Mild Traumatic Brain Injury Results in Extensive Neuronal Degeneration in the Cerebral Cortex

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Abstract
Mild traumatic brain injury (mTBI) leads to long-term cognitive and emotional difficulties and behavioral disturbances, but the diagnosis and treatment of mTBI have historically been hampered by a lack of evidence-based correlates of these clinical manifestations. Unlike moderate and severe TBI, mTBI does not show significant tissue lesions or cavities in the cortex. Moreover, neuroimaging by magnetic resonance imaging or computed tomography is usually negative, suggesting that the damage is beyond the resolution of current structure-based scanning technologies. Therefore, we investigated the morphologies of spared neurons in the mouse cortex after mTBI in a controlled cortical impact injury model. Our results indicate that, although mTBI caused only a mild extent of cell death, it led to extensive dendrite degeneration and synapse reduction in the cortex in this model. This study sheds light on the neuropathologic consequences of mTBI in humans and suggests that neurodegeneration may be a novel target for developing diagnostic methods and therapeutic approaches for mTBI.

Key Words: Dendrite, mTBI, Neural degeneration, Spine, Synapse.

INTRODUCTION
There are 1.5 million civilian traumatic brain injuries (TBIs) annually in the United States. Traumatic brain injury is conventionally graded on a severity continuum ranging from mild to severe. Because injuries at the more severe end of this continuum are associated with significant morbidity and mortality, considerable attention has been devoted to understanding and managing the most severe injuries (1). On the other hand, both clinical definitions and results of surveys indicate that mild TBI (mTBI) accounts for nearly 75% of all treated cases (2–5). Many more mild injuries undoubtedly go unreported or are otherwise unaccounted for (5, 6). Furthermore, it is estimated that more than 300,000 US veterans of the wars in Iraq and Afghanistan have sustained mTBI from improvised explosive device blast waves (20% of 1.6 million) (7, 8). Therefore, mTBI is a major public health problem.

Unlike victims of moderate and severe TBI (9–14), individuals who experience mTBI may show cognitive impairment without identifiable tissue lesions or cavities in the cerebral cortex (15). Neuroimaging by magnetic resonance imaging (MRI) or computed tomography (CT) is usually negative (16–20); therefore, mTBI has been called an “invisible wound.” Owing in part to poor understanding of the neuropathology leading to neurologic dysfunction after mTBI, there are no effective treatments. In particular, the absence of gross alterations makes the diagnosis, therapy evaluation, and outcome prediction after mTBI difficult. Here, we induced mTBI using a rodent controlled cortical impact (CCI) model and assessed neurologic changes in detail, including cortex spared volume, cell death, dendrite degeneration, dendritic spine degeneration, and synaptic loss after mTBI. The results support the concept that mTBI produces dramatic dendrite and synapse degeneration despite minimal cell death in the neocortex.

MATERIALS AND METHODS
Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were housed in groups with a 12-hour/12-hour light-dark cycle and free access to food and water ad libitum. They were used in experiments at 8 weeks of age. All procedures were performed under protocols approved by the Animal Care and Use Committee at Indiana University.

Controlled Cortical Impact TBI
The CCI injury model replicates focal abnormalities seen in many human TBI cases (21–23), and the mouse CCI model has been used to investigate posttraumatic cell death (24, 25). Here, 72 mice were subjected to mild CCI injury or sham treatment, as previously described (26, 27), with the following exceptions: the amount of deformation was set at 0.2 mm and the piston velocity was controlled at 2.8 m/s. These modifications result in a mild level of injury using an electromagnetic model (28).

Tissue Processing for Fluoro-Jade B Staining and Immunostaining
At 3 days after TBI surgery, the animals were deeply anesthetized with an overdose of sodium pentobarbital and then perfused transcardially with cold 0.9% saline, followed by a fixative containing 4% paraformaldehyde (Sigma, St

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This work was supported by grants from the Indiana Spinal Cord & Brain Injury Research Grants (SCBI 200-12), the Ralph W. and Grace M. Showalter Research Award, and Indiana University Biological Research Grant to J. Chen.
Louis, MO) in phosphate-buffered saline (PBS). The brains were removed and postfix in paraformaldehyde overnight, followed by cryoprotection with 30% sucrose for 48 hours. Thirty-micrometer-thick serial coronal sections were cut using a cryostat (Leica CM 1950; Leica, Wetzlar, Germany) and stored at −20°C. The sections were then processed for FluoroJade B (FJB) staining and immunohistochemical analysis as described (26, 27, 29–32). Briefly, to perform FJB staining (12 mice), sections were first hydrated in distilled water. The sections were then incubated in a solution of 0.06% potassium permanganate (Sigma) for 10 minutes on a rotating stage. After rinsing in distilled water for 2 minutes, the sections were incubated in a 0.0004% solution of FJB and 0.0004% 4',6-diamidino-2-phenylindole (DAPI) (both from Sigma) for 20 minutes. Sections were then rinsed in distilled water for 2 minutes and air-dried. The dry slides were cleared in xylene for 2 minutes and mounted with DPX (Sigma). For immunostaining (30 mice), sections were rinsed in PBS 3 times and incubated in a blocking solution (0.1% Triton X-100, 1% bovine serum albumin, 5% normal goat serum in PBS) for 1 hour at room temperature (RT), followed by an overnight incubation with primary antibody (1:1,000 NeuN and 1:1,000 synaptophysin; Sigma) at 4°C. Sections were then washed with PBS 3 times and incubated with the secondary antibody at RT for 2 hours. After treatment with DAPI for 2 minutes, the sections were washed with PBS 3 times and mounted with FluoroGel mounting medium (GeneTex, Irvine, CA). Secondary antibodies from Jackson Immunoresearch (West Grove, PA) were all applied 1:1,000 dilutions.

**Golgi Staining**

In total, 20 mice were used for Golgi staining (10 each for TBI and sham groups). We used the FD Rapid Golgi Stain kit (FD NeuroTechnologies, Ellicott City, MD) to perform Golgi staining following the vendor’s protocol. Briefly, the freshly dissected brains were immersed in impregnation solution (made by mixing equal volumes of solutions A and B) and stored at RT for 2 weeks in the dark. The brains were then transferred into solution C and kept for 48 hours at 4°C in the dark. They were then sliced using a horizontal sliding slicer (VT1000S; Leica) at a thickness of 150 μm and stained using standard staining procedures.

**Dendrite Analysis**

Pyramidal neurons in layers II/III of the cortex in the epicenter were analyzed as previously described (33). Briefly, the criteria used to select neurons for reconstruction were as follows: the neurons should be fully impregnated and located in layers II/III with triangular soma and apical dendrites extending toward the pial surface without truncated branches (34–36). For each selected neuron, all branches of the dendritic tree were reconstructed at 40× magnification using a motorized microscope (Olympus BX60; Olympus, Tokyo, Japan) with NeuroLucida software (Microbrightfield, Williston, VT). A 3-dimensional analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). Ten neurons exhibiting all of the criteria were randomly chosen from the 3 epicenter sections of each animal in the different experimental groups, and neurons from the same animal were averaged. Several aspects of dendritic morphology were examined. To assess overall changes, the total length of the basal and apical trees and the number of basal dendrites and apical dendritic branches were compared across groups. To assess the complexity of the dendritic tree, Sholl analysis was performed (37, 38). The numbers of intersections of dendrites were calculated with concentric spheres positioned at radial intervals of 10 μm.

**Spine Quantification**

The spine density of the dendrite branch was measured by considering only apical branches that were either parallel or at acute angles to the coronal surface of the section and did not overlap with other branches (5 mice/group). Because mTBI-induced changes in the apical dendrite branches varied depending on the distance from the soma, segments were randomly selected in the proximal and distal parts of the tree, where maximal effects were observed. To minimize bias, only spines that emerged perpendicular to the dendritic shaft were counted. To assess mTBI-induced changes in spine morphology, spines in the selected segments were classified as mushroom, stubby, or filopodia, and the proportions of spines in each category were compared between the groups. High-magnification images were captured using a camera (DXC-390; Sony Corporation, Tokyo, Japan) attached to the BX60 Olympus upright microscope under a 100× oil objective.

**Cell Number Quantification**

The optical fractionator method (39–41) was used to quantify total (DAPI-positive) and NeuN-positive cells in the epicenter in layers II/III of the cortex. Briefly, using a Bioquant stereology workstation (BioQuant Image Analysis Corp, Nashville, TN), the step size across sections was set as 100 × 100 μm². The dissector height was set as 20 μm, and a 5-μm zone at the uppermost part of the section was excluded from the analysis at every step as the upper guard zone. The upper guard zone is 5 μm, the dissector is set 20 μm, and the lower guard zone is, on average, 3.3 μm. The cells in a counting frame of 50 × 50 μm² were counted under a 40× objective, which allowed accurate recognition. Each DAPI- or NeuN-positive cell was counted when it came into focus as the plane of focus was moved up and down through the height of the dissector (20 μm) following the unbiased counting rules for the optical dissector (40). The total numbers of DAPI- or NeuN-positive cells in the epicenter were determined and normalized to the sham control.

**Synapse Assessment**

Synaptic densities were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). The images were compared to the threshold to outline immunopositive puncta, and the numbers of the puncta were determined using the “analyze particle” module of the program. These numbers were then normalized to the control to determine the synaptic density.
Statistical Analysis

Collected data were expressed as mean ± SD, and differences were analyzed using Student t test with the significant level set at p < 0.05.

RESULTS

mTBI Results in Mild Cell Death in the Injury Area of the Neocortex

To assess the neuropathologic changes after mTBI, we first analyzed mouse brains 3 days after TBI, that is, the peak of neuronal degeneration that appears after TBI (unpublished data). The brains showed very limited tissue disruption without cavitation (Fig. 1A). Staining with crystal violet showed that cortical tissue was generally intact without dramatic lesions (Fig. 1B). The volume of the spared cortex in mice with TBI was 98.3% ± 0.52% of the cortex volume of sham controls (Fig. 1C); this reduction was not statistically significant (p > 0.05). These data indicate that the cortex does not show major tissue lesions after mTBI.

Fluoro-Jade B staining is a widely used method to detect dying cells in the cortex (29, 30). Animals with a mild level of CCI injury exhibited FJB-positive neurons (green) in the epicenter on the lesioned side of the neocortex (Fig. 2A) but not in the corresponding region on the contralateral side.

![Cell death in the neocortex of mice with mild traumatic brain injury (mTBI).](image)

**FIGURE 2.** Cell death in the neocortex of mice with mild traumatic brain injury (mTBI). (A) Fluoro-Jade B (FJB)-positive cells are green in the epicenter on the ipsilateral side of the neocortex. (B-F) Distribution pattern of dying cells around the epicenter. (G) Quantitative data show that the highest number of FJB-positive cells was observed in the epicenter and gradually decreased rostrally and caudally away from it (n = 12).

![Cells lost in the neocortex of mice with mild traumatic brain injury (mTBI).](image)

**FIGURE 3.** Cells lost in the neocortex of mice with mild traumatic brain injury (mTBI). (A-D) DAPI (blue) and NeuN (red) staining revealed the nuclei of cells and mature neurons at the injury site in the cortex (A and B sham control, C and D mTBI). (E) DAPI staining followed by counting of the stained cells showed that the total cell density in the epicenter was slightly reduced to 85.8% ± 9.5% compared to the sham control (n = 10; *, p < 0.05). (F) The density of NeuN-positive neurons was dramatically reduced to 57.2% ± 5.4% (n = 10; **, p < 0.005).
The total cell and neuron density in the injury area was measured using DAPI staining and immunostaining with an antibody to NeuN, a mature neuron marker. Compared with the sham control, the total cell density based on DAPI staining was slightly reduced to 85.8% ± 9.5% (*, p < 0.05) in the epicenter. Because DAPI stains the nuclei of both neurons and glia, NeuN staining was used to assess neuron loss in the injury area. These experiments revealed highly stained NeuN in the nuclei of neurons in the cortex of control (Figs. 3A, B) and mTBI-injured brains (Figs. 3C, D). At low resolution, NeuN staining resulted in a slightly higher background in the mTBI-injured brain (Fig. 3C) compared with the sham control (Fig. 3A). At higher magnification, NeuN-stained neurons were evident in both the mTBI-injured (Fig. 3D) and the sham control (Fig. 3B). We then assessed the density of the mature neurons in layers II/III at the epicenter of the mTBI-injured cortex and the corresponding layers in the control cortex. The density of NeuN-positive neurons in the epicenter was reduced to 57.2% ± 5.4% of the density in the controls (**, p < 0.005). These data suggest that, although mTBI did not cause gross neuroanatomic changes in the brain, it did result in some neuron death in the epicenter.

mTBI Causes Extensive Neural Degeneration in the Neocortex

To determine whether mTBI causes degeneration in the spared neurons, we assessed the morphologies of the spared neurons by Golgi staining, a reliable and sensitive method for demonstrating morphologic details of individual neurons, especially dendritic spines. Golgi staining visualized individual neurons including their processes and spines at very high resolution in the control brains (Figs. 4A, C1–D3) and damaged brain after mTBI (Figs. 4B, E1–F3). The density of the Golgi-stained neurons in the outlined area of the injury brain was significantly decreased compared with the corresponding position of the control brains, consistent with the finding that mTBI induced cell death (Fig. 2) and decreased cell density in the injury area (Fig. 3). Higher-resolution images of the epicenter (Figs. 4, E1-E3) and 1 mm lateral to the epicenter (Figs. 4, F1–F3) also demonstrated that the dendrites of the spared neurons in the injured cortex were swollen with beadings, a hallmark of dendritic injury, compared to neurons in the corresponding regions of control mice (Figs. 4, C1–C3 and D1–D3). The cortex volume showed dendritic degeneration (Fig. 4B) and was surprisingly large. When degeneration was taken into account, the spared cortex decreased to 82.5% ± 4.3% (*, p < 0.05; Fig. 4G). The volume of neuronal degeneration was 10.3 times larger than the tissue lesion volume in the injured cortex after mTBI (Fig. 4H). These data indicate very extensive injury in the dendrites of the spared neurons in the injury area, that is, the actual injury was much greater than previously assessed using the percentage of spared tissue as an indicator.

mTBI Causes Dendrite Degeneration

To characterize the morphologic characteristics of neurons in the cortex after mTBI, we reconstructed dendritic morphologies of individual neurons (n = 50) of layers II/III in the epicenter or in the corresponding positions in mTBI and control mice (Figs. 5A, B). The results showed that the total length of the apical dendrites and the number of dendrite branches of pyramidal cells were reduced in mTBI (Figs. 5C, D). To characterize the dendritic branching characteristics of individual neurons in the injury area and the corresponding control regions, we performed Sholl analysis, a quantitative method for morphometric neuronal studies (Figs. 5E, F). This analysis revealed that mTBI brains showed reduced dendritic material compared with the sham controls. This decreased complexity was particularly evident at a distance between 40 and 100 μm from the soma. The difference between neurons from mTBI and sham animals peaked at 70 μm from the soma; at this distance, the total number of crossings was 6 times lower for mTBI mice (2 ± 0.7 vs 13 ± 2.5 crossings in mTBI and sham mice, respectively; 50 cells in each group, p < 0.01; Fig. 5F). These results indicate a dramatic dendritic degeneration in the neurons in the injury area after mTBI.

mTBI Leads to Spine Degeneration

To assess dendritic spines of spared neurons in the injury area, we imaged the dendritic spines in the cortices of control (Fig. 6A) and mTBI (Fig. 6B) mice and determined their density and characteristics. The density of the dendritic spines was significantly reduced from 14.48 ± 0.89 per 10 μm to 9.09 ± 0.85 per 10 μm in the spared neurons in the injured cortex (Fig. 6F). Dendritic spine protrusions were categorized into 3 types based on their shapes and lengths as follows: mushroom-shaped spines, in which the diameter of the head is much larger than the diameter of the neck, as is usually seen in mature synapses (Fig. 6C) (42); stubby-shaped spines, which are short and wide and are considered to represent either a transitional growth stage between an early to mature spine or a stage during retraction of the mature spine for elimination (Fig. 6D); and filopodia-shaped spines, which are highly...
FIGURE 5. Mild traumatic brain injury (mTBI) causes dendrite degeneration. (A, B) Neurolucida reconstruction of Golgi-stained layers II/III neurons in the cortex of sham (A) and mTBI mice (B). (C, D) The total length (C) and the number of branches (D) of the dendrites in cells from sham and mTBI mice. The total length of the dendrites in spared neurons decreased from 1905 ± 357 to 508 ± 55 μm; branches are dramatically decreased from 32 ± 5 to 10 ± 1 in the cortex after mTBI (n = 5; *, p < 0.05). (E) Sholl analysis-derived distribution of layers II/III neuron dendritic complexity based on the distance from the cell body. Mean number of intersections of dendrite branches with consecutive 10-μm-spaced concentric spheres. (F) There is a dramatic reduction of dendritic complexity in the spared neurons in mTBI mice versus sham controls (n = 5; *, p < 0.05).
mobile, long, and thin with a fine tip and are seen at the early stage of spine formation (Fig. 6E) (43–46). The densities of mushroom-shaped (8.06 ± 0.41 vs 3.36 ± 0.39 per 10 μm) and filopodia-shaped (3.31 ± 0.44 vs 1.66 ± 0.26 per 10 μm) were decreased in mTBI versus controls, whereas the density of stubby-shaped spines increased slightly (Fig. 6G).

mTBI Decreases the Number of Synapses in the Injury Area

Because a reduction in the number of dendritic branches and degeneration of the dendritic spines would decrease the number of synapses, we determined whether mTBI-induced degeneration in dendrites and dendritic spines led to a decrease in synapses by quantifying immunostaining with an antisynaptophysin antibody (47). Synaptophysin immunostaining was observed in a punctate distribution, consistent with localization of presynaptic boutons. Cell nuclei showed negligible synaptophysin immunoreactivity (Fig. 7). The densities of synaptophysin-positive puncta in layers II/III in the epicenter of the TBI-injured mice and at the same anatomic position in the brains of control mice were counted. In the TBI-injured brains, the density of the synaptophysin-positive puncta was reduced to 65.5% ± 8.1% versus the sham control cortex, indicating that mTBI decreased the number of synapses.

DISCUSSION

Although patients with mTBI show a wide range of neurologic symptoms, including headache, blurred vision, dizziness, sleep problems, subjective memory problems, and other cognitive difficulties (48–57), neuroimaging using CT or MRI does not usually identify major structural lesions (16–20). Neurological dysfunction in experimental models of TBI has been attributed to rapid cell death (12, 58–60). However, unlike moderate and severe TBI, the model of mTBI we used does not induce significant tissue lesions or cavitation in the cortex, and cell death was limited to the epicenter. Previous work in the rat also showed prolonged memory impairment in the absence of hippocampal cell death after TBI (61).

Although mTBI caused only mild cell death, it resulted in extensive dendritic degeneration and synapse reduction. The cell body and the dendrites are the major domains of neurons that receive input, and dendrites play a critically important role in providing a massive receptive area on the neuronal surface for spine formation. An important specialization of the dendritic arbor of certain neurons is the presence of large numbers of spines with as many as 30,000 to 40,000 spines on large pyramidal neurons. Maintaining such complex dendritic arbors is a prerequisite for neuronal connections and, to a large degree, neuron functions. Using Golgi staining, we found that dendrites were degenerated in the ipsilateral cortex after mTBI; the abnormalities included swelling with beading, a hallmark of dendritic injury, decreased numbers of branches, and shorter branch lengths. Dendrite degeneration around the epicenter was very extensive. Indeed, the volume of neuronal degeneration in the cortex was 10.3 times higher than the tissue lesion volume after mTBI.

![FIGURE 6. Mild traumatic brain injury (mTBI) leads to dendritic spine degeneration. (A, B) High-resolution image of a single spine in sham control (A) and mTBI mice (B). (C–E) Mushroom-shaped (C), stubby-shaped (D), and filopodia-shaped (E) spines are shown. (F) Quantitative data show that the density of the dendritic spines is significantly reduced in spared neurons in the cortex of TBI mice (n = 5; **, p < 0.005). (G) The reduction is particularly apparent in mushroom-shaped and filopodia-shaped spines (n = 5; **, p < 0.005).](https://academic.oup.com/jnen/article-lookup/10.1093/jnen/npl031)

![FIGURE 7. Mild traumatic brain injury (mTBI) decreases the number of synapses in the injury area. (A–D) Synaptophysin staining (red) reveals presynaptic puncta in layers II/III of the cortex in control (A, B) and mTBI mice (C, D). The synapse density decreased to 65.5% ± 8.1% of the control cortex in the cortex of injured mice (n = 10; *, p < 0.05).](https://academic.oup.com/jnen/article-lookup/10.1093/jnen/npl031)
Dendritic spine morphology was also significantly affected in the injured neurons in the ipsilateral cortex. In particular, mushroom-shaped spines were dramatically reduced. Because these spines form synapses, reductions in their density suggest a reduction in the number of the synapses. This was confirmed with synaptophysin immunohistochemistry. Moreover, the injury was much more extensive than assessments based on the percentage of spared tissue. Because CNS function relies critically on the establishment and maintenance of synaptic connections between the neurons, extensive degeneration of dendrites and dendritic spines and loss of synapses would likely greatly impair neurologic function.

There is increased attention directed at finding objective physiological correlates for persistent cognitive and neuropsychiatric symptoms through experimental neuroimaging techniques. Because the diameter of synapses is less than 1 μm, the formation and elimination of synapses are beyond the resolution of conventional CT and MRI. On the basis of our results, functional imaging techniques may be more promising than structural imaging techniques for the immediate diagnosis and management of mTBI. Indeed, several recent studies using newer imaging techniques such as functional MRI, positron emission tomography, and single photon emission CT showed functional impairment in the cortex after mTBI (16, 63–66). Thus, these functional techniques may be more sensitive for detecting functional impairments related to synapse degeneration.

In summary, we showed that although mTBI led to limited tissue lesion and cell death, it caused widespread and significant synaptodegeneration in the cortical neurons that were spared. This suggests dramatic neuronal degeneration after mTBI. The widespread synapse loss disrupts neural circuitry after mTBI would likely contribute to neurologic dysfunction. Furthermore, the spared neurons showed dramatic synapse degeneration without death after mTBI, suggesting that synapse degeneration is a program separate from neuronal degeneration. Brain Res 1997;751:37–46.

REFERENCES


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