Unmyelinated Axons Show Selective Rostrocaudal Pathology in the Corpus Callosum After Traumatic Brain Injury

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Abstract
Axonal injury is consistently observed after traumatic brain injury (TBI). Prior research has extensively characterized the post-TBI response in myelinated axons. Despite evidence that unmyelinated axons comprise a numerical majority of cerebral axons, pathologic changes in unmyelinated axons after TBI have not been systematically studied. To identify morphologic correlates of functional impairment of unmyelinated fibers after TBI, we assessed ultrastructural changes in corpus callosum axons. Adult rats received moderate fluid percussion TBI, which produced diffuse injury with no contusion. Cross-sectional areas of 13,797 unmyelinated and 3,278 intact myelinated axons were stereologically measured at survival intervals from 3 hours to 15 days after injury. The mean caliber of unmyelinated axons was significantly reduced at 3 to 7 days and recovered by 15 days, but the time course of this shrinkage varied among the genu, mid callosum, and splenium. Relatively large unmyelinated axons seemed to be particularly vulnerable. Injury-induced decreases in unmyelinated fiber density were also observed, but they were more variable than caliber reductions. By contrast, no significant morphometric changes were observed in myelinated axons. The finding of a preferential vulnerability in unmyelinated axons has implications for current concepts of axonal responses after TBI and for development of specifically targeted therapies.

Key Words: Axonal injury, Corpus callosum, Stereology, Traumatic brain injury, Ultrastructure, Unmyelinated axons.

INTRODUCTION
Traumatic brain injury (TBI) is an enduring public health problem, with more than 1.5 million new TBIs occurring annually in the United States alone (1). Axonal injury is a concomitant of most TBIs requiring hospitalization. Diffuse axonal injury was recently observed to occur in 72% of patients with moderate or severe TBI and was associated with worse outcome (2). This diffuse pathologic condition is also associated with postransitive coma, persistent memory deficits, and impaired information processing capacity (3). Despite these serious clinical consequences, fundamental questions remain as to whether all axons are equally susceptible to injury or whether specific populations of axons are selectively vulnerable. Moreover, the structural and functional changes initiated in axons after TBI are not completely understood.

Accumulating evidence using experimental animal models of TBI suggests that separate populations of axons undergo distinct responses to injury and to treatment with neuroprotective compounds. The longstanding assumption that axon loss is due primarily to shear and tensile forces acting at the moment of injury (4) has been supplanted by an understanding that most injured axons undergo secondary progressive alterations that include cytoskeletal changes, impaired axoplasmic transport, and axonal swelling (5–8). On the other hand, observations that some injured axons exhibit neurofilament compaction independently of impaired fast transport (9, 10) and that immunophilin ligand treatment differentially attenuates these abnormalities (11) suggest that, within the brain, there are subpopulations of axons that undergo different forms of secondary axonal injury. Post-TBI alterations in axonal function also suggest that axon phenotypes are determinants of injury outcome. Axonal excitability, assessed using compound action potentials (CAPs) evoked in the corpus callosum, was more severely suppressed in unmyelinated than in myelinated axons (12, 13). These fiber types exhibit differential degrees of functional protection after treatment with the immunophilin ligands cyclosporin-A (14) and FK506 (15) and the calpain inhibitor MDL-28170 (13). Together, these findings contrast with prevailing concepts of axonal injury being an undifferentiated pathologic disease present in varying degrees but generally affecting axons indiscriminately.

The primary objective of the present study was to investigate TBI-induced ultrastructural changes in axons of the corpus callosum; such changes have been suggested to be the morphologic correlates of electrophysiologic deficits noted in prior studies (12–17), and they reveal reactive structural alterations specific to the unmyelinated axon population. Most previous descriptions of axonal pathology after brain trauma have focused on myelinated axons, but a comprehensive understanding of axonal injury necessitates incorporation of pathologic responses specific to unmyelinated fibers.
The experimental TBI model of fluid percussion injury (FPI) in adult rats has been extensively characterized and applied to the study of a wide array of cellular alterations initiated by neurotrauma (18, 19). Our application of FPI at a moderate intensity results in diffuse injury without contusion or hematoma and does not lead to significant Wallerian degeneration. However, the injury does produce functional impairments, including electrophysiological alterations in corpus callosum (12–16) and deficits in spatial cognition without hippocampal cell death (20). Thus, this approach allows the study of nonlethal changes in unmyelinated axons, which comprise the numerical majority of fibers in parasagittal white matter of the cerebrum (21). Because degenerating axons are not salvageable, unmyelinated axons that display time-dependent changes in function and morphology represent potential targets for therapies.

MATERIALS AND METHODS

Animals

The procedures for this study followed all national guidelines for the care and use of experimental animals, and the experimental protocol was approved by the Medical College of Virginia Animal Research Committee. Male Sprague-Dawley rats (n = 54) weighing 300 to 350 g at the start of the study were housed in individual cages in a temperature- (22°C) and humidity- (50% relative) controlled animal facility on a 12-hour light/dark cycle. Rat chow and water were continually available.

Fluid Percussion Injury

Rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.), and a 4.8-mm skull craniotomy was prepared over the midline, centered between bregma and lambda. A Leur-Loc syringe hub was cemented with cyanoacrylate to the skull surrounding the craniotomy and dental acrylic was poured around the syringe hub and 2 small screws placed in the skull for implant rigidity. Bacitracin was applied to the incision, and the animals were returned to their home cages. At 24 hours after implantation of the syringe hub, rats were anesthetized with isoflurane (4% in carrier gas of 70% N2O and 30% O2) and immediately subjected to moderate intensity results in diffuse injury without contusion or hematoma and does not lead to significant Wallerian degeneration. However, the injury does produce functional impairments, including electrophysiological alterations in corpus callosum (12–16) and deficits in spatial cognition without hippocampal cell death (20). Thus, this approach allows the study of nonlethal changes in unmyelinated axons, which comprise the numerical majority of fibers in parasagittal white matter of the cerebrum (21). Because degenerating axons are not salvageable, unmyelinated axons that display time-dependent changes in function and morphology represent potential targets for therapies.

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Statistical Analysis

The effects of FPI on axonal cross-sectional area and density were analyzed with analysis of variance followed by post hoc analyses using the Dunnett multiple comparison test (SPSS v.11.5; SPSS, Inc, Chicago, IL). A \( p < 0.05 \) was considered significant for all experiments. Results are reported as mean ± SEM.

RESULTS

Before the analysis of time-dependent morphologic changes associated with FPI, it was essential to ensure that injury levels did not differ significantly among the various survival groups. The fluid percussion device was calibrated to deliver a pressure pulse of 2.0 atm, and the actual measured mean pulse magnitude was 1.98 ± 0.01 atm, averaged across all FPI rats in the study. None of the various survival groups (killed from 3 hours to 15 days) differed significantly from this magnitude (\( F_{4,12} = 0.407, p = 0.800 \)). A comparison of the duration of suppression of the righting reflex, used as an index of traumatic unconsciousness, provided additional evidence of injury uniformity among survival groups. The overall mean latency to regain the righting reflex for all animals given fluid percussion injury was 5.93 ± 0.16 minutes. None of the various survival groups (killed from 3 hours to 15 days) differed significantly from this magnitude (\( F_{4,12} = 0.407, p = 0.800 \)). A comparison of the duration of suppression of the righting reflex, used as an index of traumatic unconsciousness, provided additional evidence of injury uniformity among survival groups. The overall mean latency to regain the righting reflex for all animals given fluid percussion injury was 5.93 ± 0.16 minutes. None of the various survival groups (killed from 3 hours to 15 days) differed significantly from this magnitude (\( F_{4,12} = 0.407, p = 0.800 \)). A comparison of the duration of suppression of the righting reflex, used as an index of traumatic unconsciousness, provided additional evidence of injury uniformity among survival groups. The overall mean latency to regain the righting reflex for all animals given fluid percussion injury was 5.93 ± 0.16 minutes. None of the various survival groups (killed from 3 hours to 15 days) differed significantly from this magnitude (\( F_{4,12} = 0.407, p = 0.800 \)).

Callosal Axon Populations in Control (Sham-Injured) Rats

A total of 17,075 axonal profiles (6,659 from sham injured rats, and 10,416 from FPI rats) were digitally traced and cross-sectional areas computed. There were 13,797 unmyelinated and 3,278 myelinated axons measured. Statistical evaluations of postinjury changes to axonal morphology were based on these area measurements. Because previous studies of callosal axon size are reported in fiber diameter, cross-sectional area values were converted to diameters assuming circularity of axonal cylinders. Because the sham injury procedure dissected only the midline scalp and cranium without disturbing the dura or underlying parenchyma, we predicted that axonal morphology in sham-operated rats would be equivalent to that in naive control rats. The frequency distributions of axonal diameters from sham rats for unmyelinated and myelinated fibers combined from all regions observed (genu, mid callosum, and splenium) are shown in Figure 2A. Mean diameters of unmyelinated (0.223 ± 0.002 \( \mu \)m) and myelinated (0.568 ± 0.006 \( \mu \)m) axons corresponded closely to earlier estimates in the corpus callosum of rodents (25–27).

Morphologic parameters differed significantly among the callosal regions in sham control rats. The density of unmyelinated axons showed a striking rostrocaudal gradient, that is, highest in the splenium (mean = 6.2 × 10^5/mm²), falling off by 38.7% in the mid callosum, and by 54.8% in the genu (Fig. 2B). This regional variation in unmyelinated fiber density was highly significant (\( F_{2,24} = 19.459, p < 0.001 \)). In contrast, none of the regional estimates of myelinated axon density differed from an overall mean of 1.1 × 10^5/mm² (\( F_{2,24} = 1.627, p = 0.218 \)).
Axon diameters in sham control rats also exhibited significant spatial variation with genu fibers being largest for both unmyelinated (mean, 0.26 µm) and myelinated (mean, 0.63 µm) populations. At more caudal locations, the mean diameter decreased for both unmyelinated ($F_{2,24} = 14.645$, $p < 0.001$) and myelinated ($F_{2,24} = 13.580$, $p < 0.001$) axons (Fig. 2B). In comparison to the caliber of genu axons, axon diameters measured at more caudal regions decreased by an average of 17.7% (unmyelinated) and 14.2% (myelinated) (Fig. 2B).

The mean cross-sectional area of the total midsagittal corpus callosum, estimated from the most medial semithin sections, was $2.97 \pm 0.15 \text{ mm}^2$ in sham-injured rats. On the basis of these measurements, the overall volume of each callosal region was calculated and compared to the total callosal volume. The results indicated a significant decrease in the volume of the genu and splenium regions in comparison to the middle region ($F_{2,24} = 11.680$, $p < 0.001$). These findings suggest that the sham lesion procedure had a differential effect on the volume of callosal regions, with the greatest reductions occurring in the genu and splenium areas.

**FIGURE 2.** Characterization of axon populations in sham lesion control rats. (A) Distributions of axon diameters for myelinated and unmyelinated axons. Mean diameters and distribution shapes matched prior reports from naïve control rodents (see text), suggesting that the sham lesion procedure did not impact these parameters. (B) Separate analyses at callosal regions revealed a rostral-to-caudal (genu-to-splenium) increase in the density of unmyelinated axons but no gradient for myelinated axons (left panel). Mean axon diameter was significantly greater in the genu than in either posterior region (right panel). *, $p < 0.05$. 
of the above axonal density values and using the present definition of intact axon (continuous membrane profile, at least 1 microtubule, no degenerative debris), the estimated numbers of unmyelinated and myelinated axons in the corpus callosum were $11.55 \pm 0.73$ and $3.32 \pm 0.17 \times 10^6$, respectively. This yields a total of approximately $14.87 \times 10^6$ axons in the corpus callosum of adult male Sprague-Dawley rats, of which 77.7% are unmyelinated. These results are comparable to whole-callosum estimates provided in an early study that reported a cross-sectional area of 2.6 mm$^2$ and a total of $12 \times 10^6$ axons (25). Those authors used an essentially identical sampling scheme in rats at 60 days of age. Similarly, they reported that 80.8% of axons in the corpus callosum are unmyelinated (25), which is in close agreement with our estimates.

Effects of FPI on Callosal Axon Caliber and Density

The FPI used induces diffuse injury that results in electrophysiological (12, 14, 15) and behavioral (20) deficits with little or no cell death and only modest axonal degeneration. No areas of parenchyma contusion were observed during dissection. The ultrastructural appearance of injured corpus callosum was usually quite similar to that from control rats with the exception that most injured cases exhibited more astrocyte profiles, consistent with a postinjury activation of astroglia. Although quantitative analyses revealed significant morphometric changes to axonal dimensions at 3 days after injury, there was a qualitative similarity to controls in the general fiber architecture and the rostral-to-caudal increase in unmyelinated axon density in the injured rats at that time point (Figs. 3D–F).

Quantitative assessment of injury-related changes to cross-sectional area and density of axons was done in 2 phases. First, data from all regions were aggregated to estimate the generalized effect of fluid percussion on the corpus callosum as a whole. Then, separate analyses that focused on TBI-related changes within each callosal region were done.

Analyses of axonal cross-sectional area based on combined data from all callosal regions revealed a transient injury-induced decrease in unmyelinated axon area; this effect reached significance at 3 days (20.0% below sham levels; $p < 0.05$)
and 7 days (22.8% below sham levels; \( p < 0.05 \)) after injury. However, by Day 15 after injury, mean unmyelinated axon area was not different from that in controls (Fig. 4A). Figure 4 (and similar figures) depict postinjury values normalized to sham control means. Supplemental Digital Content 1 (http://links.lww.com/NEN/A315) contains corresponding tables of all analytic measures (mean ± SEM) in physical units (\( \mu \text{m}^2 \) for cross-sectional area and number per \( \text{mm}^2 \) \( \times 10^4 \)) for density) tabulated for each time point at each region and containing whole-callosum estimates aggregated from the regional data.

Examination of time-dependent changes in mean cross-sectional areas of myelinated axons suggested initial postinjury swelling at 3 hours and 1 day, but these increases did not reach significance (Fig. 4A). Myelinated fiber caliber overall did not significantly differ from sham control levels at any postinjury time point (\( F_{5,25} = 0.639, \ p = 0.672 \)). Area measures, which included the axon plus myelin (contour “a” in Fig. 1), also did not significantly vary from sham levels at any time point after injury (\( F_{5,25} = 0.592, \ p = 0.706 \)).

The ratio of the inner axonal diameter to the total outer diameter, commonly referred to as the “g ratio,” has been widely used as a structural index of optimal axonal myelination because the historical work of Rushton (28). The g ratio has been reported to change in some models of experimental neurotrauma, such as myelin thinning after a compressive spinal cord injury (29). In the present group of sham-injured rats, the mean g ratio was 0.708 ± 0.008, which approximates the optimal ratio for conduction velocity predicted by computer simulation (30). The g ratio was not significantly altered by FPI at any callosal regions or for the callosum as a whole.

In comparison to the FPI-induced decline in mean cross-sectional area of unmyelinated axons, postinjury changes in the density of intact callosal fibers were more variable. The experiment was designed to detect numerical losses of fibers that met the operational definition of “intact,” possibly reflecting sublethal cytoskeletal, organelle, or membranous alterations. The mean density of callosal unmyelinated axons based on combined data from all callosal regions was below the sham control level at all postinjury time points. This reduction did not reach significance in a statistical design with survival interval as a factor and which could detect time-dependent changes in density (\( F_{5,24} = 1.182, \ p = 0.347 \)) (Fig. 4B). Nevertheless, several aspects of Figure 4B suggest that the density of unmyelinated fibers was influenced by FPI to some degree as follows: 1) the mean density values were consistently below sham levels, 2) both density and area changes were maximal at 7 days, and 3) both processes trended toward recovery between 7 and 15 days. A post hoc analysis that pooled all postinjury time points did show significant injury-related reduction in unmyelinated fiber density (\( F_{1,28} = 5.855, \ p = 0.022 \)). Using similar pooling for regions showed that the greatest decrease in unmyelinated axon density was a 27.16% ± 7.13% decrease in the splenium (\( p < 0.05 \)), followed by a 23.67% ± 6.23% decrease in mid callosum (\( p < 0.05 \)); a 4.76% ± 7.72% density change in the genu was not significant. The density of myelinated axons was not significantly altered by FPI because the historical work of Rushton (28). The g ratio has been reported to change in some models of experimental neurotrauma, such as myelin thinning after a compressive spinal cord injury (29). In the present group of sham-injured rats, the mean g ratio was 0.708 ± 0.008, which approximates the optimal ratio for conduction velocity predicted by computer simulation (30). The g ratio was not significantly altered by FPI at any callosal regions or for the callosum as a whole.

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After TBI, unmyelinated axons exhibited distinct abnormalities related to their decreased density. In some cases, there were isolated unmyelinated axons exhibiting membrane discontinuities (arrowhead) and membranous folding apposed to aberrant extracellular spaces (arrow). (B) Clusters of unmyelinated fibers lacking distinct membranes (arrows), along with a myelinated axon (asterisk) with cytoplasmic abnormalities. (C) Example of vulnerability of relatively large unmyelinated axons (arrows) juxtaposed to intact small unmyelinated axons. All calibration as in C.

FIGURE 5. Representative axonal profiles that failed to meet the operational definition of “intact” in the splenium at 3 days after injury. (A) Isolated unmyelinated axons exhibiting membrane discontinuities (arrowhead) and membranous folding apposed to aberrant extracellular spaces (arrow). (B) Clusters of unmyelinated fibers lacking distinct membranes (arrows), along with a myelinated axon (asterisk) with cytoplasmic abnormalities. (C) Example of vulnerability of relatively large unmyelinated axons (arrows) juxtaposed to intact small unmyelinated axons. All calibration as in C.

After TBI, unmyelinated axons exhibited distinct abnormalities related to their decreased density. In some cases, there were isolated unmyelinated profiles that displayed membrane discontinuities or cytoplasmic abnormalities that prevented them from being scored as intact. This often took the form of atrophic changes where the axoplasm seemed to constrict, producing empty membranous folds and aberrant spaces in the adjacent extracellular compartment (Fig. 5A). In other cases, there were clusters of unmyelinated axons with degenerative changes affecting multiple fiber profiles. These groups of axons typically displayed axolemmal deterioration with untraceable membranes that prevented their designation as intact (Fig. 5B). In addition, there was an apparent selective vulnerability of relatively large axons, which often demonstrated axoplasmic constriction and membrane folding. These malformed large axons were sometimes observed within fields of intact small unmyelinated axons (Fig. 5C).

Analyses of unmyelinated axons within each callosal region revealed an FPI-induced reduction in fiber caliber that initially affected the splenium at Day 1 and then progressed rostrally to affect the mid callosal region at Day 3 and, finally,

FIGURE 6. Effect of moderate fluid percussion injury (FPI) on cross-sectional area of unmyelinated axons in the splenium, middle, and genu regions of the corpus callosum. Results are normalized to the mean axonal area measured in sham injured control rats. FPI produced a caudal-to-rostral sequence of significant axonal shrinkage expressed at 1 day in the splenium, 3 days in the mid callosum, and 7 days in the genu. Each region showed a recovery to sham control levels after the shrinkage. *, p < 0.05.
the genu at Day 7 after injury (Fig. 6). At these time points, statistically significant peak decreases in cross-sectional axon area of −30.8%, −30.0%, and −26.4% were observed for the splenium, mid callosum, and genu, respectively (Fig. 6). None of the callosal regions exhibited significant alterations in myelinated axon caliber or density (Supplemental Digital Content 1, http://links.lww.com/NEN/A315).

It is possible that postinjury changes in the cross-sectional area did not affect all unmyelinated axons uniformly, and this was also examined quantitatively. For example, relatively large unmyelinated axons may have been injured to a different extent from smaller fibers. As an initial approach to this issue, quartile values were computed for the unmyelinated axon diameters in sham injured rats, which were then used to assess injury-induced changes in the distribution of axon diameters in each callosal region (Fig. 7). Each panel establishes the quartiles for sham controls (first column) for a region, and columns to the right show how FPI altered the distribution of fiber diameters relative to the sham baseline. For example, 25.0% of the 2,603 axons in the splenium of sham rats had diameters of 0.14 μm of less, whereas among 937 splenial axons measured at 1 day after injury, 43.1% of fibers had diameters of 0.14 μm of less (Fig. 7A). The increase in the proportion of fibers with small (≤0.14 μm) diameters was accompanied by decreases in the proportion of larger fibers, particularly fibers in the top quartile (>0.27 μm), which decreased to 14.8% of unmyelinated axons measured in the splenium at 1 day after FPI. It is notable that the greatest increase in the lowest quartile occurred at Day 1 after injury in the splenium, at 3 days in the mid callosum, and at 7 days in the genu (Fig. 7, open arrows). These survival intervals match the time of greatest decrease in the mean cross-sectional areas (Fig. 6). The quartile analysis was based on axon area measurements, as well as fiber density, the latter of which was more variable after injury. This was likely the reason that significance tests for the data of Figure 7 showed that postinjury increases in the lowest quartile were only significant for the splenium (F5,20 = 3.773, p = 0.014). Finally, Figure 7 indicates that, for each region, the time of greatest increase in lowest quartile fibers was followed by a recovery to a quartile pattern very similar to that of sham controls. This is in agreement with the data in Figure 6 and probably reflects a time-dependent postinjury axonal shrinkage, followed by morphologic recovery. However, this recovery may also reflect a delayed loss of lowest-quartile fibers that no longer met the operational definition of “intact.” Thus, it is conceivable that some larger axons underwent shrinkage, thereby increasing the numbers counted as lowest-quartile fibers. These axons might have further continued to deteriorate (in membrane or microtubules) to a degree that they could not be counted as intact. Preferential loss of these lowest-quartile axons would tend to rebalance the distribution of fiber calibers; the quartile breakdown would then tend to more resemble the sham control pattern.

The preceding analysis revealed a time-dependent process wherein the proportion of unmyelinated fibers in the highest-diameter quartile transiently decreased and the smallest quartile membership increased; this effect progressed in a caudal-to-rostral pattern. Because relatively large unmyelinated axons may play a pivotal role in these changes, the largest quartile was further analyzed in detail by examining the time courses of post-TBI changes in the largest 10%, 5%, and 1% of fibers (using cutoff values based on the 90th, 95th, and 99th percentile of the sham control axons). When this analysis was conducted on the whole corpus callosum, averaging...
across all subregions, the results suggested some tendency for the largest axons to be at greater risk after TBI. The numbers of intact axons in the top 25%, 10%, and 5% of fiber diameters were generally below control values on post-TBI Days 1 and 3, although this only reached significance for the 10% subpopulation at Day 7 ($p < 0.05$; Fig. 8A). In contrast, the largest 1% was significantly less numerous at Days 1 and 3 ($p < 0.05$); this reduction remained significant even at Day 15 ($p < 0.05$), when the other fiber categories had converged toward control values (Fig. 8A). Importantly, axons in the largest 1% category were so sparse, after injury, that a time course could only be established for the whole callosum. In sham control rats, there was an average of only 5.4 fibers with diameters in the top 1% summed across all regions compared with a mean of 3.4 fibers in injured rats. A detailed time course at the regional level was not possible because of the small numbers of axons in the top 1% diameter range for at least 1 region in the injured rats: 80% of injured rats had no fibers in the top 1% in the genu (although this decrease was less severe in injured splenium and middle callosum, where 40% of injured rats had no fibers in the top 1%). Figure 8B summarizes postinjury changes in the top 25%, 10%, and 5% categories, confirming the temporal gradient of significant mean diameter decrease in the splenium at 1 day, and progressing to caliber loss in the genu at 7 days. This time course reflected significant declines in fibers in the top 25% class but that reductions in the 5% category also reached significance in the genu.

**DISCUSSION**

The findings of this study indicate that, for the specific injury conditions of moderate central FPI, unmyelinated axons undergo responses that are quite distinct from those of myelinated axons. The midline FPI model was selected for this study to match prior experiments showing functional impairments (12, 13, 20) and to explore nonlethal axonal changes in a white matter environment with minimal fiber degeneration. Although regarded as a moderate-intensity injury, the 2.0-atm midline FPI used here elicits less axonal injury and degeneration in the cerebrum than most alternative animal models of TBI, including lateral FPI, controlled cortical impact, and impact-acceleration (31, 32). The microscopic examination in the present study did not demonstrate significant degrees of axonal swelling, bulb formation, or Wallerian degeneration, which are reliably elicited in brainstem white matter tracts after impact acceleration injury in adult rats (10, 11, 33–36). Indeed, the FPI-induced morphologic changes in this study were relatively modest and required a quantitative stereological assessment for their characterization. These results may, therefore, have implications for the clinical condition of mild TBI, which often goes unreported and has been estimated to be approximately 22 times more prevalent than severe TBI (37). We observed post-TBI time-dependent cellular changes that are not necessarily lethal but may contribute to functional impairment that is concomitant with white matter subjected to mild TBI.

A key objective of the study was to establish the fiber composition of the corpus callosum in sham-injured rats as a valid estimate of the normal condition and to provide a reliable

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**FIGURE 8.** Effects of traumatic brain injury (TBI) on density of largest intact unmyelinated axons. (A) After fluid percussion injury (FPI), the mean density of the largest 25%, 10%, and 5% diameter intact fibers, combined across all callosal regions, was below sham control levels (significant for top 10% fibers at 7 days), but showed a trend toward a recovery by 15 days. In contrast, the density of the largest 1% fiber category was significantly decreased at 1 day, 3 days, and 15 days. (B) Density changes in largest unmyelinated fibers showed a caudal-to-rostral sequence spanning 1 day to 7 days, similar to postinjury diameter changes (Fig. 6). The largest 5% fiber category was significantly below control levels only in the genu at 7 days. Sham versus TBI comparisons (all $p < 0.05$): largest 25% “a,” 10% “b,” 5% “c,” and 1% “d.”
baseline against which to evaluate the effects of injury. This was warranted in view of variability among published reports regarding the percentage of unmyelinated axons in the rodent corpus callosum. Earlier studies of axon types in the rodent splenium estimated that approximately 45% of fibers were unmyelinated (38), but more recent studies have consistently estimated the proportion to be approximately 88% (25, 39, 40); our present estimate of 84% is in line with these reports. The consensus among the more recent estimates is likely due to sampling strategies that systematically cover the dorsal-to-ventral extent of the callosum. Among these prior studies, only Gravel et al (25) implemented a sampling methodology that was essentially equivalent to the present approach, including estimates at genu, mid callosum, and splenium. Averaging across all regions, those authors estimated that 81% of corpus callosum axons were unmyelinated, which corresponds closely to our estimate of 78%.

Almost all previous reports of TBI-induced changes in axon caliber have referred to focal axon swellings in myelinated axons that are usually associated with cytoskeletal degradation and resultant impairments of axoplasmic transport. Early events include focal axolemmal perturbations that are probably mechanically induced during the injury and are associated with aberrant permeability (41, 42). Subsequently, microtubule loss and altered neurofilaments lead to focal swellings in myelinated axons (33, 43, 44). These changes are observed in white matter that is subjected to greater injury forces than applied in the present study. We hypothesize that changes in the unmyelinated axon population, including the reduction in area observed in the current study, likely coexist with the abnormalities of myelinated axons in models of more severe TBI. Even within myelinated axons, a type of axonal shrinkage that takes the form of focal decreases in internodal axonal diameter has been described (41). In the current study, caliber measurements were based on sagittal sections that present axons in cross-section; these results do not address whether the observed changes in area occur at focal points along the unmyelinated axons or are expressed uniformly along the fiber length.

The finding of distinctive reactive changes in unmyelinated fibers adds to growing evidence that traumatic axonal injury has more heterogeneous features than previously considered. It is becoming clear that many injured axons do not exhibit the sequence of changes observed in myelinated axons.
For example, axons showing cytoskeletal disruption do not invariably progress to swelling (45), and neurofilament compaction is not invariably associated with impaired axoplasmic transport (9, 10). One factor underlying such divergence of reactive changes may be axon heterogeneity, that is, there likely are subpopulations of fibers with distinct molecular or structural properties. It is likely that myelinated and unmyelinated fibers are distinctive in more ways than diameter and/or the presence of myelin. Indeed, freeze fracture electron microscopy study indicated that axolemma in unmyelinated axons differs from that in myelinated axons at the macromolecular level (46). These authors found that P-face intramembranous particles were more numerous in the intermodal myelinated axolemma than in unmyelinated axolemma. Intrinsic differences in membrane constituents may influence fiber vulnerability. Indeed, the pattern and time course of structural changes that we observed in callosal fibers correspond in several respects to suppression of CAPs previously reported using the same FPI model (12). Postinjury reductions in evoked response amplitude were larger for unmyelinated, than for myelinated, CAPs. Compound action potentials evoked through unmyelinated fibers were significantly suppressed at all time points recorded (3 hours to 7 days), whereas FPI-induced impairments to the myelinated CAPs were more transient and had recovered to control levels by 7 days after injury. Previous CAPs were acquired in the mid callosal region (12, 15); more detailed analyses of CAP impairments at genu, mid callosal, and splenial regions are now in progress in our laboratory.

Differences in size and the presence or absence of myelin are likely to be primary factors in the contrasting injury responses of the axon types. Axons are the subcellular compartment with the highest membrane-to-cytoplasm ratio, which likely places them at risk to membrane-targeting pathomechanisms of TBI, including lipid peroxidation (47, 48), rapid proteolysis of voltage-gated sodium channels (49, 50), and more protracted proteolytic events attacking submembrane ankyrin (51) and spectrin (52–54). We hypothesize that, because of their higher average axolemma-to-axoplasm ratio, unmyelinated axons are at a greater risk to these processes than myelinated axons. This relationship is illustrated in Figure 9A, which models axons as uniform cylinders of arbitrary length and plots the present measured values of mean axon diameter on the curve relating surface-to-volume ratio to diameter. This demonstrates that the mean diameter of unmyelinated axons (0.22 μm) is approximately 60% smaller than the mean myelinated diameter (0.57 μm); this corresponds to a 160% increase in the surface-to-volume ratio. Intracellular calcium loading, a key TBI pathomechanism, may especially challenge small axons with less cytoplasmic volume and calcium buffering/sequestration capacity, which is known to be critical in white matter injury (55). These factors might contribute to a more significant injury response in unmyelinated axons as a class compared with the relatively mild response in myelinated fibers, which are typically much larger. Whereas it remains possible that unmyelinated axons of all sizes underwent some degree of postinjury shrinkage, the present results (Figs. 5C, 7, and 8) suggest that within the population of unmyelinated fibers, comparatively large diameter may comprise an additional risk factor.

Irrespective of axonal size, the presence of myelin itself, which provides physical support and influences fiber subtype vulnerability may account for some degree of axonal protection. In contrast, unmyelinated axolemma is extensively exposed to a posttraumatic extracellular environment with aberrant ionic composition (56), reactive matrix metalloproteinases (57, 58), and infiltrating peripheral factors after blood-brain barrier disruption (51) (Fig. 9B). The intimate exposure of unmyelinated axolemma to the extracellular compartment could also underlie differential responses to drug treatments, such as a greater functional protection for unmyelinated, over myelinated axons conferred by a calpain inhibitor (13).

Assessments of fiber density in this study also confirmed rostrocaudal heterogeneity of axonal composition, as previously described (25) and cross-sectional area measurements provided, for the first time, a stereological quantification of regional variation in mean diameter for both fiber types. The effects of FPI were regionally specific, with reduced unmyelinated size appearing first in the splenium and then progressing to more anterior zones. This injury sequence may reflect the intrinsic rostrocaudal gradient in unmyelinated axon density. This result may also in part reflect the proximity of the FPI location overlying the mid callosum and splenium (Fig. 1A), which showed axonal shrinkage before the genu. However, it would be difficult to attribute the different latencies to maximum shrinkage (1 day for splenium and 3 days for mid callosum) to different distance from the FPI location. In the cuprizone demyelination, pathologic changes were expressed in a rostrocaudal sequence (59, 60), consistent with the concept that properties intrinsic to specific callosal regions may determine the timing and magnitude of pathologic alterations.

In summary, the present results provide evidence that moderate FPI applied to the midline of adult rats induced selective morphologic changes in unmyelinated axons of the corpus callosum. A time-dependent reduction in mean axonal caliber, and its subsequent recovery, was highly significant. Reductions in unmyelinated axon density were also detected but were variable. Our findings have implications for current concepts of axonal injury that have tended to focus on pathology in larger myelinated axons. The diminutive size of unmyelinated fibers presents challenges to any monitoring of these structures in clinical, and even experimental, applications, but a comprehensive model of axonal injury must account for distinctive alterations that affect the numerical majority of cerebral white matter axons. Injury-induced reductions in average axonal size would likely reduce the mean conduction velocities in these fibers and possibly contribute to the cognitive and memory impairments that are frequently observed after TBI. Although quantitative magnetic resonance imaging has confirmed TBI-induced shrinkage of white matter area (61, 62), the basis of these changes has not been identified at the cellular level. Fiber atrophy, perhaps related to the axonal changes described here may contribute to posttraumatic the white matter shrinkage observed in patients.

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REFERENCES


44. Reeves TM, Greer JE, Vanderveer AS, et al. Proteolysis of submembrane cytoskeletal proteins ankyrin-G and cullin spectrin following diffuse brain...