Ultrastructure of Diaschisis Lesions after Traumatic Brain Injury

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Abstract

We used controlled cortical impact in mice to model human traumatic brain injury (TBI). Local injury was accompanied by distal diaschisis lesions that developed within brain regions anatomically connected to the injured cortex. At 7 days after injury, histochemistry documented broadly distributed lesions, particularly in the contralateral cortex and ipsilateral thalamus and striatum. Reactive astrogliosis and microgliosis were noted in multiple neural pathways that also showed silver-stained cell processes and bodies. Wisteria floribunda agglutinin (WFA) staining, a marker of perineuronal nets, was substantially diminished in the ipsilateral, but less so in the contralateral cortex. Contralateral cortical silver positive diaschisis lesions showed loss of both phosphorylated and unphosphorylated neurofilament staining, but overall preservation of microtubule-associated protein (MAP)-2 staining. Thalamic lesions showed substantial loss of MAP-2 and unphosphorylated neurofilaments in addition to moderate loss of phosphorylated neurofilament. One animal demonstrated contralateral cerebellar degeneration at 7 days post-injury. After 21 days, the gliosis had quelled, however persistent silver staining was noted. Using a novel serial section technique, we were able to perform electron microscopy on regions fully characterized at the light microscopy level. Cell bodies and processes that were silver positive at the light microscopy level showed hydropic disintegration consisting of: loss of nuclear heterochromatin; dilated somal and neuritic processes with a paucity of filaments, tubules, and mitochondria; and increased numbers of electron-dense membranous structures. Importantly the cell membrane itself was still intact 3 weeks after injury. Although the full biochemical nature of these lesions remains to be deciphered, the morphological preservation of damaged neurons and processes raises the question of whether this is a reversible process.

Key words: axonal injury; controlled cortical impact; electron microscopy; light microscopy; TBI

Introduction

TRAUMATIC BRAIN INJURY (TBI) is an unfortunately common source of neurological injury in civilian and military populations.^{1,2} In addition to neurological damage associated with the primary impact, brain regions neuroanatomically connected to the site of impact can undergo anterograde, retrograde and trans-synaptic degeneration, termed “diaschisis.” Initially noted in the late nineteenth century by Brown-Sequard,^{3,4} the theory of diaschisis was more broadly described by von Monakow in 1914.⁵ As our understanding of the immense interconnectivity of the brain evolved, so did the meaning of diaschisis. The development of functional imaging steered the term toward discovery of reduced metabolism or blood flow (as measured by positron emission tomography [PET] or functional MRI [fMRI]) distal to the focal

lesion. These studies were complicated by contravening phenomena of vicariation, by which intact brain regions take over function of lesioned tissues.³ More recently, attention to the brain connectome is redirecting the focus of diaschisis to assessment of how focal central nervous system (CNS) lesions distribute to anatomically connected sites.⁶ For the purposes of the current study, we will use diaschisis in its modern inception as connectional diaschisis.³ These lesions are distinct from the distal acute lesions produced by shearing forces such as diffuse axonal injury (DAI), which result from disrupted axonal membranes.

Of the variety of histological techniques that have been employed to document the extent of neuronal damage, silver stains have proved one of the most sensitive and useful.^{7,8} Brain tissue possesses abundant histological features that are naturally argyrophilic. Over the past century, methodology has been developed

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that permits silver staining to sensitively distinguish between damaged and undamaged neuritic processes.⁹ This staining depends upon specific methodology of fixation, pretreatment, and development; however, despite decades of investigation, the biophysical mechanism of silver staining remains speculative.^{8,10,11} We find that modern adaptation of the de Olmos amino-cupric-silver process labels post-traumatic lesions distant from the site of injury that are distinct from the immunohistochemically detected axonal lesions of disrupted axonal transport (amyloid precursor protein [APP] accumulation) or neurofilament compaction.^{12,13}

In our recent studies of TBI, we discovered a novel biomarker of brain injury, chitinase 3-like protein 1 (CHI3L1, aka YKL-40, breast regression protein 39).¹⁴ Severity of brain injury correlated with cerebrospinal fluid (CSF) concentration of CHI3L1, but, more importantly, *in situ* localization and temporal expression of CHI3L1 suggested that this molecule may control novel pathways that modulate brain inflammation. Discovering the inflammatory pathways controlled by CHI3L1 will permit new therapeutic interventions to augment current treatments.

To define endogenous, protective anti-inflammatory pathways, we have recently used a mouse model of controlled cortical impact (CCI).¹⁵ We compared the neuropathological outcomes of CCI in wild type (WT) mice and mice in which the murine *chi3l1* had been homozygously deleted (*chi3l1*^{-/-}). Although gross lesion size in the two mouse lines was the same, in the absence of CHI3L1 expression, *chi3l1*^{-/-} mice displayed increased glial fibrillary acidic protein (GFAP) expression in the hemisphere ipsilateral and contralateral to impact. Similarly, ionized calcium binding adaptor molecule 1 (*Iba1*) expression (as a measure of the microglial/macrophage response) was significantly increased in *chi3l1*^{-/-} versus WT mice. These findings are consistent with the hypothesis that astrocytic expression of CHI3L1 limits the extent of both astrocyte and microglial activation, and suggests a novel potential therapeutic mechanism for modulating neuroinflammation.

In the current study, we further characterize the diaschisis lesions stained with amino-cupric-silver. Silver-positive processes and cell bodies developed by 7 days post-injury (DPI) and persisted for 3 weeks. To better delineate the biological nature of these lesions, we developed a histological process that allowed us to define the lesion at the light level and then examine those regions in successive sections to delineate the ultrastructural changes associated with the silver staining. To our surprise, rather than containing tangles of filaments and tubules, these silver positive structures consisted of cell bodies and processes undergoing hydropic disintegration with loss of nuclear heterochromatin and loss and fragmentation of cytoplasmic organelles (including mitochondria, intermediate filaments, and microtubules) and gain of membranous fragments.

Methods

Mice

A total of 64 adult female WT C57BL/6 mice and their homozygously deficient *chi3l1* knockout (KO) littermates (9–12 weeks of age) were used in these studies.^{15,16} The knockout animals demonstrate a more severe neuroinflammatory lesion than do their WT littermates. However, because the ultrastructural character of the TBI lesions in both WT and KO animals was the same, they will be described together. All animal studies were approved by the University of Pittsburgh Animal Care and Use Committee (IACUC) and conducted in our *American Association for Accreditation of Laboratory Animal Care* (AAALAC) accredited facilities.

CCI

Mice were subjected to CCI as previously described, with important modifications.^{15,17,18} In brief, mice were anesthetized. A 5 mm craniotomy was performed over the left parietal-temporal cortex, and the bone flap was removed. CCI injury was produced using a 3 mm in diameter flat impactor tip (1.5 mm deformation, 6 m/sec velocity). The bone flap was replaced and sealed with dental cement, and the scalp was sutured closed. In craniotomy-only animals, surgery, anesthesia, and recovery were identical to injured mice except that CCI was not performed.

Mouse TBI tissues

On 1, 7, and 21 DPI, WT and *chi3l1* KO animals were perfused transcardially with sodium cacodylate or phosphate-buffered saline (PBS) (Electron Microscopy Sciences catalog # 1222SK, # 1219SK, Hatfield, PA) followed by 50 cc of superior reagent grade sodium cacodylate paraformaldehyde perfusion fixative or reagent grade phosphate-buffered paraformaldehyde perfusion fixative (Electron Microscopy Sciences catalog # 1223SK, # 1224SK). Brains were extracted, post-fixed an additional 24 h, and then transferred to buffer at 4°C for shipping to NeuroScience Associates (NSA, Knoxville, TN). Brains were treated overnight with 20% glycerol and 2% dimethylsulfoxide to prevent freezing artifacts, then multiply embedded in a gelatin matrix using MultiBrain™ Technology. After curing, the block was rapidly frozen by immersion in 2-methylbutane chilled to -70°C and mounted on a freezing stage of an American Optical 860 sliding microtome. The MultiBrain block was sectioned coronally at 35 µm thicknesses. All sections cut were collected sequentially into a 4×6 array of containers that were filled with Antigen Preserve solution (50% PBS pH 7.0, 50% ethylene glycol, 1% polyvinyl pyrrolidone). Sections not immediately stained were stored at -20°C. Staining methods and quantitation were as previously described.¹⁵ In addition to the 46 brains (described subsequently) embedded using the MultiBrain Technology, 16 brains (8 WT and 8 KO) with craniotomy alone were available for paraffin embedding.

Light microscopy histochemistry

Section preparation, staining, and mounting were as previously described.¹⁵ Additional antibodies and lectins were used at the following dilutions: unphosphorylated neurofilament (SMI-311; BioLegend, San Diego CA; 1:1,000); phosphorylated neurofilament (SMI-312; BioLegend, Dedham MA; 1:1,000); microtubule associated protein-2 (MAP-2) (BioLegend, San Diego CA; 1:2,000); NeuN (EMD-Millipore, Billerica, MA; 1:2,000); and Wisteria floribunda agglutinin (WFA) (Sigma, St. Louis, MO; 1:2,000). Fluoro-Jade B and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), APP (Abcam, Cambridge, MA 02139 1:200), and GFAP (DAKO, Carpinteria, CA 93013 1:1,000) staining of paraffin-embedded sections were performed as previously described.^{19,20}

Whole slide images were acquired for each slide using the Zeiss AxioScan Z1 imaging platform (Carl Zeiss MicroImaging GmbH, Munich, Germany), using a 20× Plan Apochromat objective coupled to a 3 Chip CCD Camera (Hamamatsu Photonics, Japan). All focusing and field of view assembly was handled by the Carl Zeiss Zen software (Carl Zeiss MicroImaging GmbH, Munich, Germany), which is integrated with the AxioScan device. Images were acquired using an 8 µm extended depth-of-field approach, with a stepping size of 2 µm. Each of the resultant image layers were merged into a single plane image using a variance-based technique as provided in the Zen software.

Each slide was quality checked for focus consistency, and any rescans performed. Rescanning rate was near 20% of the specimens; this is attributed to having multiple-tissue sections per slide, where a slight histology artifact such as a bubble would affect the

FIG. 1. Whole mount image of a 35 μm thick MultiBrain™ section stained with the amino-cupric-silver technique. A total of 24 mouse brains at 21 days post-injury (DPI) were fixed and embedded *en bloc*, then frozen and sectioned. Every 24th section was stained using the amino-cupric-silver technique then mounted on a 5.08 $\times$ 7.62 cm glass slide. Lesions vary in extent from minimal damage in craniotomy only (e.g., top row, second from left) to severe damage with herniation and tissue loss (e.g., top row third from left). Anatomical areas with and without silver positive lesions are identified. Subsequent 35 μm thick sections were post-fixed with glutaraldehyde and osmium tetroxide and embedded in plastic as described in the Methods section. Select silver-positive and negative regions from the amino-cupric-silver stains were matched anatomically with regions on the plastic embedded sections. These regions were then dissected out and subjected to ultrastructural analysis. Examples of selected areas included: (A,B) Silver-positive ipsilateral thalamus. (C,D) Silver-positive contralateral cortex. (E) Silver-positive corpus callosum. (F) Silver-negative contralateral cortex.

focus of nearby section(s). Once scanning was complete, a grid overlay was used with a defined ROW, COLUMN labeling system to export each brain as a separate whole slide image. The resulting image resolution used for analysis was 0.88 $\mu\text{m}/\text{pixel}$ in both X and Y direction.

The analysis was performed using IAE-NearCYTE (NearCYTE, Pittsburgh, PA), an in-house quantitative analysis system for whole slide images developed at the Thomas Starzl Transplant Institute, University of Pittsburgh. To quantify the amount of silver staining per hemisphere, each brain image was first loaded into NearCYTE and a threshold-based tissue detection was applied to find the overall convex hull of the brain section. This hull, or region, was then split into a left and right hemisphere by the software's polygonal sectioning tools. The tissue detection method also outlined large ventricle areas, or holes, in the tissue to be excluded from analysis and total area calculations.

Quantitative analysis was performed by creating a pixel-based color model that identified those pixels in the hemisphere regions matching the defined intensity characteristics. The staining characteristics were defined by first removing those pixels associated with non-specific staining (e.g., vascular structures). This was accomplished by transforming the color RGB image into an optical density image and masking those pixels that correspond to the darkest color(s) that were morphologically grouped either as a small circle or oblong structure. The remaining pixels were then thresholded to meet the specific hue, saturation, and value parameters corresponding to positive silver staining. Once the model was defined and visually qualified on various random fields, the model was applied to all pixels within each defined hemisphere region, and a total hemisphere area and "positive" silver pixel area was

generated. The output was loaded into Microsoft Excel, and the area(s) for each brain slice was summed.

Electron microscopy

Figure 1 is a low-power scan of one 35 μm thick section stained using the de Olmos amino-cupric-silver technique. Silver positive lesions are readily identified and these regions were delineated in the subsequent 35 μm thick section. This section was tacked to the bottom of a plastic petri dish with a collagen/gelatin mixture and then secondarily fixed for 30 min with 0.5% glutaraldehyde in phosphate buffer followed by 10 min osmication with 1% osmium tetroxide. The section was dehydrated in graded alcohols before infiltration with epon araldite. A blank epon capsule was then placed above the region of silver positive lesion and the plate cured. When polymerized, the capsule with attached section was cut from the petri dish and a 0.5 μm thick section was taken from the block face. This section was stained with toluidine blue. Subsequent 100 A thin sections were retrieved on carbon coated copper grids, post-stained in uranyl acetate and lead citrate, and viewed on a JEOL electron microscope.

Results

Light microscopy

Lesion severity varied from animal to animal. Craniotomy-only animals showed minimal ipsilateral cortical pathology, whereas those with CCI showed a range of lesions from contused cortical surface to frank tissue loss with minor tissue herniation.¹⁵ Our previous study showed that both WT and *chi311*^{-/-} mice had similar

FIG. 2. (A) Wisteria floribunda agglutinin (WFA staining of paraffin embedded brain from a *chi311* knockout (KO) mouse 21 days after craniotomy alone shows loss of perineuronal nets in the ipsilateral (left) compared with the contralateral (right) cortex. (B) At higher power, the most superficial cortical layer 1 demonstrates the most profound loss of WFA staining in the neuropil; however, deeper layers also show pruning of staining back to perisomal areas. (C) WFA staining of frozen brain section after craniotomy with controlled cortical impact (CCI). The residual contused tissue shows loss of WFA staining. Bar = 100 μ m.

tissue loss at 21 DPI (9%). However, *chi311*^{-/-} mice showed greater astrocytosis in the lesioned hemisphere (25.9% vs. 18.3%) and the contralateral hemisphere (17.3% vs. 11.2%) than WT mice. Microgliosis was also greater in *chi311*^{-/-} than in WT mice; however, this only reached statistical significance in the contralateral hemisphere.¹⁵ Amino-cupric-silver staining 21 days after injury demonstrated the spectrum of variation (Fig. 1). Most silver staining was observed in neuritic processes emanating from the contusion site, which descended into thalamic or striatal structures and/or crossed the corpus callosum, entering the contralateral cortex. Neuronal cell bodies in some of the ipsilateral thalami demonstrated somal perinuclear argyrophilia. We quantified silver stained regions in the ipsilateral and contralateral hemispheres of *chi311*^{-/-} and WT animals. At 7 DPI, the ipsilateral hemisphere of *chi311*^{-/-} mice ($n=3$) showed 5.51×10^6 cm² (SD 1.36×10^6) silver-positive regions, whereas the ipsilateral hemisphere of WT mice ($n=3$) showed 3.32×10^6 cm² (SD 1.13×10^6) silver-positive regions. This 160% increase in severity of ipsilateral lesions in *chi311*^{-/-} compared with WT animals was similar to the 200% difference observed in the contralateral hemispheres, where *chi311*^{-/-} mice showed 2.29×10^6 cm² (SD 0.92×10^6) silver positive regions compared with WT mice, which showed 1.13×10^6 cm² (SD 0.30×10^6) silver-positive regions. At 21 DPI, the ipsilateral hemisphere of *chi311*^{-/-} mice ($n=10$) showed 4.94×10^7 cm² (SD 0.76×10^7) silver-positive regions whereas the ipsilateral hemisphere of WT mice ($n=10$) showed 4.65×10^7 cm² (SD 0.73×10^7) silver-positive regions. This 6% increase in severity of ipsilateral lesions in *chi311*^{-/-} compared with WT animals was similar to the 16% difference observed in the contralateral hemispheres, where *chi311*^{-/-} mice showed 2.04×10^7 cm² (SD 0.45×10^7) silver-positive regions compared with WT mice, which showed 1.76×10^7 cm² (SD 0.36×10^7) silver-positive regions.

Comparison of silver staining with WFA, NeuN, MAP-2, SMI-311, and SMI-312 staining

Regions of positive and negative silver staining were reviewed and assessed with additional histochemical staining. The normal neocortex showed strong regional WFA staining, indicating presence of perineuronal nets. After TBI, the ipsilateral neocortex showed loss of perineuronal nets that persisted up to 21 DPI (Fig. 2). Because the thalamus is a region of the brain that shows little to no WFA staining normally, it was not possible to evaluate loss of perineuronal nets there. NeuN staining was more dynamic. Beginning 1 DPI, the ipsilateral cortex surrounding the tissue destruction demonstrated loss of nuclear and increased presence of cytoplasmic NeuN staining that persisted at 7 DPI (Fig. 3A–E). The normal neuronal nuclear staining pattern was retained contralaterally throughout the experiment and regained in the ipsilateral hemisphere by 21 DPI. Ipsilateral thalami with silver-positive neuronal soma staining showed loss of neuronal nuclear NeuN staining that persisted to the maximum 21 DPI studied (Fig. 3F–H). Silver-positive diaschisis lesions in the thalamus (Figs. 4A, and 5A) also showed a conspicuous loss of staining for unphosphorylated neurofilaments (SMI-311) (Figs. 4C and 5C) and MAP-2 (Figs. 4B and 5B), and a more subtle loss of staining for phosphorylated neurofilaments (SMI-312) (Figs. 4D and 5D). Silver-positive diaschisis lesions in the contralateral neocortex (Fig. 6) showed a similar immunohistochemical profile to those in the thalamus, except that there was no clear loss of MAP-2 staining (Figs. 4B and 6B). Staining paraffin sections with Fluoro-Jade B or TUNEL did not identify apoptotic neurons in either the cortex or thalamus at 21 DPI.

FIG. 3. (A–E) Frozen 35 μ m thick sections of mouse brain 7 days post-injury (DPI). (A–C) Immunoperoxidase stain for NeuN. (A) Controlled cortical impact (CCI) lesion in the left hemisphere shows loss of neuronal nuclear NeuN staining in ipsilateral hemisphere (left) with retention of neuronal nuclear staining in contralateral hemisphere (right) cortical neurons. (B) Higher power of the contralateral cortex showing preservation of neuronal nuclear NeuN staining. (C) Higher power of ipsilateral cortical neurons showing loss of neuronal nuclear NeuN staining with increased staining of surrounding neuropil. (D–E) Immunofluorescent staining for NeuN (green) with propidium iodide (PPI) counterstaining (red). (D) Nuclei of all neuroglial elements in contralateral cortex counterstained with PPI. (D') Neuronal nuclei in contralateral cortex stain with NeuN. (D'') Merged image shows costaining of neuronal nuclei with both PPI and NeuN. (E) Nuclei of all neuroglial elements in the ipsilateral cortex counterstained with PPI. (E') Neuronal nuclei in the ipsilateral cortex do not stain with NeuN; however, surrounding neuropil is abundantly stained. (E'') Merged image shows PPI staining of neuronal nuclei with surrounding cytoplasmic NeuN staining. (F–H) Frozen 35 μ m thick sections of mouse brain 21 days after mild injury (craniotomy alone) shows NeuN staining of neocortical neuronal nuclei bilaterally. However, neurons in the dorsal regions of the thalamus ipsilateral to the injury (G) show loss of NeuN nuclear staining (within dotted circle) that is retained in the thalamus contralateral to the lesion (H).

Thalamus

Using the silver-stained sections for reference, we identified thalamic and contralateral cortical areas that showed no or positive silver staining (Fig. 1). These areas were isolated in a successive section for embedding in plastic as described in the Methods section. Half-micron thick plastic toluidine blue-stained sections from silver-positive and silver-negative thalamic regions were compared. The thalamus in silver-negative regions showed excellent preservation of cell structures (Fig. 7A). Neuronal nuclei showed prominent nucleoli surrounded by abundant light

blue-stained cytoplasm. Penetrating myelinated fibers stained dark blue and were distinct from compact surrounding neuropil. There was no evidence of artifactual expansion of the extracellular space. Similar sections from regions of silver positivity also appeared well preserved, but showed an overall loss of crispness and a more “moth-eaten” appearance (Fig. 7B). Neuronal nucleoli were not as common as silver-negative regions, and neuronal cytoplasm showed a washed out, hydropic staining pattern. Myelinated fibers appeared intact, but the surrounding neuropil was less uniformly stained, and contained neuritic processes with cleared out cytoplasm.

FIG. 4. (A–D) Frozen 35 μ m thick sections. Amino-cupric-silver, microtubule-associated protein (MAP)-2, SMI-311, and SMI-312 staining at 21 days post-injury DPI in a mouse with less severe injury. (A) Amino-cupric-silver stained coronal section showing the extent of the ipsilateral cortical damage along with diaschisis lesions in ipsilateral thalamus, corpus callosum, and contralateral cortex. (B) Adjacent section immunostained for MAP-2. Loss of MAP-2 staining is seen in the thalamic area, which showed silver staining (within dotted circle). (C) Adjacent section immunostained for unphosphorylated neurofilament (SMI-311). Loss of staining is observed in the silver-positive thalamic region (within dotted circle) and in the contralateral cortical region (beneath arrows). (D) Adjacent section immunostained for phosphorylated neurofilament (SMI-312). Subtle loss of staining is noted in the ipsilateral thalamus (within dotted circle); however, more pronounced loss is observed in the contralateral cortex (beneath arrows).

At the ultrastructural level, thin sections from silver-negative regions showed excellent preservation (Figs. 8A and 9A). Extracellular space was minimal, myelin was compact and well preserved, and mitochondria showed intact cristae without dilation. The neuronal soma contained a dense complement of intact cytoplasmic organelles (e.g., Golgi, rough endoplasmic reticulum, intermediate filaments) with nuclei containing a modest amount of heterochromatin surrounded by an intact nuclear membrane. Individual cell processes showed the expected complement of intermediate filaments and microtubules. Synapses were readily identified and presynaptic vesicles and postsynaptic densities well preserved.

Ultrastructural analysis of silver-positive regions from both 7 and 21 DPI showed cytological preservation comparable to silver-negative regions (Figs. 8B and 9B). Extracellular space was minimal, and myelin was compact and uniform (Fig. 8B). Myelinated fibers had intact mitochondria with a normal complement of intermediate filaments and tubules. However, neuronal somata had

nuclei with minimal heterochromatin and hypodense cytoplasm containing a paucity of subcellular organelles (Fig. 9B). Rough endoplasmic reticulum was more fragmented (Fig. 9B). Golgi complexes were difficult to identify, and no intact intermediate filaments or microtubules were seen. Mitochondria were subjectively less common and small. Dilated neuritic processes containing cytoplasm similar to the hydropic neuronal somata were dispersed throughout the neuropil (Fig. 10). Presynaptic profiles abutted the hydropic neurites where postsynaptic densities could be resolved, but no presynaptic vesicles were identified within the hydropic neurites.

Contralateral cortex

With the exception of there not being any silver-stained neuronal somata, the contralateral cortex (Fig. 11) demonstrated similar ultrastructural changes in neuritic processes as those observed in the thalamus. Curiously, hydropic disintegration in neuritic processes

FIG. 5. Higher power view of thalamus in Figure 4: a brain 21 days after controlled cortical impact (CCI). (A) Amino-cupric-silver-stained coronal section showing intense staining of fiber tracks leading in and out of the ipsilateral thalamus in addition to cytoplasmic staining of thalamic neurons. (B) Adjacent section immunostained for microtubule-associated protein (MAP)-2. Loss of MAP-2 staining is seen in the thalamic area that showed silver staining. (C) Adjacent section immunostained for unphosphorylated neurofilament (SMI-311). Loss of staining is observed in the silver-positive thalamic region. (D) Adjacent section immunostained for phosphorylated neurofilament (SMI-312). Subtle loss of staining is noted in the ipsilateral thalamus.

showed a prominent distribution along small blood vessels. Given this distribution, it was initially thought that these profiles might represent astrocytic foot processes. However, for the following reasons, this seems unlikely. 1) GFAP immunocytochemistry at the light microscopy level did not demonstrate dilated astrocytic foot processes around cortical blood vessels (Fig. S1) (see online supplementary material at <http://www.liebertpub.com/neu>). 2) Hydropic processes extended along vessels for long distances incommensurate with astrocytic foot processes. 3) The hydropic processes were devoid of intermediate glial filaments. 4) The ultrastructural morphology of the perivascular processes was the same as those processes in the brain parenchyma in which morphological synapses could be identified.

Immunohistochemical staining for APP did not identify congested neuritic profiles or axonal retraction bulbs anywhere in the WT or *chi311*^{-/-} mouse brains (Fig. S2) (see online supplementary material at <http://www.liebertpub.com>). Conventional Bielschowsky staining of paraffin embedded material did not demonstrate neuritic tangles or abnormal profiles.

Contralateral cerebellar cortex

Crossed cerebellar diaschisis (CCD) lesions are well described, particularly in the radiology literature.^{21–23} The precise location of the cerebral cortical lesion, rather than lesion severity, is thought to be the main determinant of CCD. We noted a severe CCD lesion in only one of the 7 DPI animals (Fig. 12). With Nissl stain, the lesion showed modest gliosis in the molecular layer of the contralateral cerebellum. Immunohistochemistry for GFAP was more striking, and dramatically delineated the molecular layer lesion in addition to a deep white matter gliosis. Amino-cupric-silver technique stained processes in the molecular layer and cerebellar deep white matter. Immediately below the amino-cupric-silver stained molecular layer there was a loss of Purkinje cells. Precise delineation of the track degeneration will require further analysis; however, the pattern is consistent with degeneration of Purkinje cell dendrites and axons. Ascending mossy fiber and granule cell parallel fiber degeneration might contribute to the amino-cupric-silver-positive lesion; however, we think this is less likely, as with Nissl and NeuN

FIG. 6. High power view of contralateral neocortex in Figure 4: a brain 21 days after controlled cortical impact (CCI). **(A)** Aminocupric-silver-stained coronal section showing bands of silver staining, particularly strong in cortical layers 1–3. **(B)** Adjacent section immunostained for microtubule-associated protein (MAP)-2. No loss of MAP-2 staining is seen in silver-positive cortical regions. **(C)** Adjacent section immunostained for unphosphorylated neurofilament (SMI-311). Loss of staining is observed in the silver-positive contralateral cortical region. **(D)** Adjacent section immunostained for phosphorylated neurofilament (SMI-312). Loss of staining is observed in the contralateral cortex.

staining there was no corresponding loss of neuronal cell bodies in the pons or granule cell layer.

Ultrastructural evaluation of the CCD lesion showed findings similar to those described in the cerebral cortex and thalamus. The silver-negative cerebellar cortex showed excellent preservation of molecular, Purkinje, and granule cell layers (Fig. 13). The silver-positive cerebellar cortex showed good preservation of granule cells, but marked distortion in rare residual Purkinje cells. As seen in thalamic neurons, disturbed Purkinje neuron nuclei showed rarefaction, whereas the cytoplasm demonstrated hydropic disintegration consisting of dissolution of subcellular organelles, loss of filaments and tubules, and presence of electron-dense membrane fragments.

Discussion

Local tissue reaction

Given the mechanics of CCI, it was not unexpected to observe variation in the severity of the experimentally induced contusion. The extent of physical injury varied from surface contusion to deep

hippocampal damage. Therefore, immediately at the site of injury a variable degree of frank tissue destruction was observed, presumably dependent upon subtle variations in physical forces delivered. Even the craniotomy-alone procedure showed a degree of variability in disturbance of the underlying cortex, from no apparent local damage to mild edema. In the latter instance, at 1 and 7 DPI, there was a penumbra of neurons showing nuclear to cytoplasmic displacement of NeuN staining. In the ipsilateral lesion, the redistribution of NeuN to the cytoplasm was transient, and it regained its normal nuclear localization by 21 DPI. NeuN (Rbfox3) is a less-well-characterized neuron-specific member of the Rbfox family of RNA binding proteins.^{24,25} This class of proteins has a critical role in nuclear pre-mRNA splicing and cytoplasmic stability and translation. This family of proteins can function by direct interaction or (at least in the case of Rbfox3) through regulation of miRNA expression. They have been shown to be critically involved in global neuronal maturation programs, and are specifically involved in switching between functionally different splice variants of glutamate and γ -aminobutyric acid (GABA) receptors. The

FIG. 7. Toluidine blue-stained thick sections of thalamus from silver-negative (**A**) and silver-positive (**B**) thalamic tissue. Histology shows good preservation of tissue without expansion of extracellular space. (**A**) Silver-negative thalamus shows neurons with distinct cytoplasm and crisp nuclei and nucleoli. Neuropil is tight and crisscrossed by sharply demarcated myelinated fibers. (**B**) Silver-positive thalamus appears more “moth-eaten.” Neurons have less distinct rarified cytoplasm containing less crisply defined nuclei. Clear processes of variable diameter crisscross the neuropil.

FIG. 8. Thin sections of thalamus from silver-negative (**A**) and silver-positive (**B**) thalamic tissue. Ultrastructure shows good preservation of tissue without expansion of extracellular space. (**A**) Silver-negative thalamus shows neurons with nuclei (pink) containing fine chromatin (N), normal complement of cytoplasmic (cytoplasm green) organelles, intermediate filaments and tubules, in addition to some lipofuscin (asterisks). Neuropil is tightly packed and crisscrossed by compact myelinated fibers. (**B**) Silver-positive thalamus shows a neuron with a clefted nucleus (N colored pink). The cytoplasm (yellow) is more rarified, and dilated processes (asterisks) extend from its surface. Neuropil contains larger diameter processes containing fewer cytoplasmic organelles. Myelinated fibers show regular compact myelin. Bar = 2 μ m.

FIG. 9. Thin sections of thalamus from silver-negative (A) and silver-positive (B) thalamic tissue. Ultrastructure shows remarkable preservation of tissue without expansion of extracellular space. (A) Silver-negative thalamus shows abundant closely apposed synapses with normal complement of cytoplasmic (cytoplasm green) organelles and mitochondria. Some synaptic and dendritic processes abut the surface of the neuron cell body (C). (B) Silver-positive thalamus shows neuron with nucleus (N, colored pink). The cytoplasm (C, colored yellow) is organelle poor and without distinct filaments, tubules, or rough endoplasmic reticulum. Dilated neuritic processes (asterisks) extending from the neuronal surface also appear to be organelle depleted. Bar = 500 nm.

FIG. 10. Thin sections of thalamus from silver-positive thalamic lesions. Ultrastructure shows remarkable preservation of myelin and good compact neuropil. Dilated dendritic profiles (asterisks, colored yellow) are organelle poor and contain electron-dense lipid fragments. Mitochondria in these processes are small and occasionally tattered. Nuclei (colored pink) show a fine euchromatin pattern. Bar = 500 nm in A, Bar = 2 μ m in B.

FIG. 11. Thin sections of cortex from silver-positive cortical regions. Ultrastructure shows remarkable preservation of tissue with dense neuropil containing abundant synapses. Silver-positive cortex shows several dilated profiles with clearing of their cytoplasm (asterisks, colored yellow) and presence of electron-dense membrane fragments. Dilated neuritic processes appear to be organelle depleted. Nuclei (colored pink) show an unremarkable heterochromatin pattern. Bar = 500 nm.

cytoplasmic forms of Rbfox proteins, which are themselves splice variants, are thought to have different specificities and functions. It is likely that the population of neurons with cytoplasmic NeuN expression has an extensive network of altered protein expression that remains to be precisely defined.

Remodeling of CNS extracellular matrix (i.e., decreased WFA staining) after injury is a hallmark of synaptic remodeling and plasticity. Persistence of decreased extracellular matrix would create the morphological basis for aberrant synapse formation and potentially establish foci for subsequent seizure foci. This characteristic of the local lesion was distinct from more distal diaschisis lesions, where loss of WFA staining was not observed.

CCI diaschisis lesion variability

In this murine CCI model of human TBI, amino-cupric-silver stains demonstrated variable and widespread diaschisis lesions in ipsilateral subcortical and contralateral cortical regions (summarized in Fig. 14). Whereas in this initial study we were not able to detail a time course,⁷ the silver positive lesions present at 7 DPI were histologically similar to those present at 21 DPI. This relatively static nature was surprising, but others have reported a highly variable progression of such lesions in various human disorders and animal models²⁶ (review²⁷). We have previously shown a modest astrocytic and microglial activation in these lesions, but we found little evidence of active recruitment of systemic macrophages into regions of diaschisis.¹⁵ Others have also observed a persistence of damaged axons in animal models with a relative absence of systemic macrophage infiltration.^{28–32} (reviewed³³).

Contralateral cortical diaschisis lesion

To better characterize the diaschisis lesions, we performed a variety of histochemical stains on adjacent sections. Silver-positive

contralateral cortical regions showed loss of neurofilament staining but preserved MAP-2 and WFA staining. This would suggest that the cortical diaschisis lesions might be limited to Wallerian and retrograde degeneration of projecting axons with less clear secondary trans-synaptic damage. This limited disruption of the contralateral cortex is consistent with the clinical observation that cortical diaschisis has minimal clinical correlate compared with subcortical lesions.³

Ipsilateral thalamic diaschisis lesions

Thalamic regions showing silver staining also showed a loss of phosphorylated and unphosphorylated neurofilament staining, and additionally showed a loss of MAP-2 staining. As MAP-2 and unphosphorylated neurofilaments are predominantly distributed within neuronal somata and dendrites, we interpret this loss of staining as consistent with trans-synaptic damage to thalamic projection or interneurons. This could be the direct result of retrograde degeneration of thalamic neuron axons or an indirect result of loss of cortical/thalamic trophic innervation. The subjective observation of more severe thalamic lesions with less severe ipsilateral cortical lesions (i.e., craniotomy with CCI vs. craniotomy alone) suggests an interesting interpretation. When there is frank loss of tissue in severe cortical lesions, there is complete disruption of the thalamocortical loop. With less severe cortical damage, some of the thalamocortical connectivity is retained and able to deliver a “NoGo” degenerative signal to the thalamic neurons.³⁴ Thus the paradox of there being more severe thalamic damage with less severe cortical damage (which is more consistent with the more common, less severe form of TBI in humans). This interpretation lends histopathological support to the common clinical and radiological findings of severe ipsilateral thalamic injury following injuries as “benign” as craniotomy.

FIG. 12. Frozen 35 μm thick sections of mouse brain 7 days post-injury (DPI) (A) Amino-cupric-silver stain shows damaged processes in the brainstem and deep white matter of the cerebellum. The right, contralateral cerebellum shows strong silver staining in cerebellar white matter in addition to the molecular layer. (B) Nissl staining of the cerebellum shows only modest increased in cellularity of the right versus the left cerebellum most evident in the molecular layer of the right cerebellum. (C) Immunohistochemistry for glial fibrillary acidic protein (GFAP) shows some bilateral gliosis in the brainstem with severe gliosis in the molecular layer of the right cerebellum. (D) Higher power of immunohistochemical stain for GFAP demonstrates severe gliosis in the right cerebellar molecular layer. (E) Higher power of amino-cupric-silver-stained right cerebellar hemisphere. Two patterns of silver staining are evident. Thick silver-positive processes are seen in the deep white matter and extending through the granule cell layer. Finer silver-positive processes are oriented perpendicular to the cerebellar folia surface. (F,G) Higher power of amino-cupric-silver-stained right cerebellar hemisphere demonstrating orientation of molecular layer staining. (H) High power of amino-cupric-silver-stained right cerebellar hemisphere showing presence of Purkinje cells (green stars) in regions without silver stained molecular layer. Bar = 100 μm .

Relationship between DAI and diaschisis lesions

TBI is known to cause DAI in a distribution dependent upon nature and degree of injury. Axonal damage is associated with aberrant calcium entry and protease (specifically calpain³⁵) activation. Axonal damage can lead to aberrant axoplasmic transport that is commonly and sensitively detected using immunohistochemistry for

APP. We did not observe significant accumulation of APP in the diaschisis lesions (Fig S2), and, therefore, we presume that these two lesions are of a separate etiology. Others have described the differences between these types of axonal injuries with neurofilament compaction being attributed to changes in neurofilament sidearm structure or phosphorylation,^{36,37} possibly secondary to calpain-1-mediated degradation of cytoskeleton³⁸ (review³⁵).

FIG. 13 Plastic-embedded crossed cerebellar diaschisis lesion. **A–C** Half- μm thick, toluidine blue-stained section of the contralateral cerebellar cortex. The molecular layer (ML) on the left of **A** is separated from the granular cell layer (G) by a regular Purkinje cell layer (P), whereas the molecular layer on the right (MR) is separated from the granular cell layer by a disrupted Purkinje cell layer (P). Bar = 100 μm . Higher power of left (**B**) and right (**C**) side of the folia shows preservation of Purkinje cells (yellow stars) on the left, which are mostly lost on the right. Bar = 100 μm . (**D**) Electron micrograph of disintegrating Purkinje cell layer shows two Purkinje neurons (P) undergoing dissolution with adjacent intact granule cell neuron (G). Whereas the Purkinje cell neuron nucleus is intact, the cytoplasm is amorphous without distinct filaments, tubules, Golgi, and endoplasmic reticulum, but with well-preserved mitochondria. (**E**) Higher power electron micrograph of Purkinje cell showing dissolution of cytoplasm. Bar = 5 μm .

Comparison of thalamic and cortical diaschisis lesions

The ipsilateral thalamus and contralateral cortex are responding differently to the cortical lesion. The contralateral cortex shows long-lasting Wallerian and, possibly, retrograde degeneration of projection neurons, but maintains its dendritic arbor (as evidenced by preservation of MAP-2 staining) and extracellular matrix (as evidenced by preservation of WFA staining). More extensive changes are occurring in the ipsilateral thalamus. Wallerian and anterograde degeneration of projection axons are shown by loss of phosphorylated neurofilaments. Additionally, however, there is clear evidence of trans-synaptic degeneration with loss of Nissl-stained neuronal profiles, loss of MAP-2 staining, and hydropic disintegration of interneurons. There are multiple potential reasons for the differences in the diaschisis lesions, including differential neuronal susceptibility and differences in character of lesion (anterograde vs. retrograde).

Crossed cerebellar degeneration (CCD)

CCD is well described in the radiology literature. In its acute form, a decreased perfusion and metabolism occasionally transforms in its chronic stages to a volume loss, particularly in the lateral cerebellar hemispheres. Pathological descriptions of CCD are exceptionally rare, and with one exception,³⁹ have not been reported in >30 years.^{40–43} In the pediatric literature, CCD is frequently referred to in association with Dyke–Davidoff–Masson syndrome, and is related to perinatal unilateral hemispheric injury or seizures.⁴⁴ However, the degenerative phenomenon is not limited to the developing brain. Given the greater frequency of cortical strokes in the adult population, it is not surprising that CCD is more commonly reported in the mature brain where loss of cortical input can result in cerebellar atrophy. Nevertheless, the incidence of functional CCD (e.g., decreased metabolism or perfusion measured by PET or MRI) is low compared with the frequency of cerebral

FIG. 14. Cartoon comparing outcome of mild and severe controlled cortical impact (CCI) injury. **Top:** After severe CCI, brain tissue immediately beneath trauma is physically destroyed (red), whereas surrounding cortical tissue shows widespread dislocation of NeuN staining from neuronal nuclei to neuronal cytoplasm (brown area). By 21 days post-injury (DPI), the normal neuronal nuclear NeuN staining pattern has returned. Silver-positive diaschisis lesions (black) evident at 7 DPI persist to at least 21 DPI. In the corpus callosum and contralateral cortex, these lesions consist of hydropic disintegrating axons with loss of neurofilaments and microtubules. In the thalamus, in addition to hydropic axons, neuronal cell bodies are also silver positive at the light microscopy level and hydropic at the electron microscopy level. **Bottom:** After less-severe injury (e.g., craniotomy alone), a smaller cortical lesion is observed consisting of mild central edema with loss of Wisteria floribunda agglutinin (WFA) staining (yellow) surrounded by a zone of neurons showing loss of nuclear NeuN staining (dotted red area). By 21 DPI, the neuronal nuclear NeuN staining has returned; however, the diminished WFA staining persists (yellow). Silver-positive cortical diaschisis lesions are similar to, but less extensive than, those seen in severe CCI (black). Silver staining of thalamic neurons is paradoxically more intense after mild than after severe cortical CCI.

hemispheric damage,⁴⁵ and the incidence of structural CCD is even lower.^{46,47} The precise location rather than the size of the cerebral lesion has been hypothesized to be the key determinant of whether CCD occurs after injury,²¹ with the universal consensus that disruption of the corticopontocerebellar pathway is the primary cause of cerebellar atrophy. There is one report of a vascular malformation in the pons disrupting the pontocerebellar portion of that pathway associated with CCD.⁴⁸ An additional association with thalamic damage has been described; however, this may be an effect of cerebellar-thalamic disruption rather than causative of CCD.

Our fortunate observation of a mouse with CCD 7DPI allowed us to define the diaschisis lesion at the ultrastructural level. Purkinje cell loss was readily apparent at the light microscopic level. Silver staining demonstrates fine beaded fragmentation of the Purkinje cell apical dendrite and the coarser beaded fragmentation of either the Purkinje axon or the pontocerebellar mossy fiber projections in the middle cerebellar peduncle and cerebellar deep white matter. Ultrastructural analysis at 7 DPI confirmed presence of subcellular changes in Purkinje neurons analogous to those defined in the thalamus at the same time there was structural preservation of cerebellar granule cells.

Ultrastructure of silver-positive diaschisis lesions

Taking advantage of being able to perform electron microscopy on successive sections, we examined the ultrastructural correlates of the light microscopy findings. In some silver staining protocols, deposition of silver is associated with neurofibrillary tangles, but

the amino-cupric-silver stain is associated with cytoskeletal compaction and neuronal degeneration.^{8,10,11,49} Thick sections from silver-positive regions showed a hydropic change in both neurons and the surrounding neuropil. Ultrastructural analysis showed dissolution of thalamic neuron rough endoplasmic reticulum consistent with loss of Nissl staining observed at the light microscopy level. Additionally, intermediate filaments and microtubules were absent, mitochondria were fragmented, and abundant membrane fragments were dispersed throughout the somata and neuritic processes. Using occipital cortex ablation models, others have found ultrastructural evidence of the chromatolytic type of changes followed by apoptosis.^{29,50,51} Although we found no ultrastructural (neither Fluoro-Jade B nor TUNEL) evidence of apoptosis at 21 DPI, the hydropic changes would be consistent with some limited form of autophagocytosis. More detailed time course studies employing markers of selective cell death mechanisms (TUNEL, Fluoro-Jade, caspase staining) could be attempted to better appreciate the molecular mechanisms behind the hydropic disintegration seen ultrastructurally.

The reasons why these ultrastructural hydropic changes stain with amino-cupric-silver

The precise biophysical explanation of argyrophilia of biological structures is not well known. However, alterations in the exposure of phosphorylated and unphosphorylated amino acid side chains are thought to play a role in conferring argyrophilia.¹¹ In the case of TBI, this would suggest that physical damage of neurofilaments is either

directly associated with a conformational change or that the physical damage triggers an enzymatic process that propagates down the fiber and leads to cytoskeletal deformations.⁵² Given the distance separating and the precise neuroanatomical distribution of the lesions, we would favor the latter interpretation. Although the biophysical nature of the lesion is not known, our ultrastructural findings would suggest that the cytoskeletal scaffolding of the cell is severely disrupted in TBI. This could be hypothesized to block axoplasmic transport. Consistent with this hypothesis is our observation that staining for APP does not show the accumulations seen with DAI secondary to shearing (Fig. S2).^{13,28}

Long-term sequelae of this ultrastructural change

Using cortical ablation in the rat as a model of thalamic degeneration, Al-Abdulla and Martin²⁹ showed hydropic degenerative changes in thalamic neurons that returned to normal after 3 months. Using a trauma-based model with axonal damage of optic nerve, Povlishock and Pettus have also noted persistent cytoskeletal changes.²⁶ Our study was limited to a relatively subacute time frame of 3 weeks. Although it is impressive that extended dendritic and axonal processes are physically present, it is not clear what the functional state of these processes is, or whether their ultrastructure could be repaired. Because repairing the cytoskeleton would require new synthesis in the cell somata along with phosphorylation and transport, it is intriguing to speculate that attempted repair could lead to aggregates of phosphorylated filaments and tubules that characterize degenerative disorders. However, the presence of intact albeit hydropic processes does suggest the potential for regeneration and/or therapeutic intervention.

Relevance to human TBI

Diaschisis lesions in humans after TBI have been known of for decades and are abundantly described; however, their distribution and pathogenesis are difficult to study. In particular, human TBI is well known to mediate delayed damage to thalamic networks.^{53–57} However, the human brain is so large, and the types of brain trauma are so variable, that deciphering the pathogenesis of such lesions requires animal modeling. Even with animal models, the species,^{58,59} age, and mechanics of injury cause a broad spectrum of lesions. Nevertheless, thalamic injury is frequently observed.^{7,60–62} Using a closed head injury model in postnatal day 7 mice, Dikranian and coworkers⁶³ studied the ultrastructure of TBI. These investigators also observed cytoskeletal defects followed by apoptotic cell death 16–24 h post-injury. They hypothesized that axonal damage led to a disconnection between cortex and thalamus and delayed apoptotic death. We did not observe apoptotic death in our model; however, this could be because we sampled later and used adult animals. However, the ultrastructure we observed of amino-cupric-silver-positive lesions is similar to that previously described. A distinct advantage of the silver staining is the capacity to rapidly and sensitively document the extent and distribution of the lesions. Ultrastructural analysis advances our understanding of the pathophysiological changes, but further biochemical analysis of these lesions will be challenging because individual neuritic processes undergoing hydropic disintegration are interspersed within unperturbed neuropil. Additionally, given the capricious character of lesion severity and distribution, biochemical analysis will need to be precisely directed using technology similar to that described here. *In situ* analyses such as immune-electron microscopy might be particularly useful for these sorts of analyses.

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