Journal of
neurology
neurosurgery
& psychiatry

Editorial

The role of the axolemma in the initiation of traumatically induced axonal injury

Axonal injury is a common feature of mild, moderate, and severe head injury.[1] Damage of the axonal pathways within the brain accounts for much of the morbidity after head injury with outcomes ranging from mild concussion to profound coma and even vegetative state. Despite this, the pathogenesis of traumatically induced axonal injury remains unclear.

For many years it was assumed that the rapid acceleration and deceleration of the brain during traumatic injury sheared axons at the time of injury causing them to retract and expel a ball of axoplasm, forming a reactive axonal swelling or retraction ball, the traditional histological feature of diffuse axonal injury.[2–6] However, more contemporary investigations have not supported the basic concept of axonal shearing at the time of injury as the mode of disconnection.[7–12] Rather, they have shown that in most cases of traumatically induced axonal injury, the process of axonal disconnection is not immediate but delayed for four to 24 hours depending on the severity of injury and the species examined.[1] This process is known as delayed or secondary axotomy. The traumatic insult elicits a focal axonal abnormality that leads to the impairment of axoplasmic transport with subsequent swelling of the axon.[7] This progresses to separation of the swollen axon into a proximal segment in continuity with the sustaining soma and a distal appendage which will undergo Wallerian degeneration. When, in the above described sequence of axonal change, axonal detachment does occur, the continued delivery of axoplasmic constituents via anterograde transport allows for the continued expansion of the proximal swollen axonal segment, which matures into a retraction ball of classic description.[7 8]

Despite general agreement regarding the occurrence of secondary axotomy there has been little consensus with regard to the pathogenesis of the initiating subcellular events involved in this process. The obvious question centres on precisely what cellular component underlies the axon's propensity to fail with traumatic brain injury. In this regard, the role of the axolemma is a major topic of current investigation.

Axolemma

The axolemma or axon membrane consists of a bilipid membrane and a closely associated submembrane skeleton. This skeletal framework is essentially formed by the protein spectrin arranged in a pentagonal or hexagonal mesh lining the interior of the cell membrane, and actin, connecting via ankyrin and other components to transmembrane proteins. Transmembrane proteins such as β1-integrin bind the extracellular matrix to the cytoskeleton via the membrane skeleton.[13–15]

The hypotheses

Historically, two hypotheses have attempted to explain the biochemical and biophysical mechanisms initiating the described secondary axotomy. One has associated pathogenesis with axolemmal disruption, whereas the other describes a direct cytoskeletal insult occurring independent of any contributing axolemmal pertubation. Adams and colleagues and Bullock *et al* proposed that focal pertubation of the axolemma was the crucial initiating event.[8 16–19] Hypothetically, this was associated with increased permeability of the axolemma, allowing the influx of extracellular calcium. In the normal situation the intracellular concentration of free calcium is rigorously controlled and there is normally a steep gradient of the order of 10 000:1 in the concentration of calcium between extracellular fluid and axoplasm. In mammalian myelinated nerve fibres this gradient is largely maintained through the activity of membrane pump Ca^{2+}ATPase activity.[20] It has been posited that the increased intracellular calcium concentrations after injury may allow activation of proteolytic enzymes that cause collapse and subsequent dissolution of the axonal cytoskeleton, disrupting axonal transport as reflected in the accumulation of membranous organelles, the formation of axonal swellings, and progress towards secondary axotomy. By contrast, Povlishock and colleagues proposed that the physical forces of injury directly perturbed the intra-axonal ultrastructure, altering cytoskeletal alignment, and thereby impairing axonal transport.[7 12 21 22] The cytoskeletal changes found by this group did not include the cytoskeletal dissolution associated with the calcium activated proteolytic cascades as hypothesised by Adams and colleagues.

Recent studies have provided considerable insight into each of these mechanisms and their potential role in the genesis of the reactive axonal change associated with traumatic brain injury.

Historical review

Findings of traumatically induced axonal abnormalities in most experimental models of blunt head injury, without either myelin disruption or frank damage to the adjacent neural or glial processes, have not provided evidence for direct tearing or shearing of axons after traumatic injury.[9 10] This was supported by freeze fracture studies, conducted in the early phases of axonal injury, which failed to show either immediate disruption of the internodal myelin or early loss of the glial-axonal junctions, suggesting that the myelin sheath itself is not immediately damaged by the forces of injury.[23] At this stage both the above groups postulated that the forces of traumatic injury act first on the axolemma or the axoplasm while sparing the myelin sheath. Within this hypothesis, the mechanical forces of injury could stretch or injure the axolemma to its biomechanical limits at which point ionic homeostasis would fail and initiate reactive axonal change.

However, in a series of subsequent investigations on brain tissue from both humans[12 22] and animals[21 24] , Povlishock and colleagues provided data to support an initiating role for the intra-axonal cytoskeleton. Their studies disclosed a complex sequence of intra-axonal events which progressed to reactive axonal change. Specifically, using antibodies directed at the neurofilament component of the cytoskeleton they found that there was focal accumulation of the 68 kDa subunit (NF-L) within one hour of injury and this increase was dramatic by two hours. With increasing time the neurofilaments became disorganised and misaligned relative to the longitudinal axis of the axon. There was no loss or dissolution of neurofilaments detected. These data were therefore inconsistent with neutral protease mediated degradation of the cytoskeleton. Instead, the increase in the 68kDa neurofilament subunit supported the possibility of a traumatically induced rearrangement of the neurofilament pool. Further, no ultrastructural evidence of direct axolemmal disruption was detected in this series of experiments. Infolding and distension of the axolemma was found and attributed to the ongoing reactive axonal change. These findings suggested that a direct mechanical effect on the cytoskeleton of the axon cylinder was the pivotal event in the initiating pathogenesis of axonal injury.

Other approaches suggested that the axolemma was involved in the initiation of reactive axonal change. Maxwell *et al* used a nerve traction model to analyse the morphological changes within damaged axons.[18] These authors provided evidence that the initial sites of damage, after a non-disruptive stretch-injury, are the nodes of Ranvier, some of which develop "nodal blebs". These blebs are axolemma limited protrusions of the axoplasm into the perinodal space and are most numerous within 15 minutes of injury but less so at later intervals.The development of nodal blebs correlated with loss of the dense undercoating of the axolemma. In damaged axons with nodal blebs the neurofilaments were disorganised, deviated from the longitudinal axis of the axon, and extended into the blebs. By contrast, the microtubules maintained their longitudinal arrangement and did not deviate into the bleb. Quantitative analysis of this material showed that there was a significant loss of microtubules and an increased spacing of neurofilaments in the axoplasm of nodes with associated nodal blebs. This loss of microtubules could disrupt fast axoplasmic transport resulting in the focal accumulation of membranous organelles in adjacent paranodal regions of the axon to form axonal swellings. In addition, these workers provided the first cytochemical evidence to support the idea of calcium influx into myelinated nerve fibres injured by stretch .[25] Use of the oxalate-pyroantimonate technique for the localisation of calcium showed an increased content of pyroantimonate precipitate within nodal blebs at 15 minutes after stretch injury. This correlated with a reduction in labelling for membrane pump Ca^{2+}ATPase activity on the nodal axolemma.[26] The node of Ranvier is the specialised region of the axolemma in which groups of Na+ channels, ATPase driven pumps for calcium, and an Na^{+}/Ca^{2+} exchanger are localised.[20 27] Therefore, loss of membrane pump activity in the nodal axolemma might provide a mechanism for influx of free calcium into nodes of Ranvier after traumatic injury. This evidence supports the hypothesis that the forces of traumatic injury result in a focal pertubation of the axolemma with the resultant influx of free calcium capable of activating a subpopulation of neutral proteases.[25]

Biophysical investigations have used novel techniques to analyse the axolemma in experimental conditions analagous to axonal injury. After mild stretch injury a series of constrictions and expansions occurs in sciatic nerve fibres from the adult rat. This change in form is known as beading.[28–30] The transformation of the essentially cylindrical form of the normal nerve fibre to one that is beaded occurs rapidly, within 10 to 20 seconds on initiation of stretch. Initial experimental data suggested that beading is brought about by a mechanism related to the axolemma, or the cytoskeleton, or both. In this model, the transmembrane protein β1-integrin binds to both the extracellular matrix and the cytoskeleton.[14] Tension placed on the extracellular matrix is signalled through β1-integrin to the cytoskeleton with resultant alteration in its integrity and spatial arrangement. However, a recent study has indicated that beading does not require an interconnected cytoskeleton and concluded that the axolemma is the initiating site or locus of beading constriction.[31] It is worthy of speculation that beading may be part of the biological process that is described as reactive axonal change in the literature related to traumatically induced axonal injury.

Within the past four years the involvement of the axolemma in the pathogenesis of axonal injury has become established. Maxwell *et al* showed that severe traumatic brain injury caused by lateral acceleration was capable of directly tearing the axolemma in non-human primates.[32] This study was the first to provide ultrastructural evidence which supported the concept of axonal shearing in traumatic injury to the brain. The loss of axolemmal integrity was associated with rapid dissolution of the axonal cytoskeleton. In the axons showing axolemmal tearing or fragmentation, the filamentous organisation of the cytoskeleton was replaced by a flocculent precipitate consistent with a rapid dissolution of the underlying cytoskeletal proteins. These changes were detected within minutes of injury but only occurred in a subpopulation of fine calibre, thinly myelinated axons after severe injury. These morphological changes represented an acute response of axons to injury and was termed " primary axotomy"—defined as occurring within minutes of injury, by contrast with delayed secondary axotomy, which develops over a period of hours. In the same experimental material there was no evidence of disruption of the axolemma one hour after injury. This suggests that the disrupted axonal membrane reseals within an hour of injury.

After this publication, Povlishock and colleagues reconsidered their central hypothesis, which did not include a role for the axolemma in the initiation of traumatically induced axonal injury. On reflection, they suggested that direct tearing of the axolemma may represent the most severe end of a range of axolemmal disruption.[33] This was a major turning point, in that, for many years this research group had advocated that there was no evidence of direct alterations in the axolemma after injury in any of the numerous paradigms that they had investigated. Moreover,

they had argued that the pathobiology of traumatically induced axonal injury resulted from the direct impairment of axoplasmic transport due to the forces of injury directly disrupting the axonal cytoskeleton.[7 12 21]

In a series of experiments designed to investigate this issue, Povlishock and colleagues employed the extracellular tracer, horseradish peroxidase (HRP), to determine if direct alterations in the axolemma were detectable in traumatic brain injuries of mild and moderate severity.[33 34] This novel approach was based on the principle that macromolecular tracers such as HRP are normally excluded from the axoplasm by an intact axolemma. Therefore detection of intra-axonal peroxidase activity would constitute evidence of axolemmal disruption. Furthermore, the site of peroxidase activity would delineate the initial site of axonal pertubation, allowing insight into the initiating factors involved in the pathogenesis of secondary axotomy. Their findings showed that the pathobiology of traumatically induced axonal injury was a heterogeneous and complex process involving multiple and varied initiating pathology.

In particular the severity of the traumatic injury determined subsequent events in the axolemma and the cytoskeleton resulting in a differential response to the insult. Specifically, after moderate injury, direct pertubations of the axolemma reflected in its altered permeability to macromolecules were detected.[33–35] This was associated with a rapid local compaction of axonal neurofilaments as evidenced by a decrease in the interfilament distance. However, after mild traumatic brain injury no evidence of alterations in the axolemma were detected and a different set of cytoskeletal abnormalities, with misalignment and axonal swelling, were found. A differential response to injury had previously been described in a compression loading model of axonal injury.[36] With low tensile loading, axons showed axoplasmic disruption independent of any change in axolemmal integrity, entirely consistent with those changes described with mild traumatic brain injury. With more severe injuries, the same axons disclosed axolemmal change that correlated with dramatic axoplasmic failure. Subsequent studies have extended these findings by showing that these alterations in the axolemma and in the cytoskeleton are neither model[34 35] nor species specific.[37] Furthermore, after the demonstration of traumatically induced alterations in axolemmal permeability the studies suggested that calcium may be involved in the initiation of cytoskeletal events. This apparent contradiction with former views stemmed from the fact that they posited that calcium was acting through previously unidentified mechanisms. Instead of activating proteolytic enzymes, calcium may act less dramatically to alter the neurofilament sidearms, causing the neurofilaments to collapse resulting in their increased packing density. Conceivably these neurofilament sidearms could either be cleaved by calcium mediated processes or dephophorylated through the actions of kinases and phosphatases to result in alteration of the three dimensional spacing of neurofilaments.[1]

In conclusion, the controversy surrounding the role of the axolemma in the pathobiology of traumatically induced axonal injury has been resolved to some extent. It is now agreed that disruption of the axolemma is the initial event in certain forms of traumatic injury. However, the mechanistic basis of secondary axotomy is now seen to be increasingly complex with alterations in the axonal cytoskeleton demonstrated in the more severe forms of traumatic brain injury.

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