Therapy Development for Diffuse Axonal Injury

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Abstract

Diffuse axonal injury (DAI) remains a prominent feature of human traumatic brain injury (TBI) and a major player in its subsequent morbidity. The importance of this widespread axonal damage has been confirmed by multiple approaches including routine postmortem neuropathology as well as advanced imaging, which is now capable of detecting the signatures of traumatically induced axonal injury across a spectrum of traumatically brain-injured persons. Despite the increased interest in DAI and its overall implications for brain-injured patients, many questions remain about this component of TBI and its potential therapeutic targeting. To address these deficiencies and to identify future directions needed to fill critical gaps in our understanding of this component of TBI, the National Institute of Neurological Disorders and Stroke hosted a workshop in May 2011. This workshop sought to determine what is known regarding the pathogenesis of DAI in animal models of injury as well as in the human clinical setting. The workshop also addressed new tools to aid in the identification of this axonal injury while also identifying more rational therapeutic targets linked to DAI for continued preclinical investigation and, ultimately, clinical translation. This report encapsulates the oral and written components of this workshop addressing key features regarding the pathobiology of DAI, the biomechanics implicated in its initiating pathology, and those experimental animal modeling considerations that bear relevance to the biomechanical features of human TBI. Parallel considerations of alternate forms of DAI detection including, but not limited to, advanced neuroimaging, electrophysiological, biomarker, and neurobehavioral evaluations are included, together with recommendations for how these technologies can be better used and integrated for a more comprehensive appreciation of the pathobiology of DAI and its overall structural and functional implications. Lastly, the document closes with a thorough review of the targets linked to the pathogenesis of DAI, while also presenting a detailed report of those target-based therapies that have been used, to date, with a consideration of their overall implications for future preclinical discovery and subsequent translation to the clinic. Although all participants realize that various research gaps remained in our understanding and treatment of this complex component of TBI, this workshop refines these issues providing, for the first time, a comprehensive appreciation of what has been done and what critical needs remain unfulfilled.

Key words: animal models of injury; axonal injury; non-invasive detection methods, therapeutic targeting; traumatic brain injury

Introduction

RECENTLY, THERE HAS BEEN RENEWED INTEREST in diffuse axonal injury (DAI) and its overall implications for morbidity and mortality in traumatic brain injury (TBI). This increased attention has primarily been driven by improved clinical imaging tools, which have allowed for the identification of traumatically induced axonal change in the human brain as well as the increased recognition of the importance of DAI in both the civilian sports and military blast injury settings. Nonetheless, many deficiencies remain in our understanding of the basic pathobiology of this traumatically induced axonal damage and its overall implications in

both the clinical and laboratory settings. Most notably, despite the importance of DAI in the outcome of TBI, there have been remarkably few investigations that have specifically addressed DAI therapeutics.

Accordingly, the National Institute of Neurological Disorders and Stroke (NINDS) convened a workshop entitled *Therapy Development for Diffuse Axonal Injury* in May 2011 to examine current and emerging therapeutic approaches that target DAI. Table 1 lists the workshop participants. This review attempts to encapsulate the proceedings of that meeting by addressing our current understanding of the axon's pathobiological response to trauma. Specifically, this review focuses on the pathogenesis and

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assessment of DAI in context with potential therapeutic modification. In addition, this review identifies future directions needed to fill critical gaps in our understanding of this devastating component of TBI.

Historical Perspective of DAI

Our understanding of traumatically induced axonal damage or DAI stems primarily from the clinical setting in which this term was used to categorize the pathobiology ongoing in patients who had sustained TBI, manifesting prolonged unconsciousness, coma, and/or profound morbidity, without the presence of overt focal brain lesions.¹⁻⁸ Typically, on postmortem examination, the brains of such patients showed only limited focal change other than the isolated occurrence of petechial hemorrhage, frequently observed within the splenium of the corpus callosum and/or the dorsolateral quadrant of the rostral brainstem.⁹ When probed with various histological approaches detailed below, however, this same population of patients revealed axonal damage reflected in local axonal swelling and/or beading frequently observed within the corpus callosum, parasagittal white matter, internal capsule, and thalamus.^{3-5,9,10}

Characteristically, damaged axons were found dispersed among histologically intact fibers, thus leading to its characterization as DAI, a term that has now become embedded within both the clinical and pathological literature. While in the strictest sense DAI is reserved for humans and large animal models, similar, albeit more limited and less widespread, axonal damage has been reported in various rodent models of TBI. In recognition of the occurrence of axonal injury in animal models, the term traumatic axonal injury (TAI) is often used to distinguish this more localized axonal pathology from the various stereotypic diffuse patterns seen in traumatically brain-injured persons.

Histopathological Identification of DAI

Historically, on human postmortem examination, DAI has been recognized through multiple histological approaches including hematoxylin and eosin (H&E) and silver staining, including the Palmgren technique, to detect axonal swellings and preterminal swellings, and myelin stains to detect Wallerian degeneration.^{3,6,11} More modern approaches using antibodies to transported proteins,

FIG. 1. Examples of axonal pathology in fatal cases of traumatic brain injury (TBI) as identified using amyloid precursor protein (APP) immunohistochemistry in postmortem brain tissue. Note the accumulation of APP as varicose swellings along the length of the axon and as axonal bulbs at disconnected axon terminals in (a) a 37-year-old man who died 26 h after severe TBI caused by a fall; (b) a 20-year-old man who died 2 days after severe TBI caused by an assault, and (c) an 18-year-old woman who died 21 h after severe TBI caused by a motor vehicle collision. Images were provided courtesy of Dr. William Stewart at The Glasgow TBI Archive, Glasgow, UK. Bar = 100 μ m.

such as amyloid precursor protein (APP), however, have become the gold standard in evaluating the occurrence of DAI in both the routine neuropathologic and forensic settings as well as animal investigations.^{10,12–18} Specifically, as APP moves down the axonal cylinder via anterograde axonal transport, any focal disruption/perturbation of the axon and its transport kinetics can lead to local swelling that then can be easily identified by the pooling of APP.^{19,20} Through the use of these antibodies in both neuropathologic and forensic settings, the overall occurrence and distribution of traumatically induced axonal change has become even more apparent and has extended many of those observations initially made via the use of more traditional histological approaches (Fig. 1).^{3,14,21–26}

Characteristics and Progression of Axonal Pathology in DAI

After TBI, two general forms of axonal pathology appear.²⁶ Originally referred to as retraction bulbs, single spheroids/swellings were found at the disconnected ends of axons. Now termed axonal bulbs or reactive axonal swellings (Fig. 1), the progressive swelling of these

structures has been shown to lead to disconnection.^{3,8,21,27–30} In contrast, a series of swellings along otherwise intact axons represent “axonal varicosities.”³¹ This common pathological profile found in acute TBI may reflect a process whereby transport is only partially interrupted at multiple sites along the axon’s length, producing the string-of-beads appearance, in contrast with presumed complete interruption at one site forming singular axonal bulbs.^{3,11,24,31}

Typically in humans, reactive axonal swellings can be recognized within the first hours post-injury as large focal axonal swellings 10–20 μ m in diameter that expand in size over 24–48 h reaching up to approximately 50 μ m.^{1,3,8,15,18,25,32} During this time, both axonal bulbs and varicosities are often observed together in various ratios, presumably reflecting the different rates of swelling and progression toward disconnection in individual injured axons. It is thought that axons sustaining more severe injury manifest local calcium dysregulation that causes progressive focal dissolution of the subaxolemmal spectrin and ankyrin network. This occurs together with local mitochondrial and cytoskeletal damage that impairs axonal transport while triggering the axolemma to pinch together and rapidly seal off the distal end of the swelling.^{33–49}

Collectively, these processes result in a secondary, or delayed, disconnection of the axon, creating the classic axonal bulb profile, as extensively characterized by Povlishock and colleagues (Fig. 2).^{20,21,27,28} Interestingly, in the most catastrophic axonal injuries, there is now emerging evidence that massive local ionic dysregulation triggers a dramatic local cytoskeletal collapse so severe as to reverse axonal transport and blunt the progression of any subsequent swelling (Fig. 2).^{50–53} In contrast to these two morphological manifestations of injury, axons with significantly less severe mechanical injury may have a much slower progression of swelling in one or multiple regions along the cylinder. For these axons, it is thought that some may actually recover with the restoration of ionic homeostasis and axonal transport—a finding confirmed by recent electrophysiological studies, while others continue to slowly swell and disconnect (Fig. 2).^{23–25,44,54}

Thus, multiple phenotypes of swollen axonal profiles may be found at the same time, representing a wide range in the size of swellings, the number of swellings along individual axons, and progressive stages evolving towards disconnection and degeneration. In many cases, the type of swollen profile is difficult to determine. For example, a linear series of what appear to be discrete “axonal bulbs” may either indicate a grouping of several disconnected axons with single terminal swellings, or it could signify multiple disconnection points from varicose swellings along an individual axon. These complexities highlight some of the difficulties in quantifying the extent of axonal pathology after TBI, especially in consideration of the use of histopathological analysis for evaluating therapeutic efficacy.

In addition to acute posttraumatic changes, many evaluations report that these DAI linked swellings can be observed over months to years post-injury, with the caveat that the overall number of swellings decrease significantly over time.^{1,3,22,23,25} These observations suggest that TBI can induce insidiously progressive and long-term degeneration of white matter axons. Indeed, relatively selective atrophy of the white matter is a common neuroimaging finding years after TBI.⁵⁵ As such, there may be a very broad window of therapeutic opportunity to treat DAI spanning the acute and chronic setting. As will be discussed further, however, the exact window of opportunity in relation to specific populations of injured axons remains a matter of controversy.

Biomechanics of DAI

With its massive size and non-uniform structure, the human brain can literally pull itself apart under the physical forces sustained during TBI.^{7,8,56,57} In particular, axons in the white matter are poorly prepared to withstand damage from the rapid acceleration/deceleration of the head.⁵⁸ The principal mechanical force associated with the induction of DAI is head rotational acceleration, resulting in dynamic shear, tensile, and compressive strains of the brain tissue.^{7,8} Although these tissue deformations rarely lead to disconnection of axons at the time of injury, they nonetheless can precipitate a spectrum of focal pathological abnormalities in axons that can lead to delayed secondary disconnection or sustained dysfunction.^{3,8,18,21,27,44,51,54,59,60}

The selective injury to axons in TBI appears to be related to their viscoelastic nature reflecting a unique ultrastructure and collective anisotropic arrangement in tracts. Within axons, microtubules and neurofilaments are arranged in a linear fashion with perpendicular protein projections and cross-links. Also unique is a large surface to volume ratio of the axon membrane (axolemma) to the axoplasm. During normal daily activities, axons are supple and can accommodate substantial mechanical deformation, such as stretching.^{8,31,58,61}

Indeed, under quasistatic loading conditions, axons can easily be stretched for over 100% of their original length and spring back without any evidence of structural or functional failure.⁶¹ Constituents of axons, however, are thought to become brittle under very rapid mechanical loading conditions, predisposing vulnerable structures in the axolemma and/or axoplasm to mechanical damage, typically occurring at discrete points along the axon's length.⁶² This classic viscoelastic response to rapid deformation prompts a classification of a dynamic injury, wherein the applied forces occur in less than 50 millisecond, thereby exposing axons to high strain rates as the brain is rapidly deformed.⁸

From the perspective of potentially successful therapeutic intervention, it is also important to note that the mechanical damage to axons with TBI is the direct result of the initial axonal dynamic deformation. Importantly, this biomechanical genesis distinguishes DAI from axonopathies found in any other neurological disorder. In addition, the unique aspects of primary mechanical damage and its aftermath may provide therapeutic targets specifically for DAI. This area of investigation, however, is only in its infancy and much progress must be achieved. To sort out the unique biomechanical processes of DAI, multiple investigations have turned to clinically relevant experimental models.

Experimental Modeling of DAI and TAI

As above, the term DAI is used to denote the finding of widely distributed axonal pathology spread through the white matter domains of the human gyrencephalic brain after TBI. According to this strict definition, DAI can only be reproduced in gyrencephalic animal models. Thus far, however, there have been a paucity of large animal models of DAI because of the difficulty in replicating the brain injury biomechanics that occur in human TBI, such as the inertial loading conditions produced in the brain during automotive crashes or at the moment of head impact.⁸ Thus, various forms of small animal and *in vitro* models of TAI have been developed for expediency, including ease of use and the ability to generate higher throughput evaluations.⁵⁹ This wide range of modeling has been instrumental in identifying important biomechanical and pathophysiological aspects of axon trauma. In particular, these models have played a central role in the development of therapies aimed at reducing axonal pathology after TBI. The following is a brief account of current models of DAI and TAI:

Gyrencephalic animal models of DAI

The size of the human brain plays an important role in the development of DAI. For example, during head trauma, there can be substantial mass effects of pushing and pulling between regions of the large human brain, resulting in tissue strain at a high strain rate. In addition, the anatomical features of the human gyrencephalic brain lead to selective injury of white matter axons, potentially because of their high organization and unique cytoskeletal features as described above. Accordingly, for the first model of DAI, non-human primates were chosen because of their relatively large gyrencephalic brain with ample white matter domains. In landmark studies in the 1980s, Gennarelli and colleagues⁷ demonstrated that non-impact head rotational acceleration in non-human primates using a pneumatic actuator could induce an immediate and prolonged coma. Moreover, this model produced DAI with a microscopic character identical to that found in human TBI, the extent of which correlated with the length of unconsciousness. Notably, however, to create the same dynamic tissue deformations as calculated for human TBI, the rotational forces used for the

FIG. 2. Evolving pathophysiology of traumatic injury in myelinated axons. In this figure, we attempt, in an abbreviated fashion, to illustrate some of the key events believed to be involved in the pathobiology of traumatic axonal injury and, thereby, identify potential therapeutic targets. Although framed in the view of primary nodal involvement (A), this focus does not preclude comparable change ongoing in other regions of the axon. Panels B and C show normal axonal detail including the paranodal loops and the presence of intra-axonal mitochondria, microtubules, and neurofilaments, together with the presence of multiple axolemmal channels localized primarily to the nodal domain. Mild to moderate traumatic brain injury in panel D is observed to involve a mechanical dysregulation of the voltage sensitive sodium channels, which contribute to increased calcium influx via reversal of the sodium calcium exchanger and the opening of voltage gated calcium channels. This also impacts on the proteolysis of sodium channel inactivation that contributes further to local calcium dysregulation. Microtubular loss, neurofilament impaction, and local mitochondrial damage can follow, which, if unabated, collectively alters/impairs axonal transport illustrated in panel E. Alternatively, if these abnormalities do not progress, recovery is possible (F). When progressive, these events not only impair axonal transport but also lead to rapid intra-axonal change in the paranodal and perhaps internodal domains that elicit the collapse of the axolemma and its overlying myelin sheath to result in lobulated and disconnected axonal segments (G) that, over the next 15 min–2 h, fully detach (H). The proximal axonal segment in continuity with the cell body of origin now continues to swell from the delivery of vesicles and organelles via anterograde transport while the downstream fiber undergoes Wallerian change (I). Lastly, with the most severe forms of injury, the above identified calcium-mediated destructive cascades are further augmented by the poration of the axolemma, again primarily at the nodal region (J). The resulting calcium surge, together with potential local microtubular damage and disassembly, pose catastrophic intra-axonal change that converts anterograde to retrograde axonal transport, precluding continued axonal swelling, while the distal axonal segment fragments and disconnects (K), with Wallerian degeneration ensuing downstream (L).

non-human primate model had to be substantially scaled up to account for the over 10-fold smaller brain mass compared with humans.

In subsequent studies using the same injury device, a DAI model was developed using miniature swine, which have gyrencephalic brains of similar size to non-human primates.^{56,57} It was found that distribution of axonal pathology, relative to the plane of head rotational acceleration, was important in the induction of coma in moderate-to-severe TBI and transient loss of consciousness in mild TBI.^{63,64} In particular, damage to brainstem axons appeared to be a primary factor in the immediate loss of consciousness. This finding, however, was independent of the overall extent of axonal pathology throughout the brain hemispheres. Specifically, surprisingly extensive DAI can occur even without a marked loss of consciousness, and that loss of consciousness appears to be dependent on the density and/or distribution of axonal pathology in select regions, such as the brainstem. More recently, additional gyrencephalic animal models of DAI have been developed, such as captive bolt head impact in sheep and pigs and head rotational acceleration in neonatal pigs and adult rabbits.^{65–68}

For evaluation of emerging therapies for DAI, the large animal studies benefit from their close relation to the human brain. They suffer from limitations for determining mechanisms of action, functional analyses, and number of subjects achievable for each experimental group, however. As such, there have been very few studies using large animal models of DAI to evaluate therapeutic efficacy.⁶⁹ This has stirred renewed interest and refinements in small animal and *in vitro* models of TAI for more general screening for potential therapies.

Lissencephalic animal models of TAI

Virtually all lissencephalic rodent models of TBI produce some form of axonal pathology, which is usually found in subcortical structures such as the corpus callosum, thalamus, and brainstem. There has been much debate regarding which model is most clinically relevant, however. Important biomechanical considerations include the type of tissue deformation that leads to the axonal pathology. Notably, parameters for brain impact models (e.g., weight drop, central and lateral fluid percussion, controlled cortical impact, impact acceleration (Marmarou model) can create both stretching and compression of the brain tissue.^{59,70–75} Axonal injury resulting from brain impacts with substantial intrusion into the brain via skull depression, insertion of a mechanical impounder or fluid pulse, however, may not approximate characteristics of DAI found in human TBI. Rather, models using these parameters often display hemorrhagic contusion of the tissue below the impact site from dynamic crush injury. Focally, this injury often includes primary disconnection or even ablation of white matter axons, which does not correspond with the conventional understanding of the mechanisms or pathological features of clinical DAI.

Acknowledging that the lissencephalic rodent brain has relatively sparse white matter domains, it may nonetheless be possible to set injury parameters of current models to mimic the tensile and shear loading of axons as occurs in human DAI. Accordingly, there has been a recent shift toward modifying injury parameters in small animal models to avoid the production of contusions and, instead, focus on dynamic deformation that selectively injures the white matter. Showing signs of success, some models produce selective axonal pathology of similar appearance to that found after TBI in humans.^{74,76,77} Progress toward identifying optimal small animal models of TAI will clearly be important for the development of therapies for DAI.

A creative alternative approach for inducing direct tensile elongation of a white matter tract in lissencephalic animals is the use of dynamic stretching of the optic nerve. Thus far, optic nerve stretch injury models have been developed in guinea pigs, rats, and mice and use biomechanical loading conditions thought to be important for white matter deformations leading to DAI in humans.^{39,78,79} Owing, in part, to the isolated nature of the white matter injury, these models have proven very useful in evaluating ultrastructural changes in axons after trauma as well as identifying pathological chemical cascades that may represent important therapeutic targets.^{34,39,47,78–93} These models, however, are not widely used or practical for high-throughput therapy evaluation. Nonetheless, they represent a potential stepwise bridge between *in vitro* and *in vivo* therapy development.

Alternatively, modification of the central fluid percussion model of traumatic injury has resulted in a highly reproducible and well characterized model of scattered white matter damage that lends itself to high-throughput therapy evaluation via systemic, intrathecal, and intraocular administration strategies.⁹⁴ Importantly, with fluid percussion injury, the axonal damage occurs more remote from the retina than seen with optic nerve stretch, and this axonal injury occurs without the retinal ganglionic cell death described after stretch.⁹⁴ These findings highlight model differences, while suggesting that fluid percussion injury in the visual axis may offer even more opportunity to explore post-traumatic repair and reorganization in the visual axis.

In-vitro models of TAI

To directly assess the evolution of traumatic injury of axons, *in vitro* modeling has proven very powerful. As with optic nerve stretch injury, these models allow for controlled biomechanical input, such as setting precise levels of strain and strain rate. Two general models have emerged that induce uniaxial (one direction) stretching of axons spanning two populations of neurons via either rapid extension of an elastic substrate in the longitudinal direction of the attached axons or using a pressurized fluid jet.^{31,33,58,61,62,95–98} Important findings from these models include demonstration of primary rupture of axonal microtubules, evolving proteolysis, and loss of ionic homeostasis, collectively revealing multiple potential therapeutic targets as discussed below.

While these models are not currently used for extensive evaluation of TAI therapies, recent advances may allow for higher throughput analyses than is possible in animal models.^{31,61,99} Notably, neurons in culture can be micropatterned to create a ladder of axon fascicles spanning two populations of neurons.^{31,61} This provides a testbed for rapid automated quantitative assessment of the therapeutic efficacy on outcomes such as intra-axonal calcium concentrations and/or axonal degeneration after injury. In addition, these systems may be used to evaluate injury of both myelinated and non-myelinated axons.⁹⁹ Moreover, a multi-well system of *in vitro* TAI has recently been developed that may further expand the capacity for therapy evaluation and development.¹⁰⁰ Because the clinical relevance of *in vitro* screening systems for TAI therapies has not been established, however, it will be important to run initial studies in coordinated parallel efforts with small and large animal models.

Emerging Non-Invasive Detection of DAI

In consideration of anticipated clinical trials to address DAI, non-invasive detection of axonal injury will be a critical aspect of diagnosis and evaluation of therapeutic efficacy. Indeed, there are

mounting efforts to develop such non-invasive techniques to probe the severity, distribution, and temporal evolution of axonal pathology. Based on direct postmortem neuropathological analyses, it is already well characterized that DAI is one of the most common and important pathologies for moderate to severe TBI. As for mild TBI or “concussion,” DAI has also recently become widely accepted as the primary pathological substrate.^{101,102} Because by definition, however, mild TBI is non-fatal, there has been very little direct neuropathological evidence of DAI after mild TBI in humans. Nonetheless, the recent application of advanced neuroimaging techniques, electrophysiological examination, blood biomarker analysis, and neurocognitive evaluation may transform the detection in DAI after TBI, particularly for mild TBI.

Because of the importance of standardized data collection with clinical evaluation of TBI, including examination of DAI, a U.S. Federal Inter-Agency Common Data Elements (CDE) project was launched in 2008 and is run in parallel CDE initiatives for neuroimaging, biomarker, and neuropsychological testing of TBI (see below).

Advanced neuroimaging

Historically, the widely distributed, microscopic nature of the axonal pathology in DAI rendered it essentially invisible with conventional brain imaging. As such, DAI was often a “diagnosis of exclusion” for patients with persisting symptoms related to head injury, but no radiological findings. In some patients, minor changes in the white matter have been found with conventional imaging techniques but likely reflected associated pathologies, such as microbleeds, rather than actual axonal pathology.^{32,103}

Currently, no specific CDE guidelines were provided for advanced neuroimaging methods aimed at detecting DAI because the committee uniformly thought that these techniques are still very early in development with great incongruity in their use between centers.^{104,105} Nonetheless, there has been substantial progress using various techniques that identify white matter abnormalities in TBI not seen with conventional neuroimaging. Studies using techniques such as diffusion tensor imaging (DTI) have greatly enhanced the appreciation of the overall magnitude and distribution of axonal injury in DAI.

Most of the advanced neuroimaging techniques take advantage of the unique anisotropic nature of the white matter to indirectly probe disruption of axons. For example, DTI and other advanced neuroimaging techniques can elucidate signal changes in the white matter that have been shown to correlate with axonal pathology in animal models.^{106–110} In humans with mild TBI, DTI has also shown changes in the white matter, including both increases and decreases in fractional anisotropy and other DTI-related metrics that evolve over time.^{111–117} In some cases, these imaging changes have been linked to varying degrees of morbidity, suggesting a causal relationship.

At present, however, much remains to be further characterized with these new imaging approaches if they are to be incorporated into common medical practice. An obvious major critique is that signal changes in humans cannot be correlated with actual neuropathology. Although animal models of TBI have provided histopathological comparisons with advanced neuroimaging findings, as above, it is not always clear how these data can be extrapolated to human TBI. Thus, it is essential that neuroimaging techniques are further advanced and validated in anticipation of using them to non-invasively measure therapeutic efficacy in TBI treatment trials.

Electrophysiological aspects of DAI

Although electrophysiological detection of DAI may also be an important non-invasive clinical tool, even more limited progress has been made compared with advanced neuroimaging. Nonetheless, animal research has provided relatively compelling insight into electrophysiological changes after TAI. Following mice subjected to mild injury with TAI, Greer and colleagues¹⁰¹ identified electrophysiological abnormalities in both the axotomized and non-axotomized neuronal soma. With mild TBI, neurons sustaining TAI did not die, yet they revealed decreased excitability. Unexpectedly, in the same preparations, the non-axotomized/non-injured neurons demonstrated increased excitability over time, demonstrating that the mild TBI was associated with both structural and functional changes that most likely exerted their effects via alteration of neuronal networks and circuits, an issue of emerging interest in the field of TBI.¹⁰¹

In terms of axonal conduction itself, Baker and associates¹¹⁸ first showed, in traumatically brain-injured rats, that the presence of axonal pathology correlated with a generalized reduction in the overall compound action potentials within the corpus callosum. Reeves and colleagues^{54,119} confirmed this finding and, importantly, extended it by demonstrating within the corpus callosum that this generalized alteration in compound action potentials involved not only the myelinated axonal populations but also the unmyelinated fiber populations. Moreover, Reeves and colleagues^{54,119} found that the myelinated axonal population showed a trend for recovery over a several day posttraumatic period, consistent with little histopathological evidence of irreversibly injured myelinated axons. In contrast, the unmyelinated fiber population showed virtually no recovery over time, demonstrating that these axons were more vulnerable to irreversible damage from trauma.

These studies are important from several perspectives in that they suggest that a TBI can cause profound interruption in various conduction pathways in the early phases of injury, with a potential for partial recovery over time, at least in the large caliber myelinated fiber population, a finding that is entirely consistent with the immediate functional disruption, which initially occurs with TBI and shows partial recovery over time. The persistence of electrophysiological abnormalities within the fine fiber population, in contrast, argues that this fiber population may be more severely perturbed and, as such, a major player in continued morbidity. While the overall implications of unmyelinated fiber involvement in the corpus callosum and/or other white matter structures is not well appreciated, there is emerging thought that such fine caliber axons participate in intrahemispheric processing, perhaps explaining the intrahemispheric disconnection and circuit disruption described after TBI via functional MRI.^{120,121} While this issue obviously needs further investigation, it may explain some of the continued morbidity associated with DAI.

For general clinical evaluation, electrophysiological testing provides a non-invasive means of directly assessing brain activity and function relative to TBI. In particular, to identify the function of white matter pathways after TBI, evoked potentials have long been used to analyze the full spectrum of TBI severities. For example, graduated loss or delay of wavelets in brainstem and middle latency auditory evoked potentials after TBI are thought to be a good predictor of outcome and can identify subclinical dysfunction. Nonetheless, there is debate regarding the sensitivity of evoked potentials in TBI and, while promising, the approach has yet to be widely used or validated.

Biomarkers

Ideally, initial testing for DAI will be performed in the very acute setting similar to the current evaluation of myocardial infarction by examining troponin levels in the blood. It is anticipated that examination of “surrogate biomarkers” of TBI from body fluids would direct or supplement additional non-invasive testing. While no such test currently exists, there are many efforts under way to identify TBI protein product biomarkers released by injured brain cells using cell biological and neuroproteomic approaches in serum and cerebrospinal fluid (CSF).^{122–124} A validated and sensitive test would be especially important for mild TBI with suspected DAI, where typically there are no radiological findings. Identifying biomarkers to detect damage in mild TBI, however, has been very challenging, considering that CSF is not likely to be collected from this patient population and there is little perturbation of the blood-brain barrier that would allow biomarkers to reach the systemic circulation for routine blood analysis.

To address these challenges, several groups have characterized multiple proteins and their breakdown products as potential biomarkers for traumatic and other forms of acute brain injury in both animals and humans. Among these, recent studies have suggested that α II-spectrin breakdown products SBDP150 and 145, produced by calpain cleavage, have been linked to acute neuronal necrosis, while SBDP120, produced by caspase 3, has been linked to apoptotic cell death cascades.^{41,122,125–131} While these α II-spectrin breakdown products have been discussed within the context of neuronal damage, it is important to note that they have been associated with both TAI/DAI and its downstream target deafferentation and synaptic loss.^{38,51,132–134}

In addition to spectrin breakdown products, other potential markers of axonal injury include various phosphoforms of the neurofilament-H subunit as well as cleaved tau suggesting damage to the axon cytoskeleton.^{135–138} Also, a decrease and proteolytic breakdown of myelin basic protein may be an indicator for ongoing Wallerian degeneration and its associated axonal destruction.^{137–143} The potential utility of these biomarkers will be influenced by their different expression profiles over time post-injury because of their differential roles in the initiation of axonal damage and its progression. Despite these limitations, however, these biomarkers offer the most promise for the detection of DAI.

While not directly related to the progression of DAI, several biomarkers hold promise in understanding the evolution of focal vs. diffuse pathological changes after TBI. Using a systems biology-based approach to select top down candidate markers, ubiquitin C-terminal hydrolase L1 (UCH-L1) has been demonstrated in both CSF and serum of both animals and humans after TBI.^{135,144–152} This biomarker is associated with the occurrence of neuronal cell death, particularly in association with large contusional lesions. Likewise, glial fibrillary acidic protein (GFAP) and its breakdown products may be important companion biomarkers for distinguishing focal and diffuse pathologies in TBI. Specifically, there is a dramatic increase in serum of GFAP and its breakdown products associated with large contusional changes and the associated neuronal death. Conversely, more diffuse neuronal changes associated with diffuse brain injuries result in less elevated levels of these biomarkers. While these studies are in themselves important, they must be framed in the context of diffuse and focal neuronal loss rather than in the context of axonal injury.

In consideration of evaluating post-traumatic ischemic damage, many markers have been found in CSF and blood after cerebral ischemia. These include calpain derivatives of α II-spectrin, NFH

phosphoforms, and UCH-L1, as well as neuron-specific enolase and 14-3-3 b and z isoforms, found acutely after global or focal cerebral ischemia in both experimental animals and humans.^{123,136,145,153}

It is anticipated that TBI biomarker evaluation will play an essential role in assessing the burden of DAI and its potential therapeutic modulation. While there has been substantial progress, however, it remains unclear when a validated test will emerge.

Neuropsychological evaluation

Neuropsychological testing is unable to directly confirm the presence or extent of DAI, but it does provide an indirect measurement of the probability of DAI. DAI has been traditionally associated with impairment of consciousness in the acute and subacute post-injury phases of injury, and then poorer outcome in chronic phases of recovery. Associated cognitive deficits most often include slowed processing speed and disruption of memory and executive functioning, although these may be less persistent at the milder ranges of injury severity. Studies relating neuroradiologic evidence compatible with DAI have demonstrated chronic cognitive dysfunction, although the specific pattern of deficit may vary.¹⁵⁴ Although early studies of cognitive deficits attempted to link visible “lesions” with specific cognitive deficits, cognitive performance and functional outcome have been more recently presumed to be impacted not only by damage to specific cortical regions, but also by more general compromise of the integrity of underlying white matter, which may connect topographically distinct regions.

As with neuroimaging and biomarker analyses, an Inter-Agency CDE initiative has also addressed data archiving for the neuropsychological testing of TBI.¹⁵⁵ While significant work has been done (and is continuing) to gain consensus and recommend CDEs for outcome measures for use in clinical trials and other TBI-related research, limitations of existing outcome measures remain.^{156,157} For example, the most widely used primary outcome measures in clinical trials have been the Glasgow Outcome Scale (GOS) and the GOS-Extended. Despite continued use as primary outcome measures, they remain problematic given the limited range of values and insensitivity in a number of contexts (e.g., changes in an acute or subacute or rehabilitation post-injury interval, mild TBI, etc.). At present, there are very few measures that can be administered in an acute or subacute post-injury interval in moderate to severely injured patients and even fewer that are useful in studies that span a spectrum of injury severity or acuity. Clearly, additional test development is needed, especially with regard to achieving sufficient statistical power for therapeutic evaluation.

Evolving Pathophysiology of Axonal Trauma, Therapeutic Targets, and Rationale

While it is now clear that mechanical traumatic injury ultimately sets the stage for dysfunction, swelling, and disconnection of axons, our understanding of the initiating mechanisms involved in this sequence remains incomplete. Likewise, how primary mechanical damage can lead to multiple secondary cascades has only been partially explored. Perhaps reflecting this early state, surprisingly few therapeutic approaches specifically targeting DAI have been examined. Nonetheless, as therapeutic targets emerge, it is becoming clear that treatments must span a temporally broad window of therapeutic opportunity across a range of severities of axonal injury. The following addresses emerging therapeutic targets in context with specific pathophysiological changes:

Cytoskeleton stabilization

As noted previously, primary disconnection of axons is a rare event. Rather, at focal points along the axon's length, impairment of axonal transport can occur, leading to local axonal swelling, lobulation, and disconnection (Fig. 2). The rate at which disconnection occurs may depend on the extent of specific pathologies within the axon.³¹

At the extreme end of TAI, primary mechanical damage of axonal microtubules represents an obvious mechanism that can trigger rapid accumulation of transported cargoes. This type of mechanical disruption at the time of injury has been observed *in vitro* within fine caliber, unmyelinated axons, resulting in undulations of axons from breakage of microtubules that block complete relaxation to the pre-stretch length of the axon (Fig. 3).⁶¹ Notably, these same axonal undulations are also commonly observed acutely after TBI in humans; however, because these axons are typically large caliber with a neurofilament rich core, the extent of microtubular breakage and its role in the formation of axonal undulations is unclear.³¹

At high levels of axon stretch injury, the primary mechanical breakage of individual microtubules is followed by a secondary chemical depolymerization of the remaining microtubules in the undulations, which releases the relaxation block.^{31,61} A similar focal loss of microtubules in injured axons has also been found after optic nerve stretch injury in the guinea pig as well as rat TBI models.^{47,87,158,159} With the complete loss of microtubules, axonal transport is totally derailed, triggering unmitigated accumulation of the cargoes. In turn, the resulting swelling can lead to disconnection and degeneration.^{31,61}

Nonetheless, even this severe mechanical damage may be reduced with a therapeutic intervention. Indeed, stabilizing microtubules in injured axons *in vitro* with taxol was found to inhibit the secondary chemical depolymerization of microtubules, thus greatly reducing swelling and degeneration.⁶¹ While not yet tested in animal models, recent development of a host of taxol-like therapies (taxanes) aimed at treating Alzheimer and other neurodegenerative diseases may pave the way for this therapeutic approach for DAI as well.

Ion homeostasis

Large axonal swellings from transport interruption are considered only one end of a range of pathologic changes under the heading of "DAI." During TBI, all axons within a white matter tract are thought to suffer relatively similar dynamic deformations.²⁵ Surprisingly, however, even in severe TBI in humans, only a small percentage of axons within a given white matter tract undergo cytoskeletal disruption resulting in accumulation of transported cargoes in swellings.^{13,14,21,25} Nonetheless, it is thought that a pathological process shared by all injured axons is the loss of ionic homeostasis.

The extent of posttraumatic changes in intra-axonal ion levels is thought to range from mild and reversible to catastrophic. For myelinated axons, alterations in ionic flux are thought to occur primarily at the nodal and/or paranodal regions (Fig. 2), although recent evidence suggests they can also occur along the internodal axolemma. For unmyelinated axons, aberrant posttraumatic ionic fluctuations are thought to be more evenly distributed along the axon (Fig. 3).

Of particular focus with ionic inhomeostasis is post-traumatic increase in intra-axonal calcium concentration, which can trigger a host of deleterious cascades. Emerging evidence suggests that increases in intra-axonal calcium concentrations may result from

several sources. Maxwell and colleagues³⁴ found indirect evidence of post-traumatic calcium influx into axons via changes in calcium-ATPase activity after optic nerve stretch injury. Subsequently, using an *in vitro* model of axonal stretch injury, mechanical dysregulation of the voltage sensitive sodium channel was shown to induce progressive influx of calcium by reversing sodium-calcium exchange and opening of voltage gated calcium channels (Fig. 2D).^{33,96} In addition, proteolysis of the sodium channel inactivation gate by calcium activated protease, calpain, was found to lead to persistent increases in intra-axonal calcium.¹⁶⁰ Release of intra-axonal calcium stores after injury has also been observed.⁹⁸ These effects represent potential therapeutic targets using agents that block sodium channels, calcium channels, and sodium-calcium exchangers or by calpain inhibition as described below.

In another *in vitro* model of axon stretch injury, increases in intra-axonal calcium concentrations were inhibited by the calcium-activated phosphatase, calcineurin, demonstrating intracellular release of calcium after injury.⁹⁸ Notably, this treatment also resulted in reduced secondary axotomy. As such, further study is needed to establish whether these same events are operant *in vivo*.

It has also been suggested that increases in intra-axonal calcium concentrations from TAI can occur through frank alterations of axonal permeability, including poration of the axolemma (Fig. 2D).^{158,161–164} While there is debate of the mechanisms, poration may be a primary mechanical effect or a secondary event triggered by a chemical dissolution of the axolemma.^{58,165} In either case, therapies that address membrane fluidity and repair, such as polyethylene glycol and the surfactant, Poloxamer 188, may be beneficial for axons with modest or transient openings of the axolemma or for those that are at risk of a breach of the axolemma.^{166–169}

Protease inhibition

Several laboratories have shown that after TBI, there is local activation of the cysteine proteases calpain and caspase 3 in axons that increase over a several hour period, consistent with the progression of dissolution of the axonal cytoskeleton. In particular, activated calpain has been well studied with regard to proteolysis of subaxolemmal spectrin, with the resultant breakdown products detected in damaged axons and in the CSF.^{38,41,42,133,134,165,168,170–172} Less well known is the role of calpain on ankyrin proteolysis, which may lead to disordered sodium channel arrangement at the nodes of Ranvier, and its altered binding to neurofascin, which may contribute to axolemmal instability.¹⁷³

Several *in vivo* studies have examined various axonal protective strategies targeting calcium-mediated responses by either direct or indirect means. Calpain inhibitors such as MDL28170, 5b, AK295, and SJA-6017 have been used with multiple studies suggesting significant axonal protection after TBI, although more recently, this protective effect has been challenged.^{37,40,92,174,175} In addition, currently available calpain inhibitors lack specificity with multiple sites of action, thereby complicating the interpretation of their axonal protective effects. Moreover, the brain bioavailability of current calpain inhibitors delivered intravenously has also been questioned.

Mitochondria protection

In concert with examination of cysteine protease activation, other studies have shown that these calcium-initiated events occur in proximity with dramatic mitochondrial swelling, disruption of cristae, and mitochondrial fragmentation (Fig. 2D and E and 3F).¹⁵⁸

FIG. 3. Evolving pathophysiology of traumatic injury in unmyelinated axons (A). As established in the literature, the unmyelinated axon with a large axolemmal to cytoplasm ratio is easily susceptible to forces of injury. Panel B illustrates that along the axolemmal length, voltage sensitive sodium channels, sodium-calcium exchangers, and voltage sensitive calcium channels are found. In addition, note that in panel C, fine caliber unmyelinated axons that are typically less than $1\ \mu$ in diameter, contain primarily mitochondria that actually deform the axolemma, together with numerous microtubules distributed throughout the axoplasm (C and D). With mild through moderate TBI, calcium dysregulation occurs (E), together with potential mitochondrial damage and microtubular disruption, all of which lead to impaired axonal transport and axonal swelling (F). Such swelling can occur either in isolation or as multiple varicosities (G and H). Again, over a relatively brief posttraumatic timeframe, this focal interruption in calcium ion homeostasis, microtubular stability, and mitochondrial integrity can lead to focal disconnection of the axon cylinder with the segment in continuity with its cell body of origin continuing to swell because of delivery of organelles and vesicles via a disruption in axonal transport (I) with the detached distal segment undergoing Wallerian change (J).

While evidence to support the mechanisms involved in this mitochondrial damage is incomplete, most data point to the likelihood that the same intra-axonal calcium loads that activate the cysteine proteases also contribute to mitochondrial calcium overloading, which in the face of other factors, including the activation of oxygen radicals, trigger mitochondrial permeability transition. This leads to the demise of the mitochondria and their ability to provide the high energy phosphates needed to maintain local axonal structure and function.¹³²

To address mitochondrial damage and dysfunction after TBI, Okonkwo and colleagues^{42,45,176} first used the immunophilin ligand cyclosporin A (CsA), which protects mitochondria in injured axons by inhibiting calcineurin as well as preventing the opening of the mitochondrial permeability transition pore. In a rodent model of TBI, CsA treatment was found to reduce the axonal mitochondrial swelling and disruption, thereby attenuating the extent of axonal damage.^{42,45} Studies conducted by Büki and colleagues reaffirmed this finding, with the further observation that CsA also reduced

calcium-mediated cytoskeletal disruption.^{42,51} In addition, Colley and coworkers,¹⁷⁷ showed that CsA treatment provided preserved compound action potentials in myelinated axons. This same effect, however, did not translate to the unmyelinated axons after injury.¹⁷⁷

Subsequently, FK506, another immunophilin ligand that attenuates calcineurin but does not affect mitochondrial permeability transition, was evaluated. Surprisingly, FK506 provided dramatic axonal protection after TAI with a reduction in axonal pathology and protection of electrophysiological function, particularly in unmyelinated axons.^{44,178,179} While this may initially suggest a less important role of mitochondrial permeability in TAI pathogenesis, recent studies suggest that FK506 inhibition of calcineurin-influenced translocation of BAD to BCL-X in mitochondria may also influence the opening of the mitochondrial permeability transition pore. Thus, while controversy remains regarding mechanism, it appears that the immunophilin ligands CsA and FK506 are promising agents to preserve axons after TAI.

Hypothermia

Potentially acting as a general metabolic inhibitor, post-traumatic hypothermia treatment has also been shown to attenuate the burden of axonal damage in rodent models of TBI.^{43,79,180,181} Notably, early hypothermic intervention exerted maximal effect, which could be complemented with the use of more extended therapeutic intervals.¹⁸² Moreover, this protection was optimal when the hypothermic interval was followed by slow rewarming, whereas rapid rewarming was shown to exacerbate axonal pathology.¹⁸³ With an eye on combinational therapies, the use of either FK506 or CsA during the rewarming phases imparted further protection.^{179,184} Interestingly, both hypothermic intervention and immunophilin ligands also improved cerebral microcirculation, which may have contributed to axonal protection.^{184,185}

Unfortunately, the relatively robust protective effects of hypothermia observed in the preclinical setting did not translate in two large clinical studies of TBI patients,^{186,187} where no benefit was observed in the patient population sustaining diffuse injury, although in a subpopulation of patients who needed hematoma evacuation, some benefit was found.^{186–188} It has been suggested that the general failure of the clinical trials using hypothermia stemmed from the fact that hypothermia was initiated several hours postinjury—far later than animal studies—thereby missing the optimal window of opportunity. This negative finding points out the major challenge for pre-clinical evaluation of using realistic timing of therapies that would be applied in the clinical setting. Indeed, most agents tested in pre-clinical models display maximal efficacy only in the first hours postinjury, which decreases feasibility for clinical application.

Moving forward, it is of interest that combinational approaches incorporating hypothermic intervention with the delayed administration of immunophilin ligands may work synergistically and extend the window of therapeutic opportunity.^{185,188,189} Potentially, hypothermia impedes the progression of TAI, allowing adjunct drug therapy to superimpose protective effects at more delayed time points. Regardless, combinational therapy with hypothermia merits continued investigation.

Additional therapeutic approaches

Although less well studied, there is emerging evidence of other agents that may provide axonal protection in TBI. Several studies support the use of oxygen-radical scavengers, steroids, neurotrophic factors, or dietary supplementation,^{183,190–194} Although each of these

classes of agents shows various degrees of axonal protection, they, too, will require further development. In addition, other promising agents are in the pipeline, including erythropoietin and somatostatin, and these, too, will require detailed evaluation.

Research Gaps and Future Directions

As is clear from the previous passages, the last decade has witnessed significant advances in our understanding of the pathophysiology of DAI, which is now recognized to have multiple manifestations reflecting fiber type, severity of injury, and the temporal evolution of pathogenesis. Although daunting, our improved understanding of the diverse anatomical and physiological changes evoked by DAI in multiple fiber populations now allows us, for the first time, to turn more aggressively to the better targeting of those subcellular mechanisms that may be most amenable to therapeutic intervention.

While we have identified several candidates that hold promise in treating at least some of the components of DAI, it would be premature to suggest that these should move forward to clinical trial. These, as well as other newly identified protective agents, must undergo more rigorous preclinical evaluation to better position these or any other future agents for use in human clinical trials.

As will be soon suggested by the National Institutes of Neurological Disorders and Stroke, more attention must be focused on preclinical drug discovery and its careful evaluation and interpretation. For some of the agents identified in this communication as well as others coming online, more attention must be placed on the details of preclinical discovery. These should include appropriate power analysis to determine animal number, with incorporation of potentially multiple animal models of axonal injury to determine if any potential protection proves universal. Similarly, more focus and attention must be placed on the monitoring of general physiological responsiveness to determine if parallel changes in animal blood pressure, oxygenation, blood gas status and/or temperature would be confounds in interpreting any drug's purported beneficial effects.

Likewise, as alluded to in this article, some of the most promising candidates should be carried forward into higher order animal models of injury such as those involving rapid acceleration/deceleration of the brain. This would allow a better reproduction of the actual forces sustained with human TBI and its spatial/temporal distribution throughout the injured brain parenchyma. In addition, in these end-stage studies in higher order animals, the incorporation of appropriate imaging tools and/or the use of biomarkers could further strengthen the overall interpretation of the data generated and its overall implications for axonal protection. More importantly, the success of these surrogate markers could also provide important clues for parallel assessments conducted in clinical discovery.

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Author Disclosure Statement

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