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Review Article

All roads lead to disconnection? – Traumatic axonal injury revisited

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Summary

Traumatic brain injury (TBI) evokes widespread/diffuse axonal injury (TAI) significantly contributing to its morbidity and mortality. While classic theories suggest that traumatically injured axons are mechanically torn at the moment of injury, studies in the last two decades have not supported this premise in the majority of injured axons. Rather, current thought considers TAI a progressive process evoked by the tensile forces of injury, gradually evolving from focal axonal alteration to ultimate disconnection. Recent observations have demonstrated that traumatically induced focal axolemmal permeability leads to local influx of Ca^{2+} with the subsequent activation of the cysteine proteases, calpain and caspase, that then play a pivotal role in the ensuing pathogenesis of TAI via proteolytic digestion of brain spectrin, a major constituent of the subaxolemmal cytoskeletal network, the ''membrane skeleton''. In this pathological progression this local Ca^{2+} overloading with the activation of calpains also initiates mitochondrial injury that results in the release of cytochrome-c, with the activation of caspase. Both the activated calpain and caspases then participate in the degradation of the local axonal cytoskeleton causing local axonal failure and disconnection. In this review, we summarize contemporary thought on the pathogenesis of TAI, while discussing the potential diversity of pathological processes observed within various injured fiber types. The anterograde and retrograde consequences of TAI are also considered together with a discussion of various experimental therapeutic approaches capable of attenuating TAI.

Keywords: Caspase; calpain; spectrin; traumatic axonal injury.

Introduction

The National Institute of Health estimates that in the United States alone 2.1 million traumatic brain injury (TBI) cases occur per year causing 100,000 deaths and 500,000 hospitalizations. The TBI-related death rate of the population under 35 years of age is 3.5 times that of cancer and heart disease together [44]. The effective treatment of traumatic brain injury is considered one of the most cost-efficient medical interventions [87]. Traumatic axonal injury (TAI) – more commonly termed diffuse axonal injury (DAI) [53] in humans – is well known to be associated with TBI and accounts for at least 35% of the morbidity and mortality of TBI patients without space-occupying lesions [25]. Classical descriptions of traumatically induced axonal injury posited that axons were mechanically torn at the moment of impact, retracting to expel a portion of their axoplasm that then formed the ''axonal retraction ball'' typically described in the classic literature [1, 25, 94]. In contrast, over the last two decades it has been demonstrated that only a subpopulation of axonal profiles sustaining the most severe injury rupture at the moment of the impact [54]. Currently, TAI is considered a progressive event gradually evolving from focal axonal alteration to delayed axonal disconnection, a process potentially amenable to therapeutic intervention [75]. Recent studies have shed additional light on the complex pathobiology of TAI. While all workers in the field currently agree that axonal injury is triggered by the inertial forces, primarily the acceleration-deceleration associated with TBI, the ensuing structural and subcellular consequences of such mechanical deformation within the axon cylinder is just beginning to emerge. The following passages address the initiating features of TAI, while commenting on their implications for the injured brain as well as their potential therapeutic modulation.

Evidence for altered axolemmal permeability, calcium influx and calcium-induced proteolytic processes in TAI

Altered axolemmal permeability has long been thought to be a direct consequence of the shear and tensile forces evoked by mechanical injury.

Studies in the mid-nineties revealed focal microscopic mechanoporation of the axolemma [67, 68] reflected in the influx of normally excluded, extracellularly confined tracers, such as the plant protein, horseradish peroxidase (HRP). Such focal intraaxonal HRP passage and accumulation was found to occur within minutes of injury and persisted in the injured foci for up to six hours post-injury [68].

The significance of this mechanoporation was believed to be profound, in that it was posited this altered axolemmal permeability most likely allowed the intraaxonal influx of normally excluded ions such as Ca^{2+} [89].

While the subcellular implications of increased intraaxonal Ca^{2+} influx will be discussed in detail in the following passages, we would note, at this point, that current thought holds that the above described mechanical poration of the axolemma allows for local intraaxonal calcium dysregulation that in turn, triggers the activation of various cysteine protease pathways capable of locally degrading the intraaxonal cytoskeletal network [18]. The disruption of the microtubular and neurofilament components that participate in local intraaxonal transport, leads to organelle and vesicular accumulation. This translates into axonal swelling and detachment. Once detached from its distal, downstream segment the axonal segment in continuity with its sustaining soma continues to swell due to the continued delivery of organelles via anterograde transport. This results in the formation of a retraction ball/reactive axonal swelling or bulb of classical neuropathological description.

While most have assumed that all injured axons follow the same repertoire of Ca^{2+}-mediated damage, more recent studies from our laboratory suggest otherwise, now revealing that responses of axons to TBI are more complex than previously posited [18, 53]. Specifically, using markers of axonal injury focusing on cytoskeletal abnormality as well as impaired axonal transport and axonal swelling and bulb formation detected by the use of amyloid precursor protein, we have recently revisited this issue. Via double labeling immunohistochemical approaches, the above described scenario of cytoskeletal abnormalities and concomitant altered axonal transport were followed in the same axonal populations, with the expectation that these events would always be related. Contrary to our expectations, however, these dual labeling studies frequently showed that local cytoskeletal collapse did not translate into impaired transport and axonal swelling and bulb formation [93, 95]. In fact, in many cases, local cytoskeletal damage was detected without concomitant impaired axonal transport, although in other cases there was a direct correlation between these events as we had originally posited. These findings confirmed that the pathobiology of axonal damage was more complex than previously posited, calling into question the universal believe that the finding of axonal swelling and bulbs identifies the total population of fibers damaged by traumatic injury.

While it is uncertain why some altered axons fail to go on to swell via the continued delivery of organelles via anterograde transport, recent work conducted in other models of axonal injury provides important insight into this issue [51]. Specifically, these studies demonstrated that in more severe forms of axonal injury, massive Ca^{2+} influx triggers the conversion of anterograde to retrograde transport effectively altering local transport kinetics and thereby attenuating the development of reactive axonal swelling.

In addition to this new information emerging on the complexity of the pathobiology of TAI other new information primarily from the *in vitro* setting, has also called into question the above stated belief that alterations in the axolemmal permeability serve as the singular conduit for local Ca^{2+} dysregulation. Specifically, in fine caliber, unmyelinated axons subjected to *in vitro* mechanical loading, Smith *et al.* have found no evidence of altered axonal permeability [106]. Rather, they demonstrated that the source of local Ca^{2+} dysregulation resided in the activation of Na^{+}channels, with the demonstration that the subsequent axonal depolarization triggers the activation of voltage sensitive Ca^{2+}channels. This, together with the activation of the Na^{+}/Ca^{2+} exchangers, has been demonstrated to lead to significant increases in intraaxonal Ca^{2+}. While these *in vitro* studies, conducted in fine caliber, unmyelinated fibers are difficult to compare to *in vivo* studies that focused primarily on large caliber myelinated axons, other work from our laboratory is beginning to indirectly support these observations, particularly as they relate to fine caliber, unmyelinated axons, a previously unrecognized target of traumatically induced damage. Using

electrophysiological studies to examine compound axon potentials within the injured corpus callosum in rodents, our lab has demonstrated that the forces of injury are capable of altering the compound axon potentials originating in both myelinated and unmyelinated axons, with the suggestion that the unmyelinated axons sustained even more damage than the myelinated population [80]. Perhaps, *in vivo* in these fine caliber axons the Na^+ channels serve a more significant role in the ensuing pathological change, in a fashion consistent with that described for fine caliber fibers *in vitro*; however, this remains to be determined. Collectively, however, these findings illustrate further the complexity of traumatically induced axonal change and caution against the once over simplistic view of its pathobiology. These findings also illustrate that Ca^{2+} dysregulation may come from several sources including, but not limited to direct mechanical membrane poration, activation of Na^+ channels and/or a direct channelopathy.

Whatever mechanism ultimately is responsible for the intraaxonal accumulation of Ca^{2+}, Ca^{2+}-induced proteolytic pathways are considered key players in ensuing axonal pathology. While classical studies have suggested that the activation of these proteolytic pathways would lead to a rapid dissolution of the axonal cytoskeleton [54], ultrastructural studies of damaged axons accumulating extracellular tracers due to membrane poration have not shown this to be the case. Rather, the local axonal cytoskeleton does undergo dramatic morphological change, including neurofilament compaction and loss of microtubules, yet, despite these changes its general structural integrity remains relatively unaltered for several hours postinjury [78]. This suggests that, in most cases, Ca^{2+}-induced proteolytic alterations evoke more insidious rather than immediately catastrophic axonal change.

Calcium-induced, calpain-mediated structural proteolysis

To explore the issue of Ca^{2+}-induced proteolytic alterations our laboratory has utilized the impact acceleration rat brain-injury model [50]. This model was chosen in that it is not complicated by overt contusional lesions, yet faithfully generates focally injured axonal segments that can be easily detected within the brainstem fiber tracts where the injured axons are scattered among uninjured neighbors. The development and use of

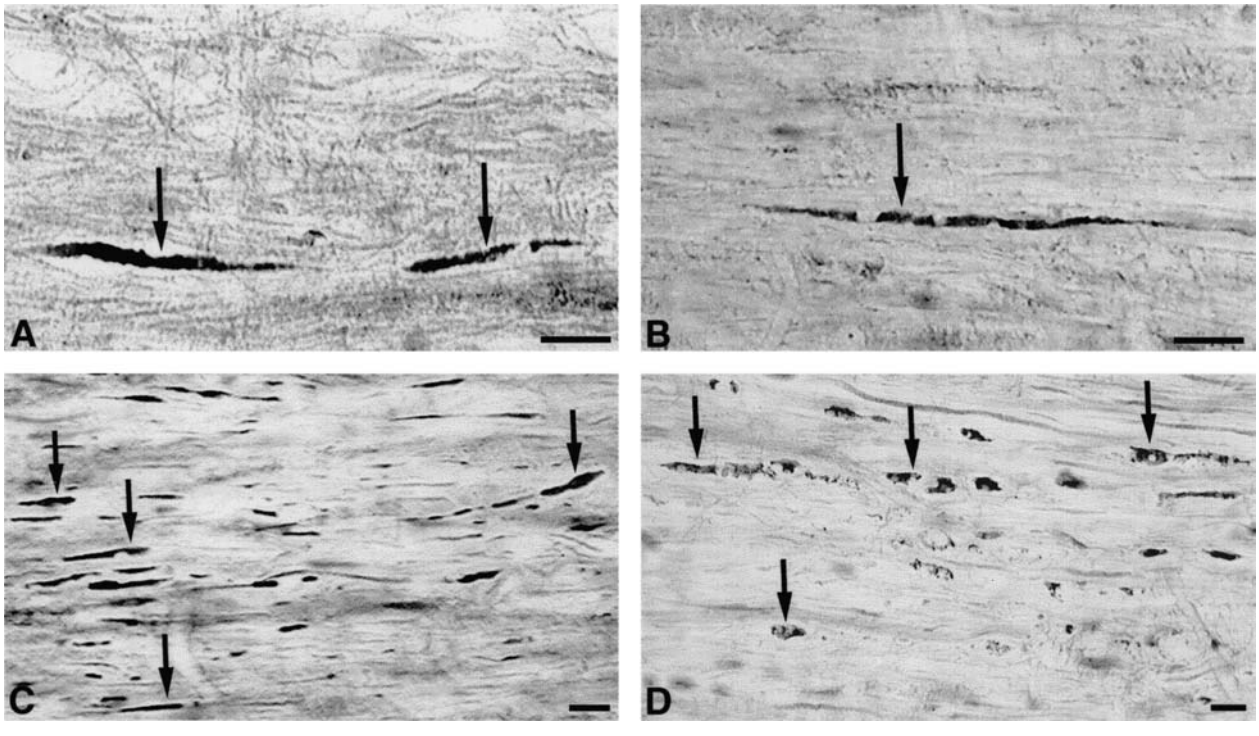

Fig. 1. Axons displaying CMSP (A, C) and SBDP-120 immunoreactivity (IR) (B, D) in rat medial longitudinal fasciculus (arrows). At 30 min postinjury (A, B), the axonal segments appear swollen, 360 min postinjury both CMSP and SBDP-120-IR axonal profiles display evidence of ongoing damage and – in some instances-imminent disconnection (C, D). Magnification bar: 10 μm

a novel set of antibodies (Ab38) targeting breakdown-products of the structural protein spectrin exclusively cleaved by the Ca^{2+}-activated neutral protease, calpain, has allowed for the specific and sensitive detection of Ca^{2+}-induced proteolytic activation within these damaged axonal segments [83, 90] (Fig. 1A, C).

Utilizing single- and double labeling light- and electron microscopic immunohistochemical studies we demonstrated the early focal intraaxonal activation (15 min postinjury) of the Ca^{+2}-induced protease calpain via the detection of calpain-mediated spectrin-proteolysis (CMSP). This colocalized with more traditional markers of TAI such as the RMO-14 antibody capable of demonstrating cytoskeletal alterations – neurofilament compaction [NFC] – long associated with TAI [42, 53, 62].

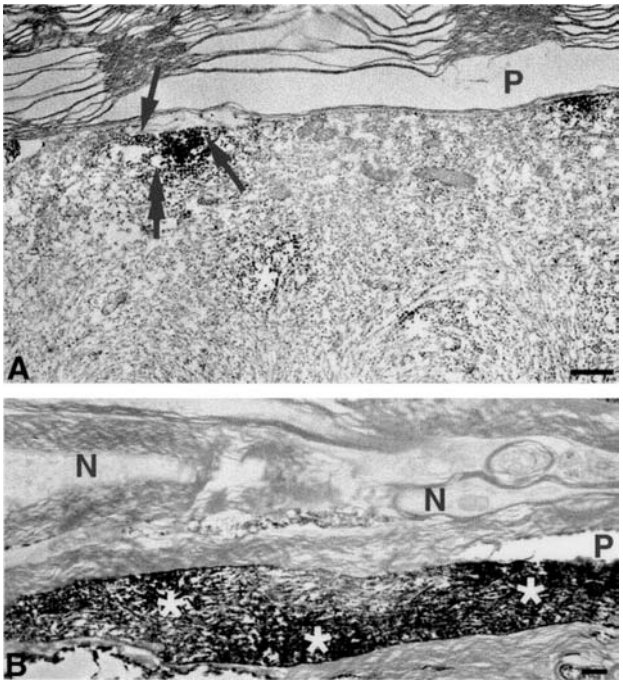

Fig. 2. Electron micrographs of axonal profiles demonstrating CMSP-IR (A–B). Immunoreactive axonal segment from the corticospinal tract thirty minutes postinjury (A). Note the loosening of the myelin sheet around the injured axonal segment with the pathologic formation of periaxolemmal space (*P*). Arrows highlight the predominantly subaxolemmal accumulation of the electron dense DAB-chromogene while a double arrow points at the remnant of a swollen mitochondrion surrounded by the immunoreactive material. Note the non-injured mitochondria in the neighborhood. White asterisks indicate sites of cytoskeletal compaction with scattered intraaxonal immunoreactivity. Immunoreactive axonal segment from the corticospinal tract two hours postinjury (B). At this magnification non-injured axonal segments lacking immunoreactivity can also be distinguished (*N*). The accumulation of the immunoreactive material in the axoplasm is now covering the compacted partially digested cytoskeletal constituents (asterisks). Again, note the formation of periaxolemmal space (*P*). Magnification bar: 1 μm

The number of CMSP- immunoreactive (IR) axonal profiles increased significantly over time postinjury in fiber tracts prone to TAI (the corticospinal tract [CSpT] and medial longitudinal fasciculi [MLF]). In this progression of Ca^{2+}-mediated change, we also noted that the calpain-mediated proteolysis of the cytoskeletal structural protein spectrin displayed different spatial compartmentalization over time postinjury [18]. Ultrastructural analysis of the focally damaged axonal segments in the early (15 min postinjury) period demonstrated a predominantly subaxolemmal and perimitochondrial CMSP-localization, followed by extensive intraaxonal protein degradation over 1–2 hrs postinjury (Fig. 2). Employing double label immunohistochemical strategies a direct colocalization of CMSP (Ab38)- and NFC (RMO-14)-IR was also confirmed [18].

These ultrastructural observations of CMSP-IR compartmentalization over time postinjury, together with the spatial/temporal colocalization of CMSP and other pathomorphological changes including microtubular loss, mitochondrial swelling and NFC, provided a solid basis for positing a novel functional role for CMSP in the pathobiology of TAI.

Spectrin is a constituent of the subaxolemmal cytoskeletal network (also referred to as cortical cytoskeleton or membrane skeleton) [4, 27, 66] and it is anchored to ankyrin that docks with various membrane proteins or other membrane constituents of the axolemma [47, 23, 66]. Additionally, spectrin is associated with the membrane through non-ankyrin-binding domains [22, 108]. Based upon the information provided in the previous paragraphs, it is likely that the initial Ca^{2+}-influx, caused by shearing forces of injury (also referred to as mechanoporation of the axolemma), modifies the subaxolemmal network via early induction of CMSP in the subaxolemmal compartment. Over time, this likely impairs the structural integrity of the axolemma thereby evoking continued and perhaps, irreversible permeability change with additional adverse downstream consequences, including an excessive activation of calpain and CMSP throughout the extent of the injured axonal segment. In this cascade of events, the calcium-influx and ongoing CMSP can also exert other devastating consequences, directly contributing to proteolytic side-arm modification resulting in NFC. Alternatively, Ca^{2+}-induced activation of calcineurin could occur, altering the NF-sidearm phosphorylation state, thereby modifying the repelling forces of the side-arms, leading to NFC [62].

One of the most intriguing aspects of the ultrastructural features associated with TAI and the above described Ca^{2+}-induced changes is the concomitant morphological change seen within the mitochondria of the injured axonal segments. As spectrin can cross-link mitochondria and other organelles [34, 66, 108] and as axoplasmatic CMSP appears on the surface of damaged, swollen mitochondria [18], this finding also raises the question whether calpain-activation and CMSP further contribute to local mitochondrial damage that then could further contribute to the pathological change associated with TAI.

Mitochondrial damage in TAI – potential causes and consequences

As noted, local morphological alterations of mitochondria including swelling and the rupture of mitochondrial cristae and membranes have been closely associated with TAI. These mitochondrial alterations are reminiscent of those evoked by Ca^{2+}-induced opening of the mitochondrial membrane permeability transition (MPT)-pore. The opening of the MPT-pore has been implicated in the pathogenesis of various diseases and is believed responsible for apoptotic neuronal death [20, 41, 60, 86]. In TAI, it is assumed that these mitochondrial alterations are mediated by the excessive sequestration of Ca^{2+} following injury.

This excessive sequestration is then believed to lead to the dissipation of the mitochondrial transmembrane potential and the opening of the MPT-pore that premealizes the mitochondrial membrane for molecules $<$1.5 kDa. This process then leads to the uptake of water and mitochondrial swelling [32, 43, 100, 110]. Also relevant to this issue is the fact that recent observations suggest that the MPT-pore can be opened directly by calpain itself [2, 28], consistent with our observations of calpain-mediated alterations in the perimitochondrial environment.

Irrespective of the initiating mechanisms, however, mitochondrial demise harbors devastating consequences for the injured axon as local axonal energy-failure will cause the dysfunction/failure of ionic pumps, leading to uncontrolled ionic homeostasis.

Another devastating consequence of mitochondrial injury and MPT is the release of pro-apoptotic substances including the apoptosis activating factor, cytochrome-c and different members of the capsase enzyme family [37, 48, 98].

These substances, either directly or indirectly, through the formation of the apoptosome – consisting of caspase-9, apoptosis protease activating factor-1 and cytochrome-c are capable of activating the caspase death cascade, particularly caspase-3, also referred to as the foot-soldier of apoptosis [20, 58, 98, 99].

Activation of the caspase death cascade in traumatically injured axons

While mitochondrial injury itself constitutes a serious energetic/homeostatic challenge for the injured axonal segment, the above described activation of the caspase death cascade due to MPT-mediated release of proapoptotic substances such as cytochrome-c and apoptosis inducing factor (AIF) could also lead to irreversible structural proteolysis and ultimate axonal demise reflected in axonal disconnection. As noted, TAI is complicated by the activation of two members of the cysteine protease family, the calpains and caspases. While collectively the calpains have been linked to controlled, limited structural proteolysis, neural plasticity and synaptic transmission [11, 65, 103]; on the other hand the caspases execute the termination of apoptotic processes, either initiated via signals from the environment, the genome or damaged mitochondria [7, 39, 60, 86, 96, 97, 105, 109].

While this apoptotic function has been extensively studied in the neuronal somata, their dendritic appendices and glial cells, their potential role in the axonal pathology associated with TAI has only been recently appreciated. Utilizing the previously described rat brain injury model we have recently demonstrated both at the light, and electron microscopic level, that cytochrome-c is realesed from damaged mitochondria within the traumatically injured axonal segments [17]. Employing double labeling immunofluorescent staining strategies we demonstrated that foci of cytochrome-c release are also labeled with CMSP-IR. Thus, the Ca^{2+}-induced, calpain-mediated proteolytic changes purported to play a pivotal role in axonal pathology are colocalized with cytochrome-c release. Other approaches also demonstrated that a signature protein of caspase-3 activation, spectrin breakdown protein (SBDP-)120 kDa-IR (Fig. 1B, D and Fig. 3), also consistently colocalized with both CMSP- and cytochrome-c-IR in TAI (Fig. 4).

Although scattered cytochrome-c-IR was detected at 30 min postinjury, the bulk of its release from damaged mitochondria occurred at 3–6 hours postinjury. SBDP-120-IR more closely paralleled the appearance of cytochrome-c-IR than that of CMSP, also reaching its maximum density at 3–6 hrs postinjury. At the ultrastructural

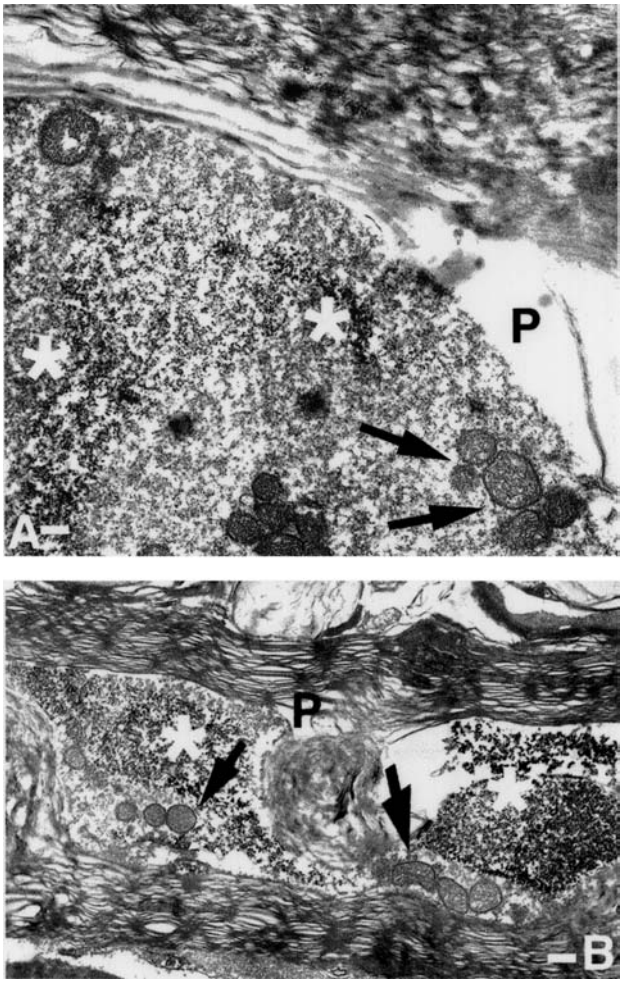

Fig. 3. Electron micrograph of caspase-associated SBDP-120 immunoreactive axonal profiles. At 30 min postinjury (A), the electron dense DAB reaction product is primarily associated with cytoskeletal elements (asterisks). Note the damaged swollen mitochondria (arrows) and the loosening of the myelin sheet with the formation of periaxolemmal space (*P*). Two hours postinjury (B) both the cytoskeletal damage (and the related SBDP-120-IR (asterisks)) and the mitochondrial swelling (arrows) and pooling is more pronounced. Magnification bar: 1 μm

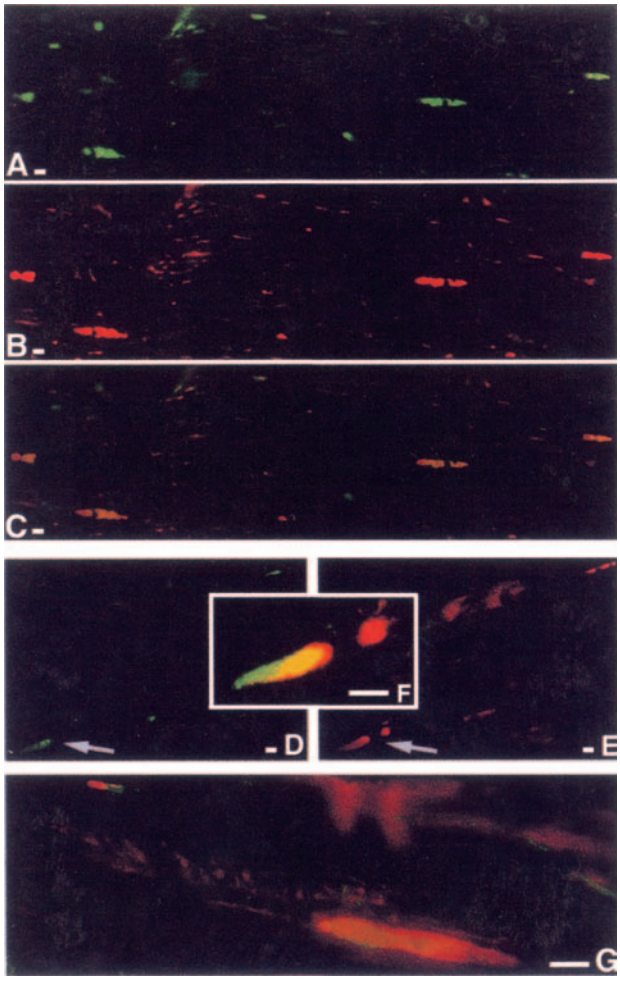

Fig. 4. (A–C): Images of Ab38 (A) and cyto-c (B) immunofluroescent damaged axonal foci and a digital overlay of the same fields (C) in rat corticospinal tracts at 60 min postinjury. Note the segmental swelling and focal vacuolization and that cyto-c and CMSP immunoreactivity are clearly colocalized in these damaged axonal segments. (D–F) Images demonstrating caspase-associated SBDP-120 (D) and cyto-c (E) immunofluroescence damaged axonal foci and a digital overlay of the immunoreactive axonal foci indicated by white arrows (F) in rat corticospinal tracts at 3 hrs postinjury. The morphology of the damaged immunoreactive axonal segment is consistent with axonal disconnection. Again, note the obvious colocalization of the immunohistochemical markers. (G) Three hours postinjury digital overlay of immunoreactive axonal foci localized at identical fields of rat MLF clearly demonstrates the coactivation of calpain and caspase-3 and resultant coexpression of CMSP-IR (red) with SBDP-120-IR (green) in the very same axonal segments. Magnification bar: 10 μm

level, SBDP-120-IR, linked to caspase-mediated spectrin degeneration failed to display the above-described compartmentalization characteristic of CMSP-IR. Rather, it revealed activation throughout the diameter of the damaged axonal segment (Fig. 4). Both quantitative analysis and CMSP-SBDP-120 double labeling studies indicated that only a subpopulation of CMSP-IR axonal profiles displayed SBDP-120-IR. Detailed analysis of ultrastructural data also indicated that the caspase death cascade was activated primarily in those axonal profiles where both the mitochondrial and ultrastructural damage appeared severe, in other words the caspases most likely contributed to the proteolysis of the most severely injured axons. In this scenario, we do not advocate that the caspases cause direct apoptotic change in the soma. Rather we posit that within the locus of axonal injury they participate in the terminal degradation of the injured axonal segments' cytoskeletal network. This premise is not revolutionary, as it is logical to assume that the activation of caspases in traumatically injured axonal segments could easily evoke devastating consequences.

The primary target of CMSP is the alpha chain of the brain-spectrin tetramer, however, caspase-3 also digests the beta chain, the integrity of which is considered crucial for the membrane-binding of subaxolemmal spectrin [104]. Conceivably, such beta-chain-cleavage should then lead to the *irreversible* collapse of the subaxolemmal membrane skeleton with devastating alterations in axonal morphology and axolemmal permeability – an event considered by many as a major pathobiological factor in neuronal degeneration in various CNS disorders [11, 104]. Recent studies have also implicated caspases in the continued activation (or overactivation) of calpains, since they are capable of cleaving the primary intracellular inhibitor of calpains, calpastatin [103, 107]. Based on the above, we consider the activation of the caspase death cascade in traumatically injured axonal segments to be an agonal event, the ''point of no return'' in the pathology that is the indicator of ultimate axonal disconnection.

Other factors that may complicate or mimic traumatic axonal injury: ischemia

Axonal injury induced by ischemic brain damage

Although the above detailed observations on the pathobiology of TAI were made in models not complicated by cerebral ischemia and/or hypoperfusion, it is of note that the cause of DAI associated with and evoked by TBI in humans is sometimes more difficult to define due to the presence of secondary insults, including but not limited to mass lesion formation and/or hypoxia/ischemia. Diffuse brain damage and DAI can be embedded in focal brain injury. Elevated intracranial pressure with resultant decrease in cerebral perfusion pressure can result in the accumulation of damaged, APP-immunoreactive axonal profiles in arterial boundary zones, at the edge of the cerebral infarction. Similar APP-immunoreactive profiles can also be seen surrounding loci of intraparenchymal hemorrhage. Major alterations in energy/blood supply of the hemispheres due to epileptic seizures or cardiac failure can lead to large, multifocal infarcts in the distribution of arterial territories. Accordingly, rigorous histological analyses guided by the clinical history are required to distinguish the axonal change seen in these areas from those wherein axonal injury was evoked primarily by the mechanical forces of injury [29–31, 56].

In this regard, it should also be noted that the repertoire of axonal change that precedes death/disconnection is limited. Thus, it is not surprising that different pathologies can evoke the same phenotypic response.

Lastly, it is of note that hypoxia and hypoperfusion can dramatically increase the magnitude of traumatic brain injury thereby facilitate those proteolytic processes that could represent the common pathway in traumatic and ischemic (diffuse) axonal injury [11]. Additionally, axonal injury can also occur as a result of elevated levels of free radical nitric oxide [69, 88, 92] which is also produced following various ischemic/hypoxic insults.

The consequences of TAI

Although the focus of this review centers on the pathobiology of TAI and its therapeutic modulation we believe that any contemporary treatment of TAI would be incomplete without a parallel consideration of the consequences of TAI in terms of its downstream (anterograde) degeneration/deafferentation and its upstream (retrograde) neuronal responses. These issues have received little attention in contemporary literature although their course is ultimately important in shaping the structural and functional responses to traumatic axonal injury and thereby influencing any subsequent morbidity. Progress in this area has been limited by the fact that the ensuing anterograde and retrograde responses are typically remote from the site of axonal injury thereby making specific linkages difficult to ascertain. Moreover, the laboratory tools to examine this remote and diffuse change are also limited thereby further limiting progress in this area.

Retrograde responses to TAI

In terms of the retrograde somatic responses to diffuse axonal injury, most have assumed that the repertoire of neuronal responses parallel those described in response to physical transsection of their axonal projections. Typically, in lesioning paradigms of CNS, the proximity of the lesion to the sustaining cell body predicts the neurons ultimate fate [8, 10]. More remote lesions translate into atrophic change whereas lesions more proximal or close to the cell body of origin translate into rapid cell death. To date, virtually all models assessing neuronal responses to axonal injury have relied on physical transsection paradigms with the implicit assumption that the subsequent somatic responses reflected those ongoing in the traumatized brain manifesting diffuse axonal injury [13, 26, 38, 45, 55, 57]. As alluded to above, the study of

the somatic responses to diffuse axonal injury is also complicated by the typical remoteness of these neuronal changes in relation to the actual site of axonal injury. Most problematic is the fact that it is virtually impossible to trace one damaged axon back to its cell body of origin and thereby follow any presumed changes occurring in that cell body in response to injury. Recently, we have been able to follow the somatic response of traumatically induced axonal injury with confidence through the recognition of perisomatically injured axons scattered throughout the neocortex, hippocampus and thalamus [93]. Typically, such perisomatically injured axons could be found within 60 microns of their sustaining somata. Based upon previous reports using experimental transsection, it was assumed that this perisomatic axonal injury would immediately translate into ultrarapid necrotic cell death [9, 38, 45]. However, contrary to expectations, in this situation cell death did not occur. Despite the finding of traumatic axonal injury within 60 microns of sustaining soma, the cell bodies did not initiate an overt necrotic cell death cascade. Rather, they revealed early chromatolytic changes associated with biochemical and immunocytochemical markers of altered protein translation. Such altered protein translation persisted over several days postinjury and remarkably was followed by a trend for recovery over a one week period. Thus, in contrast to the experimental lesioning literature, these neurons did not die. Although initially perturbed by the TAI, they appeared capable of reorganizing and mounting a reparative effect. As these findings are inconsistent with those described with physical transsection they highlight the potential differences between mechanical transsection and diffuse axonal injury [93]. Physical transsection of the axon, creates a massive renting of its microenvironment and dramatic, immediate ionic dysregulation [5]. Conceivably, the slow, progressive changes evoked by diffuse axonal injury allow the neuronal cell body to reorganize and to survive. While admittedly, the long-term fate of these axotomized neurons remains to be determined, their very persistence at seven days postinjury suggests that they may be capable of mounting various regenerative or neuroplastic rearrangements within the injured brain, issues that are currently under investigation in our laboratory.

Anterograde responses to TAI

Another important, yet poorly appreciated consequence of diffuse axonal injury is the occurrence of downstream deafferentation/denervation. Specifically, following the above described sequence of axonal damage and disconnection, the downstream axonal profile, disconnected from its sustaining soma, undergoes Wallerian degeneration that in animals proceeds over the matter of weeks. In contrast, in humans, this Wallerian change progresses over several months postinjury. Concomitant with the breakdown of the myelin sheath and the axon cylinder, the downstream nerve terminal sites also undergo neurodegenerative change. Within 24 to 48 hours of injury, they manifest increased electron density or neurofilamentous hyperplasia with detachment from their normal target sites. This process has been observed in animals and man, and although the overall degree of axonal terminal damage and deafferentation has not been quantified, there is the general impression that the amount of degenerating nerve terminals far exceeds the number of identified damaged fibers [76]. This once again adds credence to the premise that multiple fiber populations, including large caliber myelinated as well as non-neuropathologically detected fine caliber, myelinated and unmyelinated fibers may be involved. Although not well documented, the underlying assumption here is that such axonal degeneration and deafferentation contribute to the morbidity associated with DAI. Also important is the assumption that this deafferentation sets the stage for subsequent neuroplastic changes that can lead to either favorable/adaptive changes or, alternatively, less than optimal/maladaptive changes. To date, this issue has not been thoroughly investigated since neuroplastic change is difficult to follow in the human brain. Further, in the experimental setting, the diffuse nature of the injury complicates the conduct of rigorous assessment of any potential neuroplastic responses. The limited data that exists to date suggests that in the case of mild to moderate TBI, diffuse deafferentation sets the stage for the sprouting of adjacent intact nerve fibers that, in many cases, leads to the recovery of significant synaptic input to the previously deafferentated neuronal domains [24]. In contrast, with more severe injury, there is the suggestion of maladaptive change with the potential for inappropriate fiber ingrowth and/or cytoarchitectural modification [70, 71]. At present, studies are ongoing to better understand those factors involved in those neuroplastic responses triggered by the traumatic episode with manipulations of the extracellular environment now being employed to delay and/or modify the ensuing postsynaptic sprouting response. Particular emphasis has been placed on the extracellular matrix and its postinjury proteolytic modification by matrix

metalloproteinases [81]. These metalloproteinases have been posited to prepare the denervated neuropil for synaptogenesis and thereby influence the spatial distribution of any nerve ingrowth [81]. Obviously, these issues require continued investigation to fully understand and thereby treat the downstream consequences of diffuse axonal injury.

The therapeutic targeting of TAI

One important conclusion that can be drawn from the above-detailed pathobiology of TAI is that the damaging *progression* of events associated with DAI may be amenable to therapeutic intervention [73–75]. Though a small population of damaged axons may suffer devastating damage leading to ultra-early, overwhelming activation of the cysteine proteases causing rapid axonal demise [54], the majority of injured axons die through a gradually progressive cascade that could potentially be attenuated via rationally targeted therapies.

Based upon the above described findings, it would appear that four primary targets should be considered for therapeutic intervention. These would include calpain-mediated spectrin proteolysis, mitochondrial permeability transition-induced mitochondrial damage, cytoskeletal alteration caused by calcium-accumulation (activation of calcineurin) and the activation of caspase-3.

In the above described scenario of TAI the most ideal therapy should target those intraaxonal events preceding the mitochondrial release of cytochrome-c. This premise is based upon the fact that the occurrence of mitochondrial cytochrome-c release evokes in addition to the activation of caspases, severe disturbances in the electron-transport leading to bulk generation of free radicals [19, 79, 86]. Accordingly, we believe that therapeutic interventions should target the *early* phases of TAI. To this end, the calcium-activated neutral proteases and perhaps the *induction* of mitochondrial injury (MPT) would seem to represent the most rational targets for therapeutic intervention.

Although a detailed analysis of all the experimental therapeutic approaches tested to date in TAI would be beyond the scope of this review, a few important observations will be commented upon. Several experimental studies have demonstrated the beneficial effects of calpain inhibition in Ca^{2+}-induced pathology in various central nervous system disorders including ischemic brain damage [12, 33, 41, 49], spinal cord injury [6, 35, 59] and cerebral contusion [72, 84, 85].

In our hands, therapeutic intervention using calpain inhibition attenuated TAI. In accordance with our observations on the pivotal role of CMSP in the pathogenesis of TAI we demonstrated that preinjury administration of a membrane-permeable calpain-inhibitor resulted in a significant reduction in the density of traumatically injured axonal segments within the brainstem [14].

Another related, alternative therapeutic approach targets the general inhibition or slowing of the proteolytic processes via hypothermic intervention. Although the use of controlled hypothermia in the treatment of human TBI remains controversial, several studies from our group as well as others [52] have demonstrated that this therapeutic modality effectively limited CMSP and axonal disconnection, the latter indicated by a significantly decreased density of damaged axonal profiles displaying beta-amyloid precursor protein (APP)-IR a marker of impaired axonal transport and axonal disconnection [15, 36]. These effects could be explained, at least in part, via hypothermia's slowing of the calpain-mediated structural proteolysis. This could then functionally prevent or decrease secondary alterations in axolemmal permeability thereby providing an opportunity for the injured axon to preserve mitochondrial function, with the maintenance of the energy homeostasis.

Recent observations also indicate that hypothermic intervention offers beneficial effects for the cerebral microvasculature and prevents/attenuates the [101, 102] impaired vascular responsiveness associated with TBI. It is of note that in these studies of vascular and axonal perturbation, gradual posthypothermic rewarming achieved optimal benefits whereas rapid posthypothermic rewarming exacerbated any observed traumatically associated pathology. Although a recent multicenter clinical study [21] did not demonstrate the efficacy of hypothermia in the treatment of the traumatically brain injured patients, this intriguing experimental therapeutic data suggests the need for its continued evaluation.

Another potential way to influence some of the pathobiology of TAI, particularly that involving the mitochondrial change, lies in the systemic administration of cyclosporin A (CsA) that targets the MPT-pore. Utilizing this immunophilin ligand, immuno-electron microscopic studies in the rodent diffuse brain injury model have demonstrated that CsA significantly attenuated mitochondrial swelling and rupture, translating into reduced numbers of damaged, disconnected APP-IR axonal segments [63].

Subsequent experiments revealed that both pre- and postinjury administration of CsA significantly reduced

the number of damaged axonal segments detected by immunohistochemical markers of calpain-mediated spectrin proteolysis, axonal disconnection and NFC [16, 61, 63].

Corroborating these structural observations, recent studies have also shown that CsA limits/improves some aspects of functional outcome following TBI [82].

Preliminary data regarding the use of CsA in head injury in man indicate that this compound is safe and potentially beneficial [3].

While these investigations focused primarily on the beneficial effect of MPT-inhibition in TAI, other more recent observations have shed light on the therapeutic potential of another immunophilin ligand FK506, a substance that has no known effect on MPT. Rather, this agent exerts its action via inhibition of calcineurin activity [40, 46]. The beneficial effect of FK506 on TAI has been described in a preinjury administration paradigm [91] and the drug was also been shown to be effective in reducing the complications associated with rapid rewarming following hypothermic intervention for TAI [95]. In retrospect, such a favorable effect associated with the inhibition of the calcium-induced phosphatase calcineurin is not surprising as some of the cytoskeletal alterations observed with TAI may be related to calcineurin activation. Specifically, calcineurin via dephosphorylization of the neurofilament-side arms can decrease the repelling forces between the filaments thereby permitting NFC [62, 77]. Further, dephosphorylated neurofilaments would be more susceptible for proteolytic degradation by calpain and caspase [64].

Conclusions

This review has attempted to summarize contemporary knowledge on the pathogenesis of TAI and some of its consequences while also illustrating that our understanding of its detailed pathobiology remains incomplete. A detailed understanding of the mechanisms operant in TAI is of considerable importance as it is a prerequisite to the development of novel therapeutic approaches to rationally target TAI. It is anticipated that the better targeting of TAI will translate into the better care and management of brain injured patients, resulting in significantly improved outcomes.

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Appendix

Glossary of important terms

TBI	traumatic brain injury
DAI	diffuse axonal injury
TAI	traumatic axonal injury commonly termed diffuse axonal injury in humans
NFC	neurofilament compaction
MPT	mitochondrial permeability transition
Ab38	specific polyclonal rabbit antibody targeting breakdown-products of the structural protein spectrin exclusively cleaved by the Ca^{2+}-activated neutral protease, calpain
RMO-14	antibody capable of demonstrating cytoskeletal alterations – neurofilament compaction [NFC] – long associated with TAI
APP	beta-amyloid precursor protein
SBDP	spectrin breakdown protein
SBDP-120	spectrin breakdown protein (120 KDa: exclusively formed by caspase-3 mediated cleavage of spectrin)
HRP	horseradish peroxidase
CMSP	calpain-mediated spectrin-proteolysis
MLF	medial longitudinal fasciculus
CSpT	corticospinal tract
CsA	cyclosporine A
FK506	calcineurin inhibitor

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Comments

Drs. Buki and Povlishock have reviewed traumatic axonal injury, a topic analysed from many aspects in Dr. Povlishock's laboratory. This is a comprehensive review of the present understanding of the phenomenology and causes of traumatic brain axonal injury. The pathophysiology of brain injury can be studied from many aspects and most experimental paradigms allow the definition of several different trauma mechanisms which may have different relative importance in different kinds of injuries: ischaemia, inflammation, edema etc. Axonal injury is one such aspect, which is specific for traumatic injury. As such, it is neglected in comparison to injury mechanisms that are more relevant for pathophysiology of stroke or dementia. It is, however, probable that a more profound understanding of processes that are specific for traumatic injury in one factor that may allow success in preventing secondary injuries.

To get further, integration of our ''reductionist'' knowledge of specific mechanisms is necessary to define the relative importance and dynamics of different post-traumatic cellular, subcellular and molecular events.

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After recent discussions about the above manuscript I am happy if the following critique is published with my name attached.

The importance of axonal injury in determining the outcome after traumatic brain damage is now widely accepted. Increasing experience over the last 15–20 years has helped to better define its clinical correlations, neuroimaging characteristics and its recognition after post mortem. The review by Buki and Povlishock is timely and produces an up-to-date account of what is now known about this clinico-pathological entity, its time course, microscopic and ultrastructural features, and its mechanisms of causation. However, when this important work is under-

taken in controlled laboratory settings it is vitally important that recognition to given to the difficulties encountered by Forensic Pathologists in the course of their medico-legal work. This is because there are relatively few clinical examples of pure traumatic axonal injury, the great majority being complicated by hypoxia, brain swelling, the vascular complications of raised intracranial pressure, etc., and intercurrent disease all of which may closely mimic traumatic brain injury. The identification as to the cause of any axonal pathology is therefore of major importance in medico-legal work and requires careful consideration of all of the available information before an opinion can be expressed. In most instances there is more than one causative factor, and hence the need for caution. These aspects of axonal pathology after head injury require careful consideration in the evaluation of any case.

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