter. Reports in human studies indicate a diffuse and spatially-variable pattern of white matter damage following blast exposure (34). We chose to utilize fluorescent reporter mice that genetically label specific components relevant to axons and myelin that are expressed broadly throughout the brain. This experimental approach allowed us to screen a number of brain regions and white matter tracts while maintaining cellular specificity for detecting subtle pathologies. The *Thy1-YFP* reporter mouse line expresses YFP in a broad range of neurons allowing for visualization of white matter tracts across entire sagittal sections of the brain (38). This screening approach revealed pockets of white matter damage, seen as axonal varicosities or terminal end bulbs, in white matter tracts of the cerebellum, pons, fornix and CC. The ability to screen for axon damage in this manner is incredibly valuable. Research into the biomechanics of blast effects on the brain has identified brain regions that are vulnerable in both rodents and humans (31). However, inherent differences in brain structure means that identifying substrates where damage occurs, such as large white matter tracts, rather than specific regions will increase translational value. We focused our quantification of axon damage in *Thy1-YFP* mice on the CC. We chose the CC because it has been implicated as a vulnerable structure in blast TBI due to its proximity to the ventricles (127; 148). We had previously characterized axon damage in a model of repetitive concussive TBI using *Thy1-YFP* mice. The use of identical techniques across experiments lets us compare the extent of axon damage between repetitive blast and repetitive impact models (188). Following rBlast, the number of axonal varicosities was increased and was accompanied by mild neuroinflammation. The extent of axon damage

appears to be subtle compared to what was seen in our repetitive TBI work but *Thy1-YFP* mice facilitated detection of axon damage that may be missed by more traditional immunohistochemical techniques (43; 82; 113).

The second reporter line that we chose to utilize, the *NG2CreER;mTmG* fluorescent reporter mouse, was chosen to evaluate potential myelin disruptions. The fluorescent labeling of OPCs and their progeny following tamoxifen administration in these mice enable visualization of newly formed myelin which can serve as an indicator of remodeling and repair following injury (10). There was no significant change in the percentage of the CC labelled with newly synthesized myelin at either 4 or 8 weeks post rBlast exposure. Furthermore, the proportions of the CC composed of newly formed myelin compared to total myelin detected by MOG immunohistochemistry, was unchanged between rBlast and rSham groups at either time point. Our previous work using *NG2CreER;mTmG* mice showed changes in myelin synthesis at 4 weeks that plateaued by 8 weeks following a single concussive TBI (97). Comparing these findings between TBI models is important as demonstrates that the effects of this rBlast model may not be robust enough to disrupt myelin in a way that would facilitate later repair. There was also no evidence of CC atrophy following rBlast indicating that this model does not result in post-traumatic neurodegeneration.

This study highlights the value of fluorescent reporter mice as a means for combining broad screening capabilities with specific labeling of cellular components. The level of sensitivity afforded by these mice facilitated detection of subtle and diffuse axon damage after rBlast exposure that would likely have been missed using more traditional experimental techniques. The mild profile of white matter damage and the lack

of late-stage degeneration would make it difficult to thoroughly evaluate the therapeutic benefits of *Sarm1* deletion in this model.

CHRONIC POST-TRAUMATIC NEURODEGENERATION

Following the relatively mild findings of our rBlast study we looked to determine the chronic stage effects of a model of single concussive TBI using translational assessments. Our lab has extensively characterized the damage to axons and myelin at earlier time points in this TBI model (97; 105). We wanted to determine if acute white matter damage progressed to post-traumatic neurodegeneration that could be detected using clinically relevant techniques. We found on MR with DTI scans between 9-10 weeks post injury that CC microstructure integrity was reduced after TBI. This finding is significant because it demonstrates a preclinical replication of one of the major findings in human chronic TBI patients (56; 179). We also leveraged newly developed MRI techniques to obtain volumetric information about the CC. This data revealed that at chronic time points after TBI the volume of the CC is significantly reduced compared to sham mice. Post-imaging neuropathology revealed that significant CC atrophy in TBI mice was likely driving the decreases in volume seen on MRI. Taken together this data suggests that chronic post-traumatic degeneration of the CC is significant enough to induce changes in volume that can be detected by translation imaging techniques. We know from previous studies that this CC atrophy doesn't occur at earlier time points (97; 105; 162). These findings indicate a chronic profile of post-traumatic neurodegeneration that can be detected through clinically applicable imaging techniques.

The discovery that our concussive TBI model results in late-stage white matter pathology led us to investigate if persistence of axon damage resulted in functional

deficits translational to those experienced by TBI patients. We found that TBI induced a motor learning deficit on a miss-step wheel assay in the chronic stage of injury. Motor and coordination deficits are a major cause of disability in severe TBI patients but have also been identified in mild-moderate cases (177). Importantly, deficits in motor learning or performance were not detected when tested at earlier time points after TBI (188). A chronic stage deficit in motor learning after TBI is a clinically significant finding, especially when viewed in conjunction with the evidence from our neuroimaging and histopathological studies.

These data from our chronic TBI studies highlight two very important findings. The first is that there appears to be a chronic phenotype of white matter pathology following TBI. We know that there is an acute period of axon damage and demyelination followed by intervening periods of recovery after brain injury (10). We also know that CC atrophy and motor learning deficits don't appear prior to the 8th week post injury in this model, making them distinctly chronic TBI deficits. These data suggest an ongoing process of post-traumatic neurodegeneration. Therefore, our preclinical model of TBI provides a high degree of translational relevance to human TBI outcomes. The second major finding from this study is that experimental techniques with outcomes directly related to clinical measures are able to identify white matter pathology in our preclinical model. The ability to detect white matter damage by MRI and functional deficits analogous to those seen in humans position us well to utilize this model of TBI in our investigation of therapeutic strategies.

SARM1 IS A VIABLE THERAPEUTIC TARGET FOR CHRONIC TBI PATHOLOGY

A major goal of this project was to determine if blocking the SARM1 mediated axon degeneration pathway was a viable therapeutic strategy for improving outcome measures after brain injury. Following the determination that our concussive TBI model sufficiently replicated deficits seen in chronic human TBI we conducted a rigorous preclinical genetic proof-of-concept study evaluating the benefits of *Sarm1* deletion. The study was designed so that connections could be drawn between ultrastructural axon/myelin changes and clinically relevant neuroimaging and functional outcomes.

Through the use of electron microscopy we identified a number of ultrastructural pathologies that were attenuated in *Sarm1* KO mice. These findings provided an explanation of the sub-cellular changes that were driving the results seen on immunohistochemical, neuroimaging and functional assays. The most robust example of this connection between results seen on different techniques is the effect on CC atrophy. EM analysis showed that in the chronic stage of TBI *Sarm1* deletion prevented the significant loss of intact, myelinated axons that occurs in *Sarm1* WT mice. We subsequently detected, through MOG immunohistochemical analysis, an attenuation of CC atrophy in *Sarm1* KO mice. The preservation of axons seen through EM is likely contributing to the prevention of CC degeneration. Furthermore, MRI volumetric analysis of the CC showed that between baseline and 10 weeks post TBI *Sarm1* WT mice exhibit a significant reduction in CC volume that is prevented in *Sarm1* KO mice. This data provides sub-cellular, histological and neuroimaging evidence that *Sarm1* deletion prevents chronic CC atrophy after TBI. The ability connect findings across experimental techniques in this way significantly increases the translational validity of both SARM1 as a therapeutic target and our experimental design.

The electron microscopy data revealed a number of other, more specific axon and myelin pathologies that *Sarm1* deletion either greatly reduced or entirely prevented. At 10 weeks post TBI, both *Sarm1* WT and KO mice have a significantly increased number of damaged axons in the CC compared to shams. However, the number of damaged axons is significantly less in *Sarm1* KO compared to *Sarm1* WT after TBI. *Sarm1* deletion also had a therapeutic benefit on mitochondrial dysfunction and demyelination. In the chronic stage of TBI, *Sarm1* WT mice have a significantly increased number of axons with abnormal or swollen mitochondria as well as an increase in axons who have become demyelinated. *Sarm1* deletion prevented both of these deficits. These ultrastructural findings provide evidence for potential pathological underpinnings driving changes seen on our other experimental assays. Immunohistochemistry for markers of astrocytes and microglia showed reduced neuroinflammation in *Sarm1* KO mice compared to *Sarm1* WT at 10 weeks post TBI. Mitochondrial dysfunction can result in damage-associated molecular patterns (DAMPs) that initiate a pro-inflammatory response in glial cells that then leads to chronic neuroinflammation (187). Additionally, myelin debris in the extracellular matrix as the result of demyelination processes is sufficient to induce microglial activation (25). The prevention of mitochondrial swelling and demyelination in *Sarm1* KO mice may be contributing to the reduction in neuroinflammation in the CC after injury. These ultrastructural findings are also likely driving the changes seen over time in our longitudinal MRI study. Both *Sarm1* WT and KO mice show a progressive decrease in FA across scan time points. This decrease is driven by an acute reduction in axial diffusivity, a measure where an overall genotype effect of *Sarm1* is present. Reductions in axial diffusivity can be an indicator of axonal

pathology (183) and the overall effect of *Sarm1* may be the result of reduced axon damage seen in the EM analysis. Subsequently, radial diffusivity, which is sensitive to changes in myelination, is driving the chronic increase in FA (155). The prevention of demyelination in *Sarm1* KO mice seen on EM images does not translate to an attenuated TBI effect on RD. However, the presence of multiple pathologies make the interpretation of DTI results in this context more complicated.

Finally, we wanted to understand if the prevention of chronic stage axon and myelin pathology seen in *Sarm1* KO mice after TBI translated to functional improvements on outcome measures relevant to human TBI symptoms. Having these behavioral assays is crucial as it provides evidence that cellular changes are driving broader functions associated with whole neural circuits. *Sarm1* WT and KO mice performed the miss-step wheel assay from 8-10 weeks post TBI to match what was done in our study using C57BL/6 sham and TBI mice. *Sarm1* KO mice showed signs of improved motor learning compared to *Sarm1* WT mice indicating a degree of recovery after injury. The miss-step wheel assay is a CC dependent motor task and as previously described holds a high degree of translational relevance to human TBI deficits (104). The evidence that *Sarm1* deletion prevents chronic stage axon and myelin pathology in the CC supports the notion that improved performance on this assay is white matter dependent. We also chose to evaluate the effect of TBI and *Sarm1* deletion on sleep. The role of sleep in neurodegeneration has garnered a large amount of interest in recent years (116; 132). Sleep disruptions are widely reported amongst TBI patients and neuroimaging studies have correlated sleep problems with reduced white matter integrity (6; 50). We found that *Sarm1* WT mice have a significant reduction in sleep percentage during the

chronic stage of TBI that doesn't occur in *Sarm1* KO mice. Chronic sleep deficits can have profound impacts on long-term health (57). While the exact role that white matter plays in sleep remains to be more fully explained, the connection between the two has become increasingly appreciated. The evidence that preservation of white matter due to *Sarm1* deletion results in a functional benefit to sleep is a therapeutically very interesting finding.

Overall, this study demonstrates a significant reduction in chronic white matter pathologies and associated deficits when *Sarm1* is absent. Taken independently, each experiment provides strong evidence that when the SARM1 mediated axon degeneration pathways cannot initiate there is reduction in white matter injury after TBI. When considered as a collective body of scientific work this study demonstrates how specific pathologies seen at the ultrastructural and histopathological levels can contribute to significant changes on large scale imaging and functional outcomes. This preclinical approach, where experimental outcomes across techniques contribute to the interpretation of the results in complementary ways, may improve the efficacy of preclinical therapeutic development efforts. Through this experimental approach we demonstrate that SARM1 is indeed a viable therapeutic target for chronic TBI pathology.

FUTURE DIRECTIONS

This study is by no means all-encompassing. Further studies are needed to advance our understanding of chronic white matter damage in TBI and the efficacy of targeting SARM1. The studies in this thesis describe a number of experimental techniques, all of which have a great deal of value in answering specific questions. Preclinical study designs should seek to incorporate relevant techniques to best address

the hypothesis. Studies should also attempt to correlate results across techniques to most effectively increase translational value. For example, our repetitive blast study benefitted greatly from the use of fluorescent reporter mice. Some of these approaches, such as using *Thy1-YFP* mice, may have been useful tools in further describing the effect on axon damage of *Sarm1* deletion. Other approaches such as the use of *NG2CreER;mTmG* mice may not be as appropriate. For both fluorescent mouse lines the issue of genetic background being a potential confound in the breeding with a *Sarm1* genetic knockout mouse line would have to be a serious consideration. In the case of *NG2CreER;mTmG* mice, administration of tamoxifen, an estrogen receptor blocker, is required to mediate deletion of a stop codon to switch transcription from a constitutive expression of membrane localized tdTomato to NG2 driven expression of membrane-localized GFP. While no direct correlation between SARM1 and estrogen receptors has been described, SARM1 is a member of the Myd88 family of adaptor proteins whose role in modulating NF- κ B signaling has been shown to be estrogen dependent (68; 79; 83). Therefore the use of *NG2CreER;mTmG* mice could potentially insert a confound into our evaluation of *Sarm1* deletion effects. However, the conclusions that could be drawn from the use of *NG2CreER;mTmG* mice in our *Sarm1* studies would be incredibly informative. With the current techniques, we are able to determine that *Sarm1* deletion has a significant effect on TBI-induced demyelination but we are unable to elaborate on the time course of myelin pathology. In this context, *NG2CreER;mTmG* mice would provide further information on whether myelin preservation in *Sarm1* KO mice was due to protection of original myelin or remodeling and repair after demyelination. Future experiments should aim to develop techniques that provide similar information to what can be gathered from

NG2CreER;mTmG mice, without the underlying molecular mechanism concerns. These examples serve to make the broader point that preclinical study design must critically examine what their outcome measures say about the larger-scale value of their findings.

The promising data shown in genetic proof-of-concept studies focused on *Sarm1* has led to an increased effort develop small molecule inhibitors. Newly published work demonstrated axonal recovery in cultured cells administered a small molecule inhibitor of SARM1 (71). The data shows that following primary injury, preventing axons from initiating the Wallerian degeneration cascade pharmacologically allows them to recover from an intermediate, metastable state. These findings are an exciting first step towards the development of treatments for axonopathies. The translation of early stage small molecule inhibitors into clinically useful products will require rigorous and thoughtful preclinical evaluation. A number of the strategies utilized in this thesis would be beneficial for the purpose of testing of small molecule inhibitors of SARM1.

Translational outcome measures such as neuroimaging and functional assays have a high degree of relevance to deficits seen in patients with disorders characterized by axon degeneration (6; 20; 121; 132; 141; 177). Utilizing these strategies in preclinical studies will serve to mitigate some of the pitfalls of clinical translation that may occur as a drug navigates the developmental pipeline. The experimental techniques being utilized to assess axon protection are also constantly evolving and should be continually incorporated into pharmacological assessments. Outcome measures such as electrophysiology can provide information on whether axons that are preserved are still physiologically viable. This information could better inform the findings of behavioral

and functional outcomes to generate a more comprehensive and complete picture of the benefits of SARM1 inhibitors.

SUMMARY

This research demonstrated that white pathology and functional deficits can be attenuated by interrupting the SARM1 mediated axon degeneration pathway. Fluorescent reporter mice facilitated the finding that experimental repetitive blast exposure produces a subtle and diffuse profile of axon damage that requires further investigation before interventional measures can be explored. A model of concussive TBI however, produces a chronic phenotype of white matter pathology that can be detected using translational neuroimaging and functional assays. The development of this model enabled us to investigate the effect of *Sarm1* deletion on late-stage TBI deficits in a more meaningful way. *Sarm1* deletion was associated with a number of benefits on axons and myelin that ranged from ultrastructural to functional. These findings highlight the vital role of axons and myelin and support the idea that protection of white matter is a valuable therapeutic strategy following TBI.

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