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High Tolerance and Delayed Elastic Response of Cultured Axons to Dynamic Stretch Injury

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Although axonal injury is a common feature of brain trauma, little is known of the immediate morphological responses of individual axons to mechanical injury. Here, we developed an *in vitro* model system that selectively stretches axons bridging two populations of human neurons derived from the cell line N-Tera2. We found that these axons demonstrated a remarkably high tolerance to dynamic stretch injury, with no primary axotomy at strains <65%. In addition, the axolemma remained impermeable to small molecules after injury unless axotomy had occurred. We also found that injured axons exhibited the behavior of "delayed elasticity" after injury, going from a straight orientation before injury to developing an undulating course as an immediate response to injury, yet gradually recovering their original orientation. Surprisingly, some portions of the axons were found to be up to 60% longer immediately after injury. Subsequent to returning to their original length, injured axons developed swellings of appearance remarkably similar to that found in brain-injured humans. These findings may offer insight into mechanical-loading conditions leading to traumatic axonal injury and into potential mechanisms of axon reassembly after brain trauma.

Key words: axon; trauma; elasticity; dynamic deformation; tolerance; axolemmal permeability; neurofilament

Throughout the world, traumatic brain injury is a leading source of mortality and disability, particularly of children and young adults (Kraus et al., 1994; Sosin et al., 1995). Axonal injury is a common pathology resulting from brain trauma, in which the extent of axonal pathology is thought to play a major role in the outcome (Adams et al., 1982, 1989; Graham et al., 1988; Povlishock, 1992). It is proposed that the initial event in traumatic axonal injury is the deformation, or strain, of axons as the result of inertial loading commonly induced during motor vehicle crashes, falls, and assaults (Adams et al., 1984; Thibault et al., 1990; Gennarelli, 1993; Grady et al., 1993). This mechanical deformation is thought to damage the neurofilament structure in the cytoskeleton of axons, causing regional compaction and/or impaired transport. As a result, accumulation of transport material induces regional axonal swelling (Povlishock et al., 1992). The primary changes in neurofilament structure may be augmented by ionic shifts and secondary activation of proteases to degrade the axonal ultrastructure further. Ultimately, axons may disconnect at the distal border of swollen regions, forming classic terminal bulb formations followed by Wallerian degeneration. Remarkably, this process has been observed to occur over the course of hours to even months after injury (Povlishock and Becker, 1985; Povlishock, 1993; Sherriff et al., 1994; Pierce et al., 1998). Although these processes of evolving axonal pathology have been well characterized in humans and several experimental models, little is known of the immediate events during and after dynamic deformation of axons. This information may help elucidate the biomechanical factors that initiate the development of axonal pathology.

Central to understanding the induction of axonal pathology is defining the relationship between the applied mechanical force and the structural or functional response of the axon. For example, the mechanical threshold for primary axotomy (the severing of axons at the time of injury) is useful for developing preventative strategies for brain trauma. Current estimates of the mechanical tolerance for the structural and functional limit of *in vivo* axons of the CNS have been determined from *ex vivo* preparations using the squid giant axon (Galbraith et al., 1993), the frog sciatic nerve (Gray and Ritchie, 1954), the rat tibial nerve (Rydevik et al., 1990), and the pedal nerve of the slug (Jenkins and Carlson, 1904). These estimates have been complemented with data from physical and computational models (Zhou et al., 1994; Meaney et al., 1995) to develop a proposed tolerance standard of axons to mechanical damage. However, none of these studies have measured directly the pathological or mechanical changes associated with the deformation of intact CNS mammalian axons. In the present study, we examine the dynamic deformation of axons *in vitro* and report the threshold for primary axotomy, as well as novel findings on the acute temporal evolution of axonal response to stretch injury.

MATERIALS AND METHODS

Cell culture. We chose the N-Tera2 cl/D1 (NT2) cell line as our neuronal substrate because of the well-characterized ability of this cell line to differentiate into robust human neurons (Pleasure et al., 1992; Pleasure and Lee, 1993). In addition, this cell line has been shown to respond to excitatory injury in a manner similar to that of primary neuronal cell cultures (Munir et al., 1995). The NT2 cells were maintained in culture with OptiMEM (Life Technologies, Gaithersburg, MD) media supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT) and 1% penicillin–streptomycin (Pen-Strep; Life Technologies). To differentiate the NT2 cells into neurons (NT2N), we cultured NT2 cells for 5 weeks

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in DMEM supplemented with 10% FBS (HyClone), antibiotics (1% Pen-Strep; Life Technologies), and 10 μM retinoic acid (Sigma, St. Louis, MO). To isolate neurons in the culture, we trypsinized the cells, triturated them with a fire-polished Pasteur pipette, and replated them in DMEM supplemented with 5% FBS and mitotic inhibitors (10 μM 5-flouro-2′-deoxyuridine, 10 μM uridine, and 1 μM cytosine β-arabinofuranoside; Sigma) for 9 d. The cells remaining after this procedure have been determined to be 99% neuronal. These NT2N neurons were seeded on a treated (poly-D-lysine, fibronectin, and Matrigel) deformable substrate (Specialty Manufacturing, Saginaw, MI) in custom-designed culture wells. A 1.5 × 16 mm clear silicon barrier was placed on the membrane in the center of the well before plating of the NT2N cells to create a 1.5 mm "gap" through the center of the membrane. Cells were allowed to attach for 24 hr before the barrier was removed. The temporary barrier prevents neurons from seeding in the gap region, creating a cell-free area for growth of isolated axons. After barrier removal, axons begin traversing the gap, ultimately synapsing with neurons on the other side (Fig. 1). The diameter of the axons crossing the gap ranged from 0.5 to 1.5 μm, typical widths of *in vivo* human axons. These cultures were maintained in conditioned media (50% media from the first replate and 50% DMEM with 5% FBS) for 3 weeks before an experiment, because it has been demonstrated previously that NT2N cells express a mature neuronal phenotype similar to that of *in vivo* human neurons with regard to receptor function by 2–2.5 weeks after plating (Munir et al., 1995).

Stretch device. The stretch device consisted of an aluminum cover block, a stainless steel plate with a machined 1.5 × 18 mm slit, and an air pulse-generating system (Fig. 1). The culture well was inserted into the cover block and then placed on the slit plate so that the area of the deformable substrate contained the cultured axons. The cover plate was attached to the microscope stage, creating a sealed chamber. The top plate had a quartz viewing window in the center, an air inlet for compressed air, and a dynamic pressure transducer (Entran model EPX-V01-25P-/l6F-RF, Fairfield, NJ) to monitor internal chamber pressure. The introduction of compressed air into the chamber was gated by a solenoid (Parker General Valve, Elyria, OH). The solenoid and the pressure transducer were controlled and monitored by an analog-to-digital board (Keithley Metrabyte, Cleveland, OH) integrated with a computer data acquisition system (Capital Equipment Corporation, Billerica, MA). The device was mounted on the stage of a Nikon inverted microscope (Optical Apparatus, Ardmore, PA), allowing for continuous observation and photography of the axons throughout the experiments (Fig. 1).

Axonal stretch. A controlled air pulse was used to induce stretch to only the cultured axons traversing the gap in the well (Fig. 1). A rapid change in chamber pressure (rise time, 20 msec; duration, 50 msec) deflects downward only the portion of the substrate that contains the cultured axons; as a result, only these axons are stretched transiently to mimic the *in vivo* conditions of traumatic brain injury. Preliminary studies using microbeads attached to the substrate demonstrated that at static peak deflection, the axons remained attached to the membrane (data not shown). Therefore, membrane deflection correlated directly to uniaxial strain on the axons. Analysis of processes was constrained to axons within ±10° parallel to the major stretch axis. Measurement of nominal uniaxial strain (ϵ) was calculated by measuring the centerline membrane deflection (δ) and substituting into the geometric relationship:

$$\varepsilon = \frac{w^2 + 4\delta^2}{4\delta w}\sin^{-1}\left(\frac{4\delta w}{w^2 + 4\delta^2}\right) - 1.0,$$

if the measured deflection was less than one-half of the slit width (w). If the measured deflection was greater than one-half of the slit width, then the applied stretch was calculated from a second geometric relationship:

$$\varepsilon = \frac{w^2 + 4\delta^2}{4\delta w}\left(\pi - \sin^{-1}\left(\frac{4\delta w}{w^2 + 4\delta^2}\right)\right) - 1.0.$$

A strain value (ϵ) of 0.5 indicates that the axon was stretched to 50% above its initial length. For the experiments presented here, internal chamber pressure was 5–7 psi, correlating to a uniaxial strain on the axons of 0.58–0.77, or 58–77% beyond its initial length. In all cases, the rate at which this strain was applied to the axon was between 26 and 35 sec^{-1}, well within the range for traumatic injury. Over 50 separate experiments using these injury parameters were performed for this study.

Microscopy. Phase and fluorescent microscopy and photomicrography were performed on a Nikon Diaphot microscope with a Nikon 8008 camera. Confocal microscopy was performed with a Zeiss LSM5 (Heidelberg, Germany). Deconvolution microscopy (Hiraoka et al., 1987) was performed on a Zeiss Axiovert 100 microscope equipped with a cooled CCD (Princeton Instruments, Trenton, NJ) and DeltaVision constrained iterative deconvolution software (Applied Precision, Issaquah, WA).

Measurement of morphological response to injury. In each experiment, we used time-lapse phase-contrast micrographs to measure the change in axonal geometry after stretch. Micrographs were recorded before and after stretch at regular intervals (2 min) in a selected set of experiments. The micrograph set was transferred to digital format, and an image analysis program (ImagePro, West Chester, PA) was used to measure the length of the axonal processes within the field of view. Because a preliminary study showed that the axons did not detach from the membrane below the primary axotomy threshold, the axons appearing in the field before the stretch were also present at all times after stretch. Moreover, regular morphological markers on the axons in the field allowed the direct tracking of a portion of an axonal segment within the field of view. Therefore, these data reflected the true length change of the axonal process after a controlled stretch.

To quantify the morphological response over time, we defined a distortion (distension) parameter [$D(t_i)$] that reflected the change in geometry from the initial axon segment length before stretch (L_o) to the length of the same segment at a time after stretch [$L(t_i)$]:

$$D(t_i) = L(t_i)/L_o.$$

By definition, before stretch the distortion of the process is 1.0 (i.e., there is no change in length). After the stretch, values of $D(t_i)$ increased above 1.0 because there was a net increase in axon segment length in these samples.

Immunohistochemistry. To evaluate the disruption of both the axonal cytoskeleton and the axonal transport after trauma, we used immunohistochemical techniques to stain neurofilament protein. The cultures were fixed 2 hr after injury in 2% paraformaldehyde and 0.05% gluteraldehyde for 30 min, permeabilized with 0.5% Triton X-100 for 1 min, and then labeled with monoclonal antibodies (RMO-281; 1:5) against phosphorylated neurofilament medium side arms (Lee et al., 1987). These antibodies were colocalized with fluorescent label conjugated to anti-mouse IgG (Alexa 488; Molecular Probes, Eugene, OR).

Axolemmal permeability. The permeability of axonal membranes (axolemma) after stretch injury was evaluated using Alexa 488 hydrazide (Molecular Probes), a membrane-impermeant fluorescent dye with a molecular weight of 570 Da. Accordingly, intracellular accumulation of this dye demonstrates the membrane permeability of small molecules. Axons were deformed with a strain of 60 or 75% in the presence of 200 μl of 580 μM Alexa 488 hydrazide dissolved in control saline solution (CSS; 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, and 25 mM HEPES, pH 7.4, at 335 mOsm). The dye solution was immediately rinsed off after injury and replaced with CSS. The axons were then evaluated with phase and fluorescence microscopy and photographed at 5 min after injury as described above. As a positive control, the NT2N cultures were permeabilized with a single rinse of 0.005% Saponin (Sigma) in CSS, which was washed off with CSS, and then incubated for 2 min with dye solution. As a negative control, the cultures were incubated in the dye solution without injury or Saponin for 2 min.

RESULTS

Using NT2N human neuron cultures (Pleasure et al., 1992; Pleasure and Lee, 1993), we developed an *in vitro* model system of isolated axonal stretch. This system uses an injury device that can precisely control the biomechanics of axon stretch (i.e., strain and strain rate) and can be integrated with an inverted microscope. Accordingly, we were able to observe continuously the acute temporal response of axons to uniaxial stretch at specific strains and strain rates.

The threshold for primary axotomy in cultured neurons

We found that the axons remained attached to the substrate until levels of strain inducing primary axotomy were reached. Examination of axons with phase microscopy during and immediately after stretch revealed that axons experienced strains that corresponded to the underlying substrate until primary axotomy occurred. We determined that the uniaxial strain on the substrate at

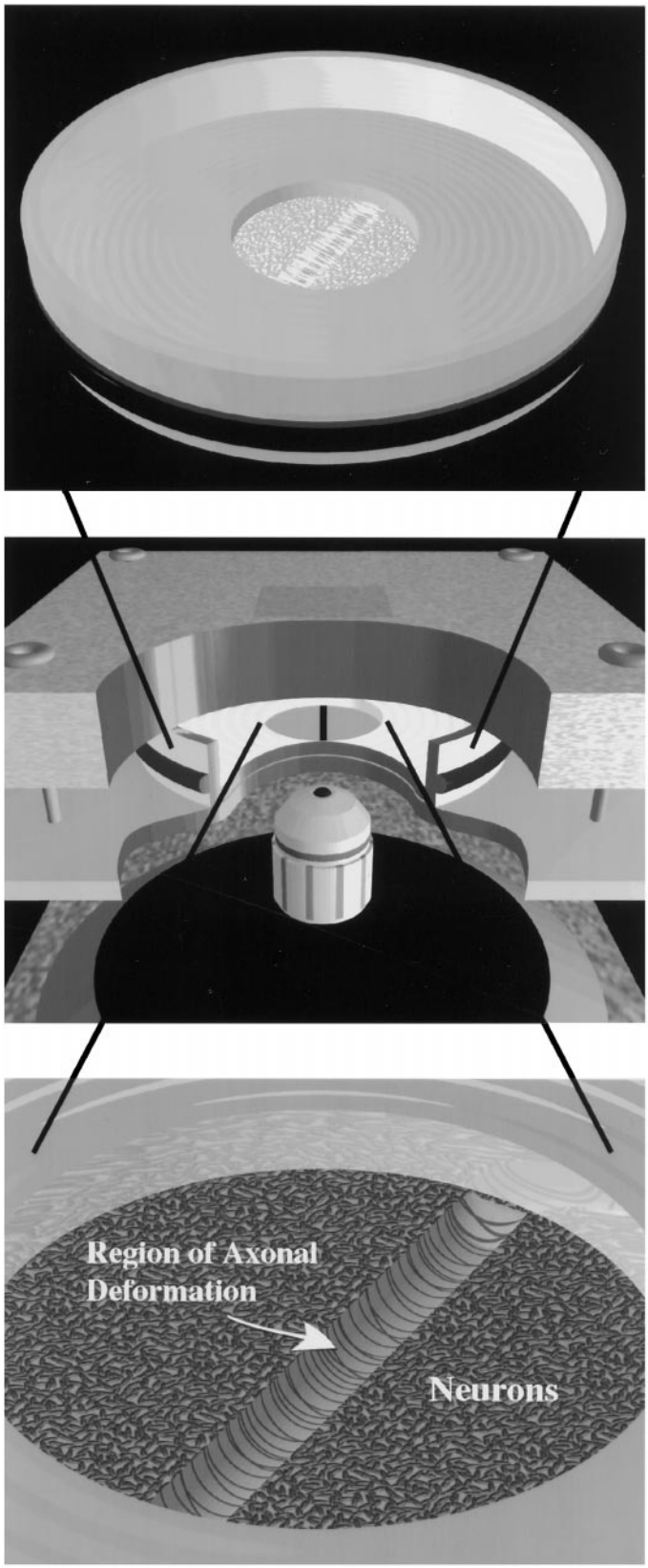

Figure 1. Schematic illustration of the technique and apparatus used to induce dynamic stretch injury to axons spanning two populations of human neurons. *Top*, Stainless steel well with a thin transparent deformable membrane in the center on which neurons (NT2N) are plated is shown. A 1.5 × 18 mm cell-free gap is formed in the middle of the membrane that is bridged only by axons. *Middle*, The well is placed in the stretch device (cut away in the figure to reveal components) that consists of an aluminum cover block with a quartz viewing window and a stainless steel plate on the bottom with a machined 1.5 × 18 mm slit that aligns with the gap region on the membrane. For continuous observation of the axons via an inverted microscope, the apparatus is bolted to the microscope stage that also creates a sealed chamber. *Bottom*, Compressed air is introduced into the chamber causing a downward deflection of only the gap region of the membrane, thereby inducing uniaxial stretch of the axons.

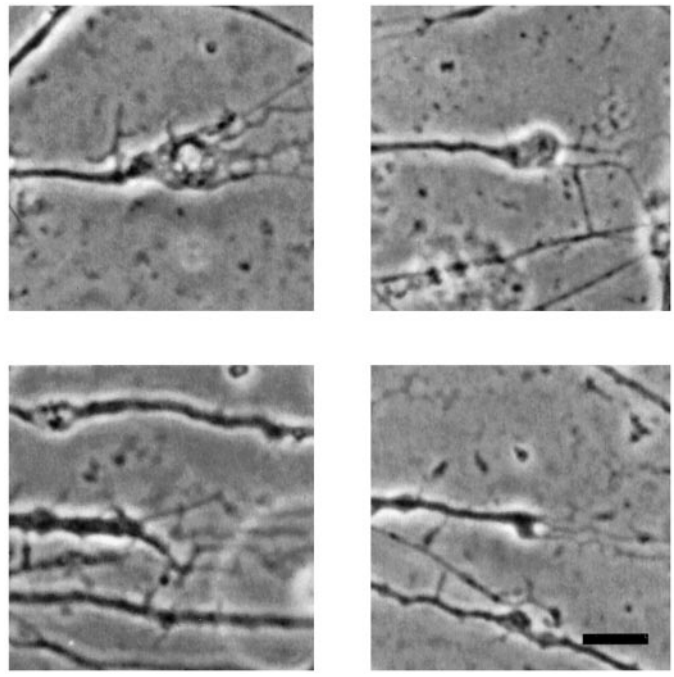

Figure 2. Phase-contrast photomicrographs of four examples of primary axotomy immediately after dynamic stretch injury. Primary axotomy, found only at strains >65%, is represented by severed axons shown in the middle of each *panel*. Note the torn or shredded appearance of the axonal terminal stumps with possible cytoskeletal remnants trailing out. Scale bar, 10 μm.

which primary axotomy could be found was >65%. However, even strains of 77% did not sever all axons. We also observed that primary axotomy resulted in the appearance of a shredded or torn terminal end of the severed axons (Fig. 2). Extruding from these ends were thin fibrils that may represent cytoskeletal remnants trailing out.

The morphological response to stretch injury

Below the level of strain for primary axotomy, axons maintained their overall position on the substrate but were consistently found to have undulating distortions at periodic points along their length immediately after trauma (Fig. 3). Although the extent and number of these distensions increased with an increase in the applied axonal strain, it is important to note that these distensions were consistently observed at all strains and strain rates used in the present study (i.e., 0.58–0.77 strain; 26–35 sec^{-1} strain rate). Independent of the shape or extent of distortion of the elongated regions, the axons gradually recovered their straightened pre-stretch shape over a period of 45 min, with most of the relaxation occurring in the first 10 min (Fig. 3). It is important to note that the delayed elastic response of regions of injured axons was consistently observed in >50 separate experiments using the described techniques.

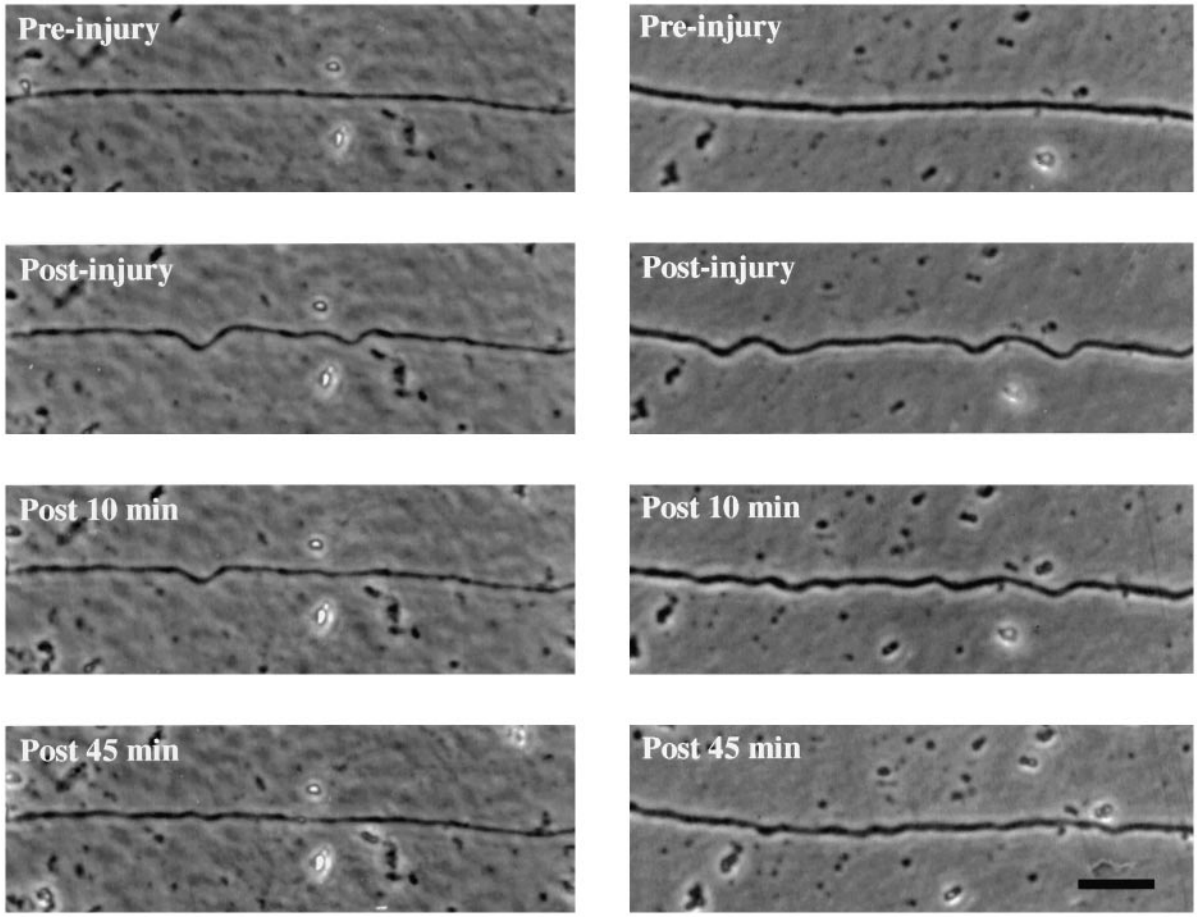

Figure 3. Phase-contrast photomicrographs of two examples (*left*, *right*) of the temporal evolution of the delayed elastic response of axons to dynamic stretch injury. As labeled, axons change from a straight orientation before injury to developing large undulations immediately at the time of injury yet gradually reassume their original orientation within 1 hr. Scale bar, 10 μm.

Because the periodic distortions occurred at selected points along the axon, we examined the regional geometric change in injured axons over time using serial photomicrographs to quantify the immediate increase and gradual recovery in length after stretch. Focusing on subregions of processes that showed geometric changes after stretch, we measured the regional distortion that occurred in these regions as a consequence of stretch over axon lengths of ~200 μm. We found that the overall length increased almost 8% immediately after stretch and that the greatest reversal of this increase occurred within the first 5 min after injury. This increased average length remained significant until 30 min after injury (Fig. 4). However, although the total length increased <8% after injury, the increase in length of many individual distortions (undulations) approached the level of the applied substrate stretch. For example, many of the local distortions had a half circle or triangle appearance immediately after injury (as illustrated in Fig. 3), in some cases measuring up to a remarkable 60% increase in length compared with their preinjury status. This observation is supportive evidence that the substrate strain was translated to the tissue. Taken together, these data also show the nonuniformity of the distortion phenomena of axons after dynamic stretch injury. Some axon regions appeared to have an elastic recovery after injury (i.e., immediately returning to their preinjury length), whereas other regions of the same axon demonstrated a delayed elastic response.

Changes in neurofilament structure after stretch

By 2 hr after stretch injury, multiple swellings along the length of many axons could be observed with phase microscopy. We subsequently found that these swellings could also be consistently observed at 2 hr after injury in fixed preparations of axons stained with antibodies targeting neurofilament sidearms. Confocal and deconvolution microscopy of these samples demonstrated that the accumulation of neurofilament in the axonal swellings appeared strikingly similar to swollen axons described in human brain injury. In addition, both confocal and deconvolution microscopy revealed that a central core of neurofilament was still apparent in some of these swellings represented by a more intense immunoreactivity to the neurofilament antibody. In some cases, this central core appeared disturbed from its original orientation with a tortuous course, even though the axon as a whole had returned to its preinjury orientation (Fig. 5).

Axolemmal permeability after stretch injury

With no stretch injury or chemical permeabilization, axons did not take up the 570 Da Alexa 488 dye (i.e., the axolemma was impermeant to small molecules) (Fig. 6*A*,*B*). However, chemical permeabilization of nonstretched axons did induce substantial uptake of the Alexa 488 dye (Fig. 6*C*,*D*). In comparison, 5 min after stretch injury to axons, a 60% strain (which produces no axotomy) did not induce uptake of dye into any axons, all of which

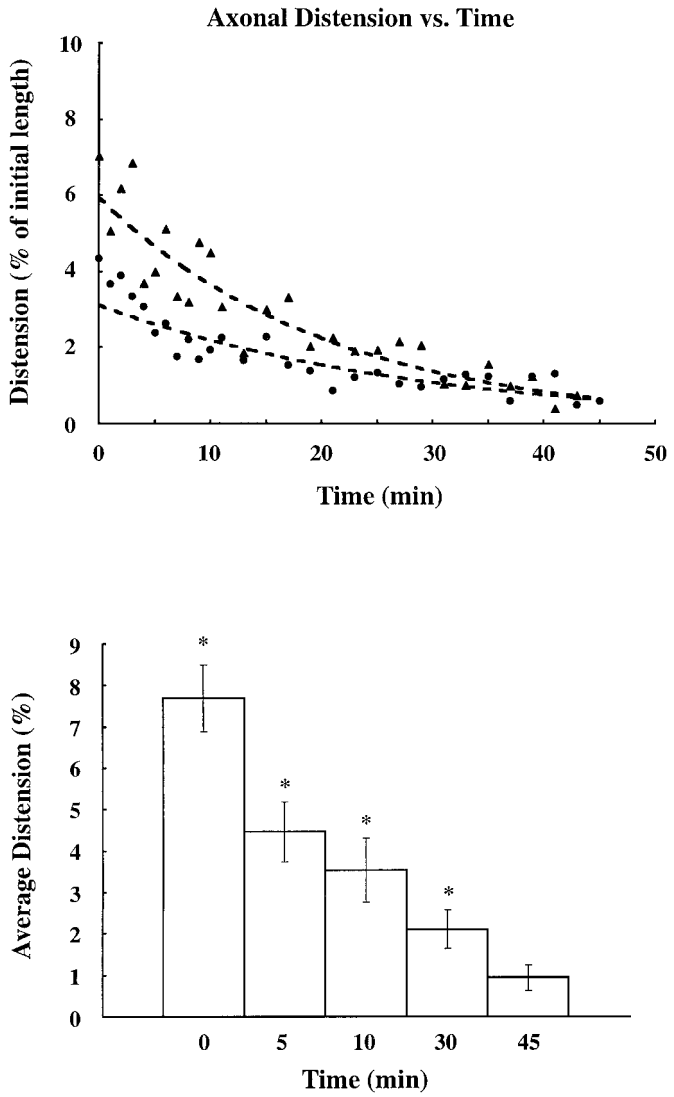

Figure 4. Quantitative temporal maps of the delayed elastic response of axons to dynamic stretch injury. *Top*, The temporal change in percent initial total length of two individual axons (*circles*, *triangles*) after stretch injury. *Bottom*, Average percent change in length of 11 axons over time after injury (*$p < 0.001$ compared with preinjury length).

demonstrated the typical post-trauma undulated orientation (Fig. 6*E*,*F*). At the strain of 75%, there was modest uptake of dye, only in axons that had been severed (primary axotomy) (Fig. 6*G*,*H*). Axons not severed at these higher strains were not permeable to the dye. In severed axons, the extent of dye uptake appeared to be less than that for chemically permeabilized axons on the basis of the general intensity of the fluorescence signal. Therefore, after stretch injury, dye was not taken up by the axons unless axotomy had occurred.

DISCUSSION

In the present study we developed an *in vitro* model system that can deliver dynamic uniaxial deformation at specific strains and strain rates to axons spanning two populations of human neurons. The application of tensile strain to axons in this model, designed as a replication of the mechanical loading experienced by axons *in vivo* during traumatic brain injury, resulted in the temporal development of multiple foci of swelling along the axons. In these

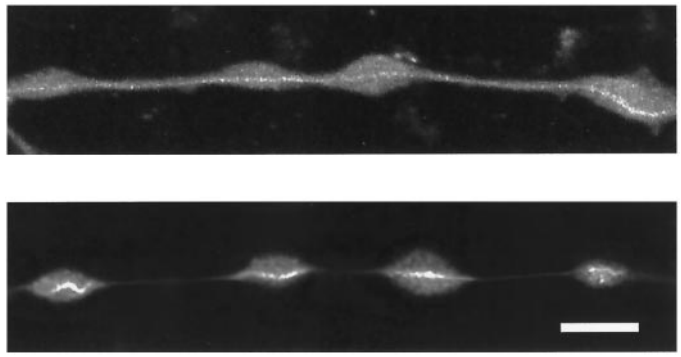

Figure 5. Accumulation of neurofilament in axons by 2 hr after dynamic stretch injury. Confocal (*top*) and deconvolution (*bottom*) microscopy demonstrates multiple swellings along injured axons immunostained to reveal neurofilament protein. Both confocal and deconvolution microscopy reveals what appears to be a central core of neurofilament in the swellings represented by more intense immunostaining. In some of the swellings, this central core appears disturbed from its original orientation with a tortuous course, even though the axons as a whole had returned to their straight preinjury orientation.

axonal swellings, we found accumulation of neurofilament proteins, potentially demonstrating impaired axonal transport or local disassembly of the cytoskeleton. Notably, this pathology has a remarkably similar appearance of axonal swellings found after diffuse brain injury in humans suffering brain trauma (Adams et al., 1989; Povlishock, 1992). Thus, this *in vitro* model seems to reproduce the most salient pathological feature of traumatic axonal damage found clinically. Because of our ability to control the applied stretch precisely and to observe directly the temporal evolution of pathology after stretch, three additional and potentially important observations were made. (1) Axons demonstrated a remarkably high tolerance to tensile strain even under dynamic loading conditions, with no primary axotomy observed from applied strains below 65%, (2) axons exhibited the behavior of "delayed elasticity" after dynamic deformation, going from a straight orientation before injury to developing an undulating course immediately at the time of injury, yet gradually recovering their original orientation and morphology, and (3) despite the internal damage resulting from the stretch injury, the axolemma was not permeable to small molecules unless primary axotomy had occurred.

Previously, thresholds for primary axotomy were thought to be 25–30% tensile strain when the axon was stretched in <50 msec. Data from the tensile elongation of excised giant squid axons suggested that axons would structurally fail with strains of ~25–30% at strain rates in excess of 10 sec^{-1} (Galbraith et al., 1993). Functional deficits, as measured by changes in resting membrane potential, appeared when dynamic strains were >5%. In peripheral nerve stretch studies using the dissected sciatic nerves from frogs, the structural failure limit was found at 33% at similar strain rates (Gray and Ritchie, 1954) and did not show changes in electrophysiological measures at levels 5–7% strain in contrast to the squid giant axon. However, the axons used in these previous studies had relatively large diameters (5–1000 μm), were not of mammalian origin, and were evaluated under the *ex vivo* conditions. Taken together, these potentially important considerations may have contributed to failure at much lower thresholds than that observed in the intact mammalian CNS axons used in the present study.

Evidence developed in parallel has suggested that the tolerance

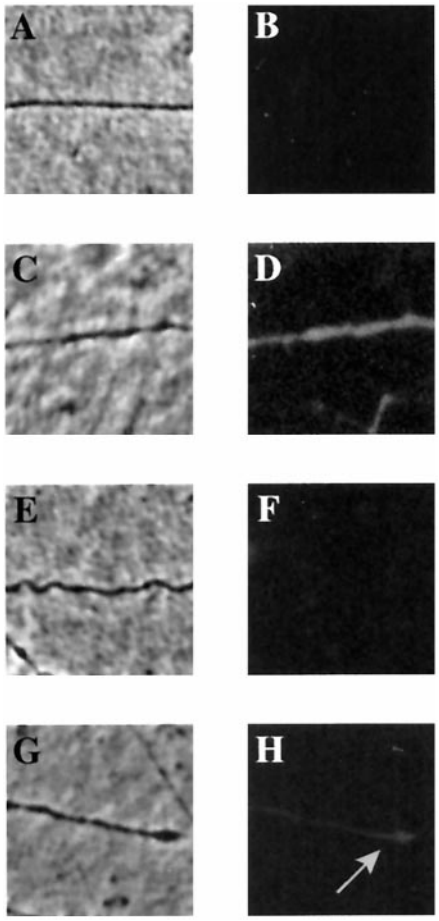

Figure 6. Representative images of axolemmal permeability shown under phase (*left*) and fluorescence (*right*) microscopy. These axons were exposed to a fluorescent dye, the uptake of which demonstrates the membrane permeability of small molecules. *A*, *B*, No dye is taken up in the absence of either stretch injury or chemical permeabilization of membranes. *C*, *D*, Dye is readily taken up after chemical permeabilization of the axolemma of axons. *E*, *F*, No dye is taken up by stretch-injured axons that are distorted but not severed. *G*, *H*, A detectable amount of dye is taken up in stretch-injured severed (primary axotomy) axons.

for primary axotomy is closer to the value reported herein rather than to previous data from squid and peripheral nerve studies. Recently, we developed a model of inertial brain trauma in pigs that produces selective injury to axons in the white matter (Smith et al., 1997). This model is accompanied by complementary physical and computational models used to determine the strain in regions shown to develop pathology (Zhou et al., 1994; Meaney et al., 1995). The estimated thresholds for shear strain associated with the appearance of axonal pathology (i.e., below the level of primary axotomy) in these collective models were between 15 and 24%, higher than that reported to produce primary axotomy in earlier *ex vivo* studies. Our findings in the present study of primary axotomy tolerances of 65% tensile strain support our observations in the pig inertial brain trauma models of a substantially higher tolerance of axons than believed previously.

Our present results represent the first observation of a characteristic delayed elastic response of axons to dynamic deformation. The phenomena of delayed elasticity is commonly demonstrated in viscoelastic solids and is generally described as the complete recovery of shape for a material that has been deformed by a mechanical force (Flügge, 1975). The delayed elastic response of the axons to mechanical stretch in the present study included an immediate change in length of the axon and subsequent gradual recovery of the axon to its original length. Although it has been found previously that neurites demonstrate elastic responses under mechanical stress (Zheng et al., 1991), the ability of axons in the present study to resume original orientation gradually after potentially devastating deformation was unanticipated. Distortions of axons after brain trauma have been described at single time points after axonal trauma in animal models and in humans. However, it has been presumed that these distortions represented irreversible progression of cytoskeletal disruption and degeneration (Povlishock and Becker, 1985), underscoring the limitation of the *in vivo* studies to examine the temporal evolution of pathological changes in individual axons. We propose that our *in vitro* model replicates axonal distortions found after injury *in vivo* and demonstrates that, despite marked deformation, the axons can eventually regain their original (prestretch) geometry. The identification of a post-traumatic geometric change in the axon is an unprecedented phenomenon for neural tissue and may reflect a molecular-based process that can have important clinical implications in developing therapeutic strategies for traumatic axonal injury.

Although the source for the delayed elastic response of damaged axons is not presently clear, delayed elasticity is a well studied phenomenon in inert viscoelastic materials and is often attributed to one or more of the following factors: (1) the viscous flow of molecules in a network or dilute solution, (2) the self-diffusion or "reptation" of molecules within a constraining molecular network, or (3) the active reformation of a molecular structure via new network connectivity (De Gennes, 1979). Because the axonal cytoskeleton has a high degree of interaction and cross-linking between its structural protein constituents (Burgoyne, 1991) and is a dynamically adaptable structure, these factors all may provide a basis for the mechanism(s) of delayed elasticity observed in the present study. Owing to the observation that axons return eventually to their original length after stretch, it seems that some part of the cytoskeleton is preserved sufficiently to provide an entropically driven restoring force. More precisely, the rate of recovery after stretch may be driven by the remaining elastic integrity of the network. Ongoing studies are evaluating the mechanical response of individual cytoskeletal constituents to mechanical deformation (Leterrier et al., 1996).

A major paradox of axonal injury found *in vivo* is the observation of extremely damaged axons among normal-appearing axons in the same white matter tracts after trauma. It is unclear why some axons regain their structure and function despite evidence of severe trauma, especially axons of similar size and orientation as that of neighboring axons that go on to degenerate. Although axonal bulb formation after axotomy is most likely an end stage event, it has not been resolved whether swollen yet still connected axons may recover. On the basis of our current results, we propose that axons have intrinsic mechanisms that facilitate recovery. The behavior of delayed elasticity in traumatized axons may reflect a "molecular memory" for axon shape such as has been posited for polymer dynamics (Sperling, 1992). Accordingly, cytoskeletal repair may be facilitated by further driving intrinsic molecular repair strategies to restore the initial cytoskeleton network geometry. Specifically, the viscoelastic reorientation of injured axons potentially brings back together regions separated by trauma and, thus, may improve the likelihood of cytoskeleton reassembly. Moreover, realignment of injured axons may help reinstate transport, which would further facilitate repair. It may

also be important to consider that rather than being a detriment, the limited accumulation of neurofilament and other transport materials in damaged axons may actually provide the substrate for cytoskeletal reassembly after trauma.

Another potentially important finding in the axonal stretch model was that despite a 60% strain and substantial distortion of the axon, the axolemma appeared to remain impermeant to small molecules because no low molecular weight fluorescent dye was taken up in the cytosol. Modest uptake of dye into axons was only found at higher strains and only in axons that had been severed. These data suggest that substantial axolemmal permeability changes are not produced unless there is disconnection of the axon. These observations are consistent with the results of multiple previous studies using hematopoietic cells that have established the remarkable ability of the cell membrane to "flow" as an accommodation to substantial distention or deformations without changing permeability (Evans, 1973). In apparent contrast with these observations, studies of axonal damage in experimental animal models of brain trauma have demonstrated altered axolemmal permeability acutely after injury (Pettus et al., 1994; Povlishock et al., 1997). However, only a subpopulation of damaged axons was shown to have permeability changes in these animal studies. Therefore, changes in axolemmal permeability after trauma in animal models may also depend on the relative extent of axonal damage. Because contiguous regions of membranes rapidly adapt to high strain and distortions, the substantial and acute increases in axolemmal permeability shown in animal models of brain trauma models may reflect partial or complete axotomy, as demonstrated in the present study.

In summary, the present results suggest that axons not only have remarkably high resiliency to primary axotomy and axolemmal disruption from dynamic deformation but also demonstrate a unique ability to resume relatively normal shape and orientation. These findings may offer important considerations for the understanding of mechanical-loading conditions that lead to axonal injury. Moreover, these data may allow us to understand mechanisms of axon reassembly and regeneration after trauma using the model system described here.

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