Identification and Characterization of Heterogeneous Neuronal Injury and Death in Regions of Diffuse Brain Injury: Evidence for Multiple Independent Injury Phenotypes

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Diffuse brain injury (DBI) is a consequence of traumatic brain injury evoked via rapid acceleration–deceleration of the cranium, giving rise to subtle pathological changes appreciated best at the microscopic level. DBI is believed to be comprised by diffuse axonal injury and other forms of diffuse vascular change. The potential, however, that the same forces can also directly injure neuronal somata *in vivo* has not been considered. Recently, while investigating DBI-mediated perisomatic axonal injury, we identified scattered, rapid neuronal somatic necrosis occurring within the same domains. Moving on the premise that these cells sustained direct somatic injury as a result of DBI, we initiated the current study, in which rats were intracerebroventricularly infused with various high-molecular weight tracers (HMWTs) to identify injury-induced neuronal somatic plasmalemmal disruption. These studies revealed that DBI caused immediate, scattered neuronal somatic plasmalemmal injury to all of the extracellular HMWTs used. Through this approach, a spectrum of neuronal change was observed, ranging from rapid necrosis of the tracer-laden neurons to little or no pathological change at the light and electron microscopic level. Parallel double and triple studies using markers of neuronal degeneration, stress, and axonal injury identified additional injured neuronal phenotypes arising in close proximity to, but independent of, neurons demonstrating plasmalemmal disruption. These findings reveal that direct neuronal somatic injury is a component of DBI, and diffuse trauma elicits a heretofore-unrecognized multifaceted neuronal pathological change within the CNS, generating heterogeneous injury and reactive alteration within both axons and neuronal somata in the same domains.

Key words: cortex; fluorescence; hippocampus; horseradish peroxidase; neuron; necrosis; traumatic brain injury; diffuse brain injury

Introduction

Diffuse brain injury (DBI) is a significant clinical problem that contributes to poor outcome (Adams et al., 1977; Lobato et al., 1983; Cordobes et al., 1986; Shigemori et al., 1992). In contrast to focal brain injury, in which the brain sustains direct impact, DBI occurs via rapid cranial acceleration–deceleration with or without impact (Adams, 1992). Although the lesions associated with focal injury are readily detectable, DBI generates little overt pathology (Meythaler et al., 2001; Hardman and Manoukian, 2002). What little macroscopic pathology is evident with DBI does not accurately reflect the extent of damage, which can be assessed only at the microscopic level (Adams, 1992).

DBI is potentially composed of four separate pathologies, diffuse axonal injury, diffuse vascular injury, diffuse hypoxic–ischemic injury, and brain swelling (Adams, 1992). The potential,

however, that DBI can also directly affect neuronal somata has received little attention. This limited understanding of potential neuronal cell perturbation and loss occurring after DBI stands in contrast to the well documented cell death that occurs with focal injury (Graham et al., 2000). Isolated descriptions of scattered necrotic and apoptotic cell loss occurring after diffuse insults can be found in the literature, yet the mechanisms involved in the pathogenesis of these diffuse changes have not been characterized (Smith et al., 1997; Finnie et al., 2000; Lin et al., 2001; Runners-tam et al., 2001; Cernak et al., 2002).

Although our current understanding of the neuronal somatic fate after DBI is incomplete, we recently demonstrated that neuronal somata linked to diffusely injured axons undergo a series of changes consistent with transient perturbation rather than cell death. In concert with these nonlethal somatic changes, we also identified in these same loci scattered necrotic neurons that were not axotomized. Apparently, the forces of injury directly damaged these neurons independent of axotomy (Singleton et al., 2002).

To understand the potential neuronal somatic damage caused by diffuse injury, we used a well characterized model of moderate traumatic brain injury (TBI) (Dixon et al., 1987) that reproducibly generates DBI (Yaghai and Povlishock, 1992; Yamaki et al.,

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1994; Qian et al., 1996; Murakami et al., 1998; Singleton et al., 2002). Direct neuronal somatic plasmalemmal disruption was evaluated via the use of different high-molecular weight tracers (HMWTs), normally excluded by intact neurons. Double- and triple-label fluorescent immunocytochemical and histochemical strategies were used to assess the relationship between neurons demonstrating plasmalemmal perturbation and those manifesting delayed pathological alteration or death.

Through this approach, we identified widespread DBI-mediated perturbation of neuronal somatic cell membranes within the early postinjury period. In some neurons, the tracers denoting somatic membrane disruption were associated with markers of neuronal death, whereas other tracer-flooded neurons did not reveal comparable linkage. At the same time, and in the same brain loci, different populations of injured neurons revealed no evidence of membrane disruption yet demonstrated either perisomatic axonal injury or a non-axotomy-related induction of heat shock protein (HSP) expression. Collectively, these novel findings speak to the diverse neuronal somatic responses to diffuse trauma and illustrate that direct somatic injury is a component of DBI.

Materials and Methods

The current study was designed to identify traumatically induced diffuse neuronal somatic damage after DBI. Because diffuse injury does not generate focal change that can be targeted by routine morphopathological strategies, we used different approaches, exploiting the potential for mechanically induced neuronal somatic plasmalemmal perturbation. Specifically, this study explored the potential for DBI to mechanically disrupt neuronal cell membranes, the potential for delayed, reactive pathological responses in these disrupted and other neuronal populations, and the spatiotemporal relationships between these ongoing changes. To visualize neurons sustaining cell membrane disruption or perturbation, several different HMWTs were used. Their use was based on the recognition that, in sham-injured animals, they are excluded from CNS neurons and glia by their intact cell membranes, whereas in brain-injured animals, at least in the case of diffusely injured axons, they can pass through the damaged plasma membrane, the axolemma, to reach the intracellular front (Pettus et al., 1994; Pettus and Povlishock, 1996). To determine whether comparable membrane disruption occurs at the level of the neuronal soma after DBI, the 40 kDa plant protein horseradish peroxidase (HRP) was used along with a second, confirmatory tracer of the same molecular weight (MW), 40 kDa dextran (dex40). Additionally, a smaller 10 kDa dextran (dex10) was used to determine whether any observed DBI-induced neuronal somatic membrane alteration involved either bulk flow, with flooding by both the dex10 and dex40 tracers in severely disrupted cells, or, alternatively, a more graded passage, with the lower-weight tracer occurring only in those neuronal somata sustaining a lesser degree of membrane damage.

Several companion immunocytochemical and histochemical markers were also used to characterize further the reactive neuronal somatic responses associated with any potential membrane alteration. Given the established utility of intra-axonal β -amyloid precursor protein (APP) pooling to identify axonal injury (Blumbers et al., 1994; Sherriff et al., 1994a,b; Gentleman et al., 1995; Bramlett et al., 1997; Geddes et al., 1997; Okonkwo and Povlishock, 1999; Buki et al., 1999; Finnie et al., 2000; Stone et al., 2000; Singleton et al., 2001), antibodies targeting the C terminus of the APP protein were used as previously described to identify perisomatic axonal injury (Singleton et al., 2002) and to explore the relationship, if any, between those cells manifesting axotomy and those revealing somatic membrane perturbation. Because our previous study also identified the presence of terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end-labeling-negative, necrotic neurons in close relation to other neurons sustaining in perisomatic axotomy, we also used the histochemical marker Fluoro-Jade (FJ), which selectively stains degenerating neurons independent of the mechanism of cell death (Schmued et al., 1997; Schmued and Hopkins, 2000). Antibodies to the

70 kDa HSP (HSP70) were also used to identify neuronal stress or injury. To determine the relationship between those neurons demonstrating alteration of their somatic plasmalemma (via HRP, dex10, and dex40 labeling) and those neurons revealing other reactive changes (via APP, HSP70, and FJ labeling), double- and triple-label fluorescent studies were performed using various combinations of these markers, with parallel electron microscopic (EM) assessment.

Surgical preparation and injury induction. Adult rats were infused with extracellular tracers and subjected to moderate central (midline) fluid percussion injury (FPI) consistent with previously described protocols (Sullivan et al., 1976; Dixon et al., 1987). Briefly, 23 male Sprague Dawley rats weighing 390 ± 22 gm (mean $\pm$ SD) were surgically prepared for the induction of FPI. Each animal was initially anesthetized in a bell jar with 4% isoflurane in 70% N₂O and 30% O₂. After induction, each animal's head was shaved and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) fitted with a nose cone to maintain anesthesia with 1–2% isoflurane in 70% N₂O and 30% O₂. A feedback-controlled heating pad (Harvard Apparatus, Holliston, MA) was used to maintain the body temperature at 37°C during surgery. A midline sagittal incision was made to expose the skull from bregma to lambda. The skull was cleaned and dried, and two 1 mm holes were drilled in the right frontal and occipital bones 1 mm rostral and caudal to bregma and lambda, respectively, for the subsequent insertion of fixation screws. A 4.8 mm circular craniotomy was then made along the sagittal suture midway between bregma and lambda, taking care not to damage the underlying dura and superior sagittal sinus. A blunt 25 gauge needle was then lowered through a burr hole into the left lateral ventricle (0.5 mm posterior, 1.4 mm lateral, and 4.0 mm deep relative to bregma) to infuse the specified HMWT. The tracers infused through the needle were horseradish peroxidase (type VI; Sigma, St. Louis, MO), 40 kDa dextran conjugated to fluorescein, and 10 kDa dextran conjugated to Texas Red (Molecular Probes, Eugene, OR). In both sham-injured and injured animals, a 16.7 mg/kg dose of each tracer, which was dissolved to a final concentration of 50 mg/ml in saline for a final infusion volume of 333 μ l/kg, was infused over 20–30 min. In cases in which two tracers were simultaneously infused, the concentration of each tracer was doubled so that the final volume remained consistent. All tracers were infused under isobaric conditions. The needle was allowed to remain in place for 10 min after completion of the infusion, after which the needle was removed. Although induction of FPI typically involves an intact dura, the intracerebroventricular infusion did not cause persistent breach of the dura at the needle penetration site. The injection site resealed because of the movement of the arachnoid and dural membranes. Furthermore, because dental acrylic was used to seal the overlying burr hole site, the biomechanical properties of the model were not altered. A Leur-Loc syringe hub was then cut away from a 20 gauge needle and affixed to the craniotomy site using cyanoacrylate. After confirming the integrity of the seal between the hub and the skull, two fixation screws (round machine screws; $\frac{3}{16}$ inch long) were inserted into the 1 mm holes. Dental acrylic was then applied over the screws, around the hub, and over the burr hole site to provide stability during the induction of injury. After the dental acrylic hardened, the animal was then maintained on anesthesia for 2 hr after completion of the infusion. At the end of the 2 hr postinfusion period, the male end of a spacing tube was inserted into the Leur-Loc hub. The hub-spacer assembly was filled with normal saline, and the female end of the spacing tube was inserted onto the male end of the FPI apparatus, ensuring that no air bubbles were introduced into the system. The animals were then injured at a magnitude of 2.00 ± 0.05 atmosphere (ATM), corresponding to a brain injury of moderate severity (Dixon et al., 1987). The pressure pulse measured by the transducer was displayed on a storage oscilloscope (5111; Tektronix, Beaverton, OR), and the peak pressure was recorded. Injury preparation and induction were completed before recovery from anesthesia. After injury, the animals were monitored for recovery of spontaneous respiration and, if necessary, ventilated to ensure adequate postinjury oxygenation until spontaneous respiration was regained. Animals that did not demonstrate return of spontaneous respiration within 15–20 sec of injury were rapidly intubated and ventilated with 100% oxygen for 30–60 sec until spontaneous breathing resumed, at which point the endotracheal tube was removed. The hub, dental acrylic, and screws were re-

moved en bloc, and the incision was quickly closed with sutures before recovery from unconsciousness. The duration of unconsciousness was determined for all animals by measuring the time needed for the recovery of the following reflexes: toe pinch, tail pinch, corneal blink, pinna, and righting. After recovery of the righting reflex, animals were placed in a holding cage with a heating pad to ensure the maintenance of normothermia and monitored during recovery. For animals receiving a sham injury, all of the above steps were followed minus the release of the pendulum to induce the injury. Animals with a short postinjury survival time (≤ 2 hr) were removed from the holding cage for perfusion, whereas animals with longer survival times (≥ 4 hr) were returned to the animal housing facility after recovery until the designated perfusion time. Central FPI caused transient unconsciousness in all injury groups when compared with sham-injured animals ($p < 0.01$; df between groups = 3; df within groups = 17; data not shown), as determined by a one-way ANOVA of righting reflex times with Tukey *post hoc* analysis. No significant difference in transient unconsciousness was noted between injury groups ($p > 0.05$; data not shown), indicating that all groups received injuries of comparable severity.

Tissue processing. After injury, animals were allowed to recover for 5 min or 2, 6, or 24 hr. The rats were then intraperitoneally injected with an overdose of sodium pentobarbital and transcardially perfused with 4% paraformaldehyde in Millonig's buffer. After perfusion, the brains were removed, immersed in the perfusion fixative for 24 hr, and then coronally blocked at the optic chiasm and the midbrain to include the parietal and temporal cortices, hippocampus, and thalamus. The blocked brains were then postfixed in the perfusion fixative for another 24 hr. The brain blocks were then flat-mounted on the sectioning disk with cyanoacrylate and embedded in agar. Blocks were coronally sectioned in 0.1 M phosphate buffer with a VT1000S vibrating blade microtome (Leica, Bannockburn, IL) at a thickness of 40 μ m. Sections were serially collected in alternating wells, such that each well consisted of adjacent sections, which, after immunocytochemical processing, could be compared to permit spatiotemporal characterization of the chosen markers. The tissue was stored in Millonig's buffer in 12-well culture plates (Falcon, Newark, DE).

Bright-field visualization of HRP. The methods used to identify HRP flooding into neuronal somata have been described (Povlishock et al., 1978, 1983). Briefly, sections from animals infused with HRP were incubated in 0.5% CoCl₂ in 0.1 M Tris-HCl buffer, pH 7.6, for 10 min. After three 5 min rinses in 0.1 M Tris-HCl buffer and two 5 min rinses in 0.1 M Millonig's phosphate buffer, sections were incubated two times for 45 min in a solution of 0.05% 3–3'-diaminobenzidine (DAB), 0.2% β -D-glucose, 0.04% NH₄Cl, and 0.00041% type II glucose oxidase in Millonig's buffer while slowly agitating in a 37°C oven. After rinsing thoroughly in Millonig's buffer, sections were either mounted on slides, dehydrated, and coverslipped or prepared for EM evaluation as described below.

Bright-field visualization of HSP70, APP, and dex40. Endogenous peroxidase activity within the tissue was first blocked with 0.5% H₂O₂ in PBS for 30 min. Sections were then processed using the temperature-controlled microwave antigen retrieval approach described previously (Stone et al., 1999). After microwave antigen retrieval, sections were preincubated for 60 min in 10% normal horse serum (NHS) with 0.2% Triton X-100 in PBS. The tissue was then incubated overnight in a 1:2000 dilution of the mouse anti-HSP70 1° antibody (Ab) (Santa Cruz Biotechnology, Santa Cruz, CA) in 1% NHS in PBS. Sections were then incubated for 1 hr in biotinylated rat-adsorbed horse anti-mouse 2° Ab (IgG; Vector Laboratories, Burlingame, CA) diluted 1:200 in 1% NHS in PBS (Vector Laboratories) and then for another 1 hr in a 1:200 dilution of an avidin–horseradish peroxidase complex (ABC Standard Elite kit; Vector Laboratories). The reaction product was visualized with 0.05% DAB, 0.01% hydrogen peroxide, and 0.3% imidazole in 0.1 M phosphate buffer for 10–20 min. Sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. To visualize APP, the above protocol was used with the following modifications: normal goat serum (NGS) was used in place of NHS; the APP 1° Ab was used at a concentration of 1:2500 (Zymed, San Francisco, CA); and the 2° Ab was a 1:200 dilution of biotinylated goat anti-rabbit Ab (Vector Laboratories). For bright-field visualization

of dex40, the above APP protocol was used with the exception that the 1° Ab was a 1:1000 dilution of rabbit anti-fluorescein IgG (Molecular Probes).

Electron microscopy. Tissue from sections to be treated with antibodies to HSP70 and dex40 or those processed for the visualization of HRP via the cobalt-glucose oxidase technique was postfixed for 60 min in 2% paraformaldehyde and 2% glutaraldehyde in Millonig's buffer at room temperature. The sections were then reacted for bright-field visualization as described above. The tissue was then osmicated, dehydrated, and flat-embedded between plastic slides in Medcast resin (Ted Pella, Redding, CA). The embedded slides were then scanned to identify immunoreactive neuronal somata within the specific region(s) to be explored. Once identified, these sites were removed, mounted on plastic studs, and thick-sectioned to the depth of the immunoreactive sites of interest. Serial 70 nm sections were cut and picked up on to Formvar-coated slotted grids. The grids were then stained in 5% uranyl acetate in 50% methanol for 2 min and 0.5% lead citrate for 1 min. Ultrastructural analysis was performed using a JEOL 1200 electron microscope.

Multiple-label fluorescent analysis. Most of the double- and triple-label studies described below were performed on brain tissue from animals surviving for 2 hr after injury. This early time point was chosen to ensure that any potential degenerative events or cell loss that could interfere with marker identification would be minimized. Most fluorescent labeling studies were performed in animals infused with dex40, dex10, or both. Because these tracers were conjugated to fluorescein and Texas Red, respectively, no additional steps were required to visualize the markers after tissue processing. Sections assessed for the relationship between HRP and dex40 were first rinsed in PBS for three times for 5 min and then subjected to temperature-controlled microwave antigen retrieval as described above. After retrieval, sections were again rinsed in PBS three times for 5 min and treated for 60 min in 10% normal donkey serum (NDS) with 0.2% Triton X-100 in PBS. The tissue was then incubated in a 1:300 dilution of goat anti-HRP (Biomedical, Foster City, CA) in 1% NDS and PBS overnight. The following day, sections were rinsed three times for 5 min 1% NDS and PBS and then incubated for 2 hr in a 1:200 dilution of Alexa 594 donkey anti-goat IgG (Molecular Probes) in 1% NDS and PBS. After rinsing three times for 5 min in PBS, sections were mounted on gelatin-coated slides, coverslipped with antifade (ProLong; Molecular Probes), and sealed with nail polish. Sections from dex40 animals reacted with antibodies to APP followed the above protocol, using NGS rather than NDS, a 1:200 dilution of rabbit anti-APP 1° Ab, and a 1:200 dilution of Alexa 594 goat anti-rabbit Ab (Molecular Probes). Tissue from animals infused with HRP was double-labeled to visualize the relationship between the tracer and APP. To this end, two rounds of the above immunocytochemistry protocols were performed, with HRP visualized in the first round (Alexa 488 Ab used rather than Alexa 594) and APP reacted in the second round, with omission of microwave antigen retrieval during the second round. In those sections in which dex40, APP, and HSP70 were visualized, the above protocol for labeling dex40 and APP was performed. However, subsequent to the APP labeling, sections were rinsed three times for 5 min in PBS and then incubated in 10% NHS and PBS with 0.2% Triton X-100 for 1 hr. Sections were then incubated overnight in a 1:5000 dilution of mouse anti-HSP70 primary Ab in 1% NHS and PBS. The following day, sections were rinsed three times for 5 min in PBS and incubated for 2 hr in a 1:200 dilution of biotinylated rat-adsorbed horse anti-mouse secondary Ab. Sections were then rinsed in PBS three times for 10 min and then incubated in ABC for 1 hr. To visualize the HSP70 marker, tyramide amplification was used to produce a blue fluorescent signal (kit NEL703; PerkinElmer Life Sciences, Boston, MA). Sections were first rinsed for 20 min in the proprietary blocking buffer, during which time the tyramide solution was prepared. The blue tyramide, amplification, and PBS were mixed at a ratio of 1:50:250 and then applied to the sections for 20 min after rinsing two times for 10 min in PBS. The sections were then rinsed three times for 15 min in PBS and then mounted and coverslipped as above.

Fluoro-Jade single labeling and double labeling. Sections were first rinsed three times for 3 min in deionized H₂O (dH₂O), then incubated for 15 min in 0.06% KMnO₄ while shaking gently, and rinsed again three times for 5 min in dH₂O. After this point, while taking care to protect the

Figure 1. Passage of the HRP tracer through the brain parenchymal extracellular space. *A*, Coronal brain section from a rat that was not infused with HRP. At 2 hr after sham injury (4 hr after infusion), the tracer extends into the deeper layers of the neocortex and also encompasses the majority of the hippocampus (*B*). At 6 hr after sham injury (8 hr after infusion), the tracer has diffused throughout the majority of the brain parenchyma (*C*). By 24 hr after sham injury (26 hr after infusion), extracellular HRP is not readily seen, most likely because of its clearance from the CSF (*D*).

tissue from exposure to light, the sections were rinsed for 30 min in 0.001% FJ (Histo-Chem, Jefferson, AR) in a 0.1% solution of acetic acid and dH₂O, after which rinsing in dH₂O was again performed three times for 5 min. The tissue was then mounted on gelatinized slides and allowed to dry overnight. After immersion in Hemo-De (Fisher Scientific, Pittsburgh, PA) three times for 15 min, the sections were coverslipped with DPX (Electron Microscopy Sciences, Fort Washington, PA). Those sections requiring double labeling were first reacted with the non-FJ marker. For those animals infused with fluorescent dex10, no first-step labeling was required, whereas sections labeled for APP or HRP were labeled as above, with the exception that an Alexa 594 donkey anti-goat Ab (Molecular Probes) was used for HRP labeling. After application of the first label, the above FJ protocol was used, except that the KMnO₄ treatment was limited to 3 min, and the FJ solution was applied at 0.0001% for 1 hr at 4°C.

Digital image acquisition and analysis. All qualitative single- and double-label light microscopic analysis and capture were performed using a Nikon (Tokyo, Japan) Eclipse 800 microscope fitted with a Spot-RT digital camera (Diagnostic Instruments, Sterling Heights, MI). Appropriate excitation and emission filters were used with fluorescent specimens. Fluorescent images were then analyzed, and overlays were obtained with Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

Results

Gross pathological observations

Moderate central FPI (2.00–2.05 ATM) generated virtually no gross parenchymal brain alteration. Although the meninges directly under the craniotomy site typically demonstrated modest subarachnoid hemorrhage, the underlying cortex revealed no signs of compression, contusion, or tissue loss indicative of necrotic change. Consistent with diffuse injury, the brain parenchyma demonstrated no evidence of overt pathological alteration, with the exception of occasional isolated petechial hemorrhages in the corpus callosum underlying the craniotomy site.

Evidence for neuronal somatic membrane disruption: horseradish peroxidase

Sham-injured animals receiving preinjury HRP infusion demonstrated interstitial diffusion of the marker into the periventricular layers of the neocortex and hippocampus 2 hr after sham injury (Fig. 1*B*). Consistent with previous control observations (Turner and Harris, 1974; Povlishock et al., 1978), light microscopic (LM) analysis revealed that the HRP diffused through the extracellular space and perivascular sleeves of the brain without evidence of neuronal or glial uptake (Fig. 2*A*). By 6 hr, the HRP staining had diffused throughout the extent of the neocortical and hippocampal extracellular compartment (Fig. 1*C*) without any evidence of cellular flooding. Twenty-four hours after sham injury, the extracellular and perivascular HRP staining was greatly reduced compared with the previous two time points (Fig. 1*D*), most likely because of diffusion of the tracer out of the brain into the CSF, or via endocytosis of the marker by neuronal somata and their axon

Figure 2. Neurons in multiple loci demonstrate altered plasmalemmal permeability to HRP and dex40 after traumatic brain injury. In animals infused with HRP and subjected to sham injury (*A*, neocortex), no neuronal flooding is observed, although HRP is seen in relation to the vasculature (arrowheads). In contrast, injured animals demonstrate dramatic neuronal HRP flooding in multiple sites at 2 hr after injury (*C*, neocortex). Flooded neuronal somata are also visible at 6 hr after injury scattered throughout these same anatomic loci, whereas at 24 hr after injury, HRP-positive neurons were few. Similarly, in sham-injured animals infused before injury with dex40, no neuronal flooding is observed in the neocortex, the CA1 region of the hippocampus (*B*), or any other brain region. In contrast, 2 hr after TBI, scattered neocortical neurons demonstrate striking dex40 labeling within somatic and dendritic domains. Similar findings are noted in scattered CA1 pyramidal neurons (*D*), as well as in other regions of the hippocampus and dentate gyrus. At a higher magnification, microvacuolization, suggestive of mitochondrial swelling, can be noted in the dendrites of dex40-flooded neurons (*D*, inset, arrows). Scattered neurons flooded with dex40 remained visible in the same anatomic loci at 6 and 24 hr after injury. Scale bars: *A–D*, 100 μ m; *D*, inset, 25 μ m.

terminals, pericytes, and endothelial cells, or a combination thereof (Turner and Harris, 1974; Deurs, 1977). At this time, the tracer could be seen as a punctate reaction product scattered throughout the cytoplasm of these cells and synaptic terminals. This vesicular HRP uptake is entirely consistent with normal endocytotic mechanisms (Heuser and Reese, 1973; Kristensson, 1977; Povlishock et al., 1978). The spatiotemporal pattern of HRP diffusion in injured animals was identical to that observed in shams.

In contrast to these microscopic findings, HRP-flooded neuronal perikarya and their processes were prevalent 2 hr after moderate central FPI. These neurons revealed an HRP reaction product within the cytoplasm of their somatic and dendritic domains, suggesting bulk diffusion of the tracer from the extracellular to the intracellular compartment across a wounded plasmalemma. Such HRP-laden neuronal profiles were seen spanning the deep layers of the neocortex (Fig. 2*C*), in the suprapyramidal and infrapyramidal layers and hilus of the dentate gyrus, as well as in the CA1–CA3 subsectors of the hippocampus (results not shown). These HRP-containing neurons and appendages varied in their overall distribution but were found consistently in both cerebral hemispheres of all sections harvested between the anterior commissure and caudal thalamus. Although quantitative analyses were not conducted, the overall distribution of these flooded neurons paralleled the dex10 labeling illustrated in the camera lucida images described below (see Fig. 5). Remote neuronal somatic labeling was also noted in the cerebellum, where scattered HRP-laden Purkinje neurons were observed, and in the brainstem, where isolated axons in the pontomedullary corticospinal tract also demonstrated focal HRP flooding (results not

Figure 3. Neurons flooded with HRP demonstrate various forms of pathological change. In injured animals surviving for 2 hr after injury, one can identify scattered HRP-flooded neurons demonstrating overt necrosis at the LM level. One such neuronal soma is shown in *A*, with disruption of the normal neuronal profile, pyknosis, and perinuclear vacuole formation (arrow). Also note the lucent areas in the perisomatic domain, indicative of synaptic terminal loss with glial ensheathment. The surrounding neuropil and neuroglial nuclei demonstrate little, if any, overt pathological change (arrowheads). These LM findings were confirmed at the ultrastructural level (*B*), with scattered HRP-flooded neurons revealing perisomatic glial ensheathment, microvacuolation, and pyknosis. In contrast to the relatively rapid cell death observed in association with permeability to HRP, other tracer-flooded neurons were found to demonstrate relatively little overt ultrastructural pathology. The neuron shown in *C* reveals only modest swelling of the Golgi apparatus (large arrowhead), whereas the remainder of the cytoplasm appears intact. Surrounding this neuron, one can identify HRP in the extracellular space (small arrowheads), as well as a degenerating presynaptic bouton (arrow). Scattered HRP-flooded neurons revealing intermediate levels on injury were also identified, as can be seen in *D*, which demonstrates an injured neuronal soma with mitochondrial swelling (arrowheads) and perisomatic glial infiltration. In all of these cases, evidence of somatic HRP influx was confirmed in adjacent unstained sections. Scale bars: *A*, 50 μm ; *B*, *C*, 4 μm ; *D*, 2 μm .

shown). The flooded neurons were most striking in the neocortex because of the presence of HRP within long apical dendrites and the somata of pyramidal neurons in lamina V (Fig. 2*C*) and in CA1–CA3 of the hippocampus. At 6 hr after injury, neuronal HRP flooding patterns comparable with the 2 hr time point were observed, with the suggestion that increased numbers of HRP-containing neurons were noted in the more superficial layers of the neocortex. Traumatized rats surviving 24 hr after injury demonstrated low background and extracellular levels of HRP, whereas astrocytic HRP was more pronounced because of the presence of reactive gliosis within the above-mentioned regions sustaining DBI. Compared with 2 and 6 hr after injury, few HRP-laden neurons could be identified at this time (results not shown).

In the above-described progression of HRP influx, routine LM analysis revealed a population of neurons that were flooded with the extracellular tracer 2 hr after injury. On LM observation of the 1 μm plastic sections at selected time points, some of the

HRP-flooded neurons appeared morphologically intact, whereas others revealed overt necrotic change. The necrotic subpopulation of cells revealed irregular, distorted profiles, microvacuolation, and concomitant perisomatic infiltration by glial processes, suggestive of synaptic stripping (Fig. 3*A*), findings that were subsequently confirmed by ultrastructural analysis (Fig. 3*B*). In other neurons, this tracer flooding was associated with more limited subcellular change, typically associated with limited dilation of mitochondria and the endoplasmic reticulum at the EM level (Fig. 3*D*), all consistent with a pre-necrotic state. Other scattered neurons showed overt HRP flooding with only minimal evidence of subcellular change (Fig. 3*C*). In these neurons, the tracer was distributed throughout the cytosol with occasional vesicular uptake, a finding confirmed in both stained and unstained sections. Importantly, all the above events occurred throughout an otherwise structurally unaltered brain parenchyma.

Evidence for neuronal somatic membrane disruption: dextran

To confirm the presence of DBI-mediated neuronal somatic plasmalemmal disruption, a second independent HMWT was used. Those animals infused with dex40, a polysaccharide similar to HRP in molecular weight, revealed LM changes comparable with those described in animals receiving HRP (Fig. 2*A,C*). At all time points in sham-injured animals, no dextran flooding was seen either in neuronal somata or their processes (Fig. 2*B*). At 2, 6, and 24 hr after injury, somatic flooding with dex40 was noted in the same locations previously associated with HRP influx (Fig. 2*D*). Ultrastructural analyses using antibodies targeting fluorescein-labeled dex40 yielded findings similar to those seen in HRP-

flooded neurons, identifying separate populations with overt necrosis, subtle neuronal perturbation, or little to no alteration (results not shown).

To assess whether the somatic plasmalemmal disruption was graded, another subset of animals was simultaneously infused 2 hr before injury with both dex10, a 10 kDa dextran conjugated to a Texas Red fluorescent marker, and dex40, which was conjugated to fluorescein. In brain sections harvested from these animals, complete overlap of the two tracers was noted in all flooded neurons at all time points (results not shown), indicating that the disruption of the somatic plasmalemma occurring as a consequence of DBI was not graded. Rather, it allowed for the free passage of different MW tracers from the extracellular to the intracellular space. On the basis of this finding, both dex10 and dex40 were subsequently considered equivalent markers of altered plasmalemmal permeability to dextran.

Consistent with the premise that the observed neuronal somatic tracer flooding was caused directly by membrane injury

Figure 4. Neuronal somata demonstrate altered membrane permeability and flooding with dex10 within 5 min of injury. In this low-power image of the rat brain 5 min after moderate midline central fluid percussion injury, neurons flooded with dex10 tracer are prevalent throughout the mediodorsal neocortex (demarcated by the dotted line). The CA1 region of the hippocampus is seen at the bottom left. Inset, At a higher magnification, neuronal profiles flooded with the dex10 tracer are evident scattered among other neurons demonstrating no evidence of tracer flooding. The deep layers of the neocortex can be seen at the bottom left, whereas the more superficial cortical layers can be observed at the top right. Note the areas of very intense dex10 labeling seen in relation to the vasculature (arrowheads). Scale bars: 300 μ m; inset, 200 μ m.

rather than delayed cellular degeneration, rats infused with dex10, subjected to moderate central FPI, and killed within 5 min of injury also revealed neuronal flooding. The distribution of neuronal flooding identified at this time was similar to that observed at 2 hr, with dex10-filled neurons noted in the mediodorsal neocortex (Fig. 4) and multiple regions of the hippocampus. LM analysis of tracer-filled neurons revealed little overt evidence of cell injury at this early postinjury time point.

Although quantitative analyses were not performed because of the difficulties associated with quantitative fluorescent analysis, camera lucida drawings of three different coronal brain levels from an animal infused with dex10 and allowed to survive for 2 hr after injury illustrated the spatial distribution and relative numbers of neurons with plasmalemmal wounding (Fig. 5). Fewer dex10-flooded neurons were present in more rostral sections, whereas brain sections at the level of the injury and more caudal sites demonstrated increased numbers of tracer-labeled neurons. No dex10-flooded neuronal somata were identified directly under the craniotomy or injury site.

Evidence for neuronal somatic membrane disruption: horseradish peroxidase and dextran

Single-label evaluation of HRP and dextran flooding suggested that the two tracers labeled neurons in the same brain regions at comparable postinjury time points. In animals simultaneously infused with both HRP and dex10, incomplete correspondence was found between the two markers (Fig. 6), suggesting different populations of injured neurons. Neurons flooded with dex10 consistently revealed simultaneous flooding by HRP (Fig. 6, arrowheads). Some of these dual HRP- and dex10-flooded neurons

Figure 5. Extent and relative burden of neuronal somata demonstrating plasmalemmal injury and flooding with dex10 2 hr after moderate central fluid percussion injury. Coronal brain levels rostral (-0.80 mm relative to bregma), at the level of (-3.60 mm), and caudal (-5.30 mm) to the injury site are depicted. Each dot represents five dex10-flooded neurons within the specific brain region. As can be observed, most of the flooded neurons were noted at the level of the injury and in more caudal sites; less damage was noted in more rostral brain regions. In addition, no dex10-flooded neurons were observed directly under the injury site (-3.60 mm, dorsal midline neocortex).

Figure 6. Neurons flooding with HRP and dex10 reveal partial overlap. Animals were infused with HRP and dex10, subjected to moderate central FPI, allowed to survive for 2 hr, and then reacted with antibodies to HRP. In sections from multiple brain regions, including the neocortex (pictured) and the hippocampus, some neurons flooded with dex10 also demonstrated cytoplasmic HRP (arrowheads). In addition to these double-labeled cells, however, a second neuronal population was observed to flood only with HRP but remained impermeable to the dextran tracer (arrows). Those neurons observed to flood with both HRP and dex10 appear overtly pathological, with irregular profiles, whereas other neurons flooding with HRP alone do not demonstrate any other pathology at the LM level. Scale bars, 100 μ m.

appeared pathological at the LM level, manifesting somatic changes similar to the above-mentioned single-labeled HRP- and dex40-flooded neurons undergoing necrotic cell death. Other neurons were found to flood with HRP alone (Fig. 6, arrows), with no evidence of concomitant dextran uptake. These neurons consistently appeared unaltered at the LM level. Unfortunately, technical limitations prevented reliable simultaneous ultrastructural EM visualization of both the HRP and dextran tracers.

Neuronal somatic responses to DBI

Complementing the above-described tracer exclusion studies, other markers of neuronal perturbation and reactive change were

Figure 7. Neuronal HSP70 expression is induced after diffuse traumatic brain injury. No HSP70 expression is evident in sham-injured animals. At 2 hr after injury, no upregulation of HSP70 is noted, whereas at 6 hr, scattered HSP70-positive neurons are localized in the mediadorsal neocortex and several regions of the hippocampus and dentate gyrus. Expression of this cell stress marker increased further by 24 hr after injury, at which time marked immunolabeling of scattered neuronal somata and their dendritic processes can be noted in the same regions in which HSP70 expression was seen at 6 hr, including the neocortex (pictured). Scale bars, 100 μ m.

Figure 8. Neuronal degeneration or death occurs after diffuse brain injury. In rats subjected to sham injury, FJ staining reveals only low levels of background fluorescence throughout the brain (*A*). In contrast, brain sections from rats subjected to moderate central fluid percussion injury reveal scattered, brightly stained, FJ-positive neuronal profiles at 2, 6 (*B*), and 24 hr after injury in the neocortex and hippocampus and dentate gyrus. Many of these labeled neurons are overtly pathological, demonstrating irregular somatic profiles. Scale bars, 100 μ m.

assessed within the same injured brain loci. Staining for the HSP70 revealed no immunocytochemical labeling in sham-injured animals (results not shown). Similar to sham animals, rats sustaining diffuse TBI and surviving for 2 hr after injury revealed no evidence of upregulation of this marker (results not shown). However, at 6 hr after injury, immunoreactive neuronal profiles could be observed in the mediadorsal neocortex, the hilus, suprapyramidal and infrapyramidal blades of the dentate gyrus, and CA1–CA3 of the hippocampus (results not shown), the same anatomic loci in which somatic plasmalemmal alteration was observed via the use of extracellular tracers. Those neurons demonstrating HSP70-IR at this time appeared intact at the LM level, with no overt evidence of cell death. At 24 hr after injury (Fig. 7), neuronal HSP70 staining was increased compared with the previous time point with respect to both the intensity of labeling and number of immunoreactive profiles. Similar to 6 hr after injury, HSP70-labeled neurons at 24 hr after injury revealed no LM evidence of pathological alteration. No HSP70 expression was observed at any time in the neocortical domain directly underlying the impact site.

FJ, a purported marker of neuronal degeneration, similarly demonstrated no staining in sham-injured animals (Fig. 8*A*). At 2 hr after injury, histochemical staining with FJ labeled scattered neurons in the same diffusely injured brain regions demonstrating reactivity for all of the above-described markers (Fig. 8*B*). Although the LM appearance of some FJ-labeled neurons appeared normal at this early time point, other FJ-positive cells did appear pathological, with distorted somatic profiles and tortuous

Figure 9. Neurons demonstrating perisomatic axotomy reveal no evidence of somatic plasmalemmal disruption. In animals infused with dex40 (green), subjected to TBI, and killed 2 hr after injury, tracer-flooded neurons (arrowheads) do not colocalize with other neurons linked to adjacent APP-positive reactive axonal swellings (red, arrow). The heterogeneous nature of diffuse injury is also depicted, in that one can observe two directly adjacent neurons manifesting distinctly different injury phenotypes, with an overtly pathological, microvacuolated, dex40-flooded neuron adjacent to an axotomized neuron demonstrating no other evidence of perturbation. Scale bar, 50 μ m.

apical dendritic processes, similar to those cells flooded with HRP and dex40. At 6 hr after injury, FJ-labeled neurons persisted in the same regions, with a greater proportion of FJ-positive cells assuming a pathological phenotype, demonstrating irregular membranes and dendrites. Brain sections from animals killed at 24 hr after injury again revealed FJ-stained neurons in the above-mentioned regions, although at this time, most profiles appeared overtly pathological (results not shown).

LM findings in sections from injured animals stained with antibodies targeting APP to identify neurons sustaining perisomatic axonal injury at 2, 6, and 24 hr after TBI (results not shown) were consistent with previous observations (Singleton et al., 2002). Briefly, APP-positive axonal injury was not identified in sham-injured rats. At 2 hr after injury, scattered neuronal somata linked to small, APP-positive spheroidal axonal swellings were distributed throughout the previously identified regions of DBI, including the mediadorsal neocortex, hilus of the dentate gyrus, and dorsal thalamus, with the localization of neurons sustaining axonal injury unchanged at 6 hr. Twenty-four hours after injury, the localization of axotomized neurons remained consistent with previous time points. The size of the reactive axonal swellings was noted to progressively increase from 2 to 24 hr after injury.

Relationship between membrane alteration and the neuronal somatic response to DBI

Given that all of the above-noted markers of neuronal somatic membrane disruption and reactive change were spatiotemporally related, multiple labeling studies were performed to assess the relationship between these different injured phenotypes. Specifically, single-, double-, and triple-labeling strategies were used to detect reactive somatic change within regions of diffuse injury and then to assess whether such change was linked to parallel somatic plasmalemmal wounding. Double labeling with HRP or dex40 with APP revealed no evidence of overlap at any time point in either the somata or in their proximal disconnected axon stumps (Fig. 9). This indicated that neither neuronal somata linked to injured axons nor the injured axons themselves sustain either immediate or delayed plasma membrane disruption. Sections triple-labeled for APP, HSP70, and dex40 at 2 hr after injury revealed no overlap between any of these markers in the neocor-

Figure 10. Reactive neuronal somatic expression of HSP70 is independent of both cell membrane permeability alteration and perisomatic axotomy. In this triple-labeled section from an animal infused with dex40 and surviving for 24 hr after injury, fluorescent labeling for dex40 (green), APP (red), and HSP70 (blue) can be observed. As noted above, no overlap occurs between those axotomized neurons linked to disconnected, APP-positive reactive axonal swellings (triple arrowhead) and neurons flooding with dex40 (arrowheads). HSP70-positive neuronal somata (double arrowhead) do not reveal any colocalization with either axotomized neurons or those neurons demonstrating altered plasmalemmal permeability. Intense non-neuronal dex40 staining can be seen, attributable to diffusion of the tracer along the perivascular sleeve (arrows). Scale bar, 50 μ m.

Figure 11. Neuronal somata demonstrating permeability to HRP reveal incomplete overlap with FJ. This section from an animal surviving for 2 hr after injury demonstrates a lack of complete concordance between these two markers, with some HRP-flooded neurons (red) in the neocortex staining positively for FJ (green; arrow) and others revealing no evidence of FJ labeling (arrowheads). Scale bars, 100 μ m.

tex or hippocampus and dentate gyrus at any postinjury time point (Fig. 10), suggesting that neuronal injury sufficient to cause expression of HSP70 is associated with neither axonal injury nor plasmalemmal disruption.

Double-labeling studies, however, did reveal colocalization of FJ-stained neurons with many neuronal somata demonstrating plasmalemmal perturbation and flooding with extracellular dex-tran or HRP tracer. Specifically, FJ and dex10 were found to colocalize in many neurons in brain sections from all three postinjury time points (results not shown), with FJ likely identifying those dex10-flooded neurons progressing to necrotic cell death. Consistent with the previous suggestion that not all HRP-containing neurons progress to cell death, incomplete neuronal overlap was noted between the HRP- and FJ-containing neurons at 2 hr after injury, with some FJ-positive neurons demonstrating HRP flooding, yet other HRP-flooded neurons were devoid of FJ reactivity (Fig. 11). In accordance with our previous findings that perisomatic axonal injury does not result in neuronal cell death (Singleton et al., 2002), FJ-stained sections double-labeled with

APP identified the two markers in distinct populations of neurons at all postinjury times (results not shown). In addition, other FJ-stained brain sections from all time points did not reveal any overlap between HSP70-expressing neurons and those positive for FJ (results not shown), suggesting that the neuronal injury responsible for upregulating this cell stress marker is not sufficient to cause cell death within the first 24 hr after injury.

Discussion

This study, which builds on previous work identifying distinct populations of injured neurons after diffuse injury (Singleton et al., 2002), is novel in several respects. Specifically, we now describe diffuse neuronal somatic membrane perturbation as a consequence of DBI, with varied pathological responses identified in those neurons sustaining membrane wounding, ranging from rapid cell death to apparent recovery. Furthermore, we demonstrate other forms of reactive neuronal pathology not related to somatic disruption, collectively illustrating the diversity of the neuronal response to DBI. Last, we demonstrate that many of these injury phenotypes occur independently, with neurons in close proximity manifesting varied forms of injury-induced change. Taken together, this work describes, for the first time, a complex and multifaceted neuronal somatic pathological response to DBI and also shows that diffuse neuronal somatic injury is a component of DBI.

Historically, those involved in the study of TBI have focused on sites of focal injury wherein the progression of necrotic and apoptotic cell death has been described (Dixon et al., 1991; Clark et al., 1997; Conti et al., 1998; Newcomb et al., 1999; Raghupathi et al., 2000). Evidence for diffuse involvement of the neuraxis in terms of injured neuronal somata has been limited in humans and animals (Lin et al., 2001; Runnerstam et al., 2001; Cernak et al., 2002). Although it is recognized that DBI evokes diffuse axonal damage, the neuronal somatic consequences of this event are not well understood. Additionally, it is unknown whether the cell death that has been observed after DBI follows from the axonal injury or reflects direct neuronal somatic damage. Recently, we have demonstrated that axonal injury even adjacent to the neuronal soma does not translate into immediate cell death (Singleton et al., 2002). Rather, it triggers transient neuronal perturbation consistent with a reparative process. The current study confirms this finding and also shows that the same forces of injury can also damage other neuronal soma, independent of axotomy. Previous investigations have also revealed the occurrence of diffuse neuronal damage and necrosis in regions remote from focal injury (Dietrich et al., 1994; Rink et al., 1995; Sato et al., 2001). This work extends these findings, demonstrating that DBI elicits varied neuronal somatic alterations, ranging from necrotic change to less severe forms of neuronal pathology, potentially associated with neuronal survival.

The current observation of diffusely injured somata demonstrating rapid flooding (<5 min) with large-MW tracers, independent of any axotomy-mediated change, argues for an injury-induced disruption of the neuronal cell membrane, allowing for the rapid intracellular passage of large-MW tracers. The rapidity of tracer passage suggests direct diffusion across a damaged membrane rather than an event secondary to traumatically induced neuroexcitation or ischemia, which could not elicit scattered changes within this short time span. We have explored blood flow changes in this model and have found no ischemia (Povlishock et al., 1992). As noted, the peroxidase and dextran tracers used often colocalized, suggesting that the membrane disruption allowed for bulk flow, with no discrimination based on tracer mass. LM anal-

yses typically revealed pathological changes consistent with necrosis in those neurons flooded with both tracers, a finding also confirmed via parallel FJ studies. The EM findings of mitochondrial damage, organelle disruption, and nuclear condensation in these tracer-laden neurons support this premise because they are consistent with a process of cell membrane disruption, ionic dysregulation, and the activation of neutral proteases, leading to rapid degradation of the neuron (Kampf et al., 1997; Barros et al., 2002).

The possibility that mechanically induced cell membrane disruption, allowing for the passage of extracellular HMWTS, may or may not progress to cell death is an unexplored concept in the CNS. However, cells in cardiac muscle (Clarke et al., 1995), skeletal muscle (McNeil and Khakee, 1992), vascular endothelium (Yu and McNeil, 1992), epithelial skin (McNeil and Ito, 1990), the gastrointestinal tract (McNeil and Ito, 1989), and alveoli (Vlahakis and Hubmayr, 2000), which normally experience mechanical stress and tensile forces, have been found to sustain physical membrane disruption, demonstrated by their uptake of HMWTS on both physiological and suprathreshold mechanical distortion (McNeil and Steinhardt, 1997; McNeil and Terasaki, 2001). In these non-neuronal cells, membrane disruption is typically followed by rapid resealing, accomplished via calcium-mediated vesicular fusion (Terasaki et al., 1997). Vesicular fusion with the plasmalemma involves lipid trafficking (Vlahakis et al., 2002) and reestablishes cellular integrity in a matter of seconds (McNeil and Baker, 2001). No comparative data exist for the neuronal somata, although some suggest that CNS postinjury resealing proceeds more slowly than reported in non-neuronal cells (McNeil and Terasaki, 2001), at least in the context of disrupted neurites.

Although tracer flooding was, on many occasions, associated with neuronal cell death, some HRP-flooded somata demonstrated neither concomitant dextran uptake nor morphological changes consistent with overt cell death, at least within the time frames assessed. Similarly, some of the dextran-containing neurons did not reveal changes consistent with cell death. The mechanism(s) by which these cells allowed tracer entry, while maintaining structural integrity, is unclear. Conceivably this HRP and dextran passage was linked to initial plasmalemmal disruption with rapid postinjury membrane resealing, a point to be addressed in future investigations.

In addition to this suggestion of membrane resealing and recovery, the findings that some neurons revealed flooding with only one rather than two different high-MW tracers introduces other possible explanations, including the possibility that these differential responses were based on the net charge or conformation of HRP versus dextran or both. The globular HRP protein has a Stokes radius of 30 Å (Rennke and Venkatachalam, 1979), whereas the pseudolinear dex10 and dex40 have radii of ~20 and 70 Å, respectively (Nicholson and Tao, 1993). In addition, the isoelectric point (pI) of HRP is basic, approaching 9 (Welinder, 1979), whereas the pI of polyanionic dextrans is much more acidic (Yamagata et al., 1994). It is unknown whether differing functional tracer size or overall charge or both could account for the observed differential neuronal influx.

Paralleling our descriptions of neurons with somatic membrane perturbation, we also found, in the same anatomical loci, other forms of reactive neuronal alteration not associated with membrane disruption. These somata were immunoreactive for HSP70, which is not constitutively expressed, but induced in times of cell stress or injury (Abravaya et al., 1992; Nowak, 1993; Planas et al., 1997). Although HSP70-IR has been observed in

neurons that progress to cell death, it is also transiently expressed in recovering neurons (Nowak, 1993). HSP70 expression was found in neurons associated with neither membrane injury nor FJ staining, consistent with its temporary expression in transiently perturbed neurons. Similarly, neuronal somata linked to APP-positive reactive axonal swellings were also identified independent of either somatic membrane alteration or induction of HSP70. Consistent with previous observations that those neurons axotomized by TBI survived axotomy and reorganized over time (Singleton et al., 2002), no colocalization with the FJ marker was found.

In accordance with other observations using the FJ marker to identify cell death occurring after lateral FPI (Sato et al., 2001), we did discern scattered FJ-positive neurons after DBI. FJ-positive neurons were noted within regions of diffuse injury and also demonstrated alteration of their plasmalemmal integrity, evidenced by the colocalization of FJ with dextran flooding, HRP flooding, or both. The utility of FJ is based on its ability to identify degenerating neurons independent of either the initiating mechanism or mode of cell death (Schmued et al., 1997; Schmued and Hopkins, 2000), although the precise mechanism by which this labeling occurs has yet to be identified. In light of the absence of apoptotic cell loss within diffusely injured domains after central FPI (Singleton et al., 2002), coupled with the present ultrastructural observation of neuronal necrosis in the same areas and the current evidence of membrane integrity loss, it is likely that FJ-positive neurons are dying a necrotic death.

In sum, the current study provides new insight into the pathology of diffuse trauma, identifying direct neuronal injury as a component of DBI and demonstrating for the first time the complexity and heterogeneity of the injury-induced neuronal somatic alterations. We believe that these findings have increased significance to those conducting preclinical studies that typically consider the response of the brain to injury in the context of a singular pathological response.

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