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INTRODUCTION
Concussive traumatic brain injury (TBI) has been defined in many ways, but common elements include temporary impairment in cerebral function after traumatic biomechanical forces without evidence of gross structural injury to the brain. Most of the 1.7 million or more TBIs that occur each year in the United States are “mild,” uncomplicated injuries (1–3). Although most patients with concussion recover well, growing evidence indicates that multiple concussions are less benign (4–8). Perhaps the best-documented examples are in chronic traumatic encephalopathy patients. These are typically athletes in contact sports who sustain several concussive brain injuries and subsequently demonstrate cognitive deterioration, emotional instability, and other symptoms (7, 9). The problem of repetitive concussive TBI is of great interest to the military because many military personnel sustain multiple combat-related concussive injuries during their service (10, 11).

Little is known about the pathology of concussion or the mechanisms underlying the adverse effects of multiple concussions. To begin to address this gap, several groups have produced animal models of repeated mild TBI. In an early study involving 7 injuries repeated at 24-hour intervals in rats, neuronal cytoskeletal protein abnormalities were prominent (12). A single closed-skull injury caused subtle and transient behavioral and immunohistochemical abnormalities in mice, whereas 2 such injuries 24 hours apart caused more persistent behavioral impairments and axonal injury in the thalamus but no gross pathological injury or neuronal cell loss (13). In another mouse model, there were behavioral deficits but no apparent neuronal loss observed after 4 mild injuries at 24-hour intervals (14). Repetitive mild TBI was shown to accelerate β-amyloid deposition in Tg2576 transgenic mice, a model of Alzheimer disease–related amyloidosis (15). Vitamin E pretreatment prevented this β-amyloid deposition and improved behavioral performance (16). A window of vulnerability lasting approximately 5 days between injuries was described (17). Contraconup histologic abnormalities were found after 3 mild impacts accompanying deficits in spatial learning in another mouse model (18). In all of these studies, the pathological features of the injury were relatively subtle in comparison to the extent of the behavioral deficits.

This mismatch between structural injury and performance suggests 2 non–mutually exclusive hypotheses: 1) functional disturbances in electrical or chemical signaling could underlie...
the performance impairments or 2) previously used histologic assessments may not be sufficiently sensitive to detect structural injuries responsible for the impairments. The primary objective of the current study was to elucidate the structural consequences of closed-skull mild repetitive TBI. We hypothesized specifically that traumatic axonal injury might be more severe and widespread than has been previously recognized. This hypothesis was based on recent imaging-based findings of white matter abnormalities in human patients with mild TBI (19, 20). Silver staining, Iba1 immunohistochemistry, and electron microscopy after 2 impacts were used to test this hypothesis. Mice subjected to 1 impact and 1 sham procedure or 2 sham procedures were used as controls. Using these methods, we found extensive and persistent multifocal argyrophilic abnormalities, ultrastructural evidence for axonal cytotoxic changes, marked reactive microgliosis after 2 impacts. Conventional histologic methods markedly underestimated these pathologies.

MATERIALS AND METHODS

Animals

All experiments were approved by the Washington University Animal Studies Committee. Male C57BL/6J wild-type mice were purchased from Jackson Labs (Bar Harbor, ME) at 6 to 8 weeks of age. They were housed with siblings at 2 to 5 mice per standard cage for at least 1 week before experiments. They were fed standard mouse chow and given water ad libitum. Ambient temperature was controlled at 20 to 22°C with 12-hour light/12-hour dark cycles. A total of 147 mice were used.

Electromagnetic Repetitive Closed-Skull TBI

When mice were 2 to 3 months of age, they were anesthetized with 5% isoflurane and placed in a stereotoxic frame with rounded Kopf head holders (David Kopf Instruments, Tujunga, CA). Pointed ear bars were not used. Temperature was controlled at 37°C using a feedback temperature controller (Cell Microcontrols, Norfolk, VA). Isoflurane was delivered by nose cone at 2% in air. The heads were shaved and prepared with povidone-iodine. A midline skin incision was made, and the skull was exposed. No craniotomy was performed.

A rubber tip (Precision Associates, Inc, Minneapolis, MN) was mounted on an electromagnetic stereotaxic impact device in place of the metal tip used in previous controlled cortical impact studies (21). The rubber tip was 9 mm in diameter and the rubber had a spring constant of 3.01 N/mm. The tip was fully extended and lowered at a 20-degree angle until the vertex touched the skull at 1.8 mm caudal to bregma and 3.0 mm left of midline. This was confirmed with a hand lens in all cases. The tip was then retracted automatically. The stereotoxic device was moved by 3.3 mm, and the electromagnetic device was triggered, driving the tip 3.3 mm into the exposed skull at 5.0 m/s with a dwell time of 100 milliseconds. The deformation of the rubber tip spread the impact force over the skull. There were fewer than 3% skull fractures and no immediate fatalities after these injuries. Mice with skull fractures were killed and were not used in any experiments. After impact, the skin was sutured and the mice were allowed to recover from anesthesia on a warming pad and then returned to their home cages. After 24 ± 1 hours, a second identical closed-skull TBI procedure was performed. For sham injuries, the same procedure was performed except that the impact device was discharged in the air; the handling of the mice and duration of anesthesia were the same for TBI and sham procedures.

Impacts and sham procedures were performed by 2 independent investigators (Y.S. and R.E.B.) without direct contact during the procedures. Behavioral performance, silver staining, and Iba1 immunohistochemical appearance were essentially indistinguishable for injuries performed by the 2 investigators. The mice used for behavioral assessments were not used for histologic analyses. Nonetheless, after completion of behavioral testing, these mice were killed and their brains were examined for hemorrhage. Mice with hemorrhages were excluded from the behavioral analyses.

Behavioral Testing Using the Morris Water Maze

In the first experiment, a total of 26 mice were randomly assigned to TBI-TBI, TBI-Sham, or Sham-Sham groups and tested 2 days after the second TBI or sham procedure. In the second experiment, a total of 20 mice were randomly assigned to TBI-TBI or Sham-Sham groups and tested 2 days after the second TBI or sham procedure. In the third experiment, 18 mice were randomly assigned to TBI-TBI or Sham-Sham groups and tested 7 weeks after the second TBI or sham procedure. The examiners were blinded to group identity, and all mice had indistinguishable sutures on their heads.

The water maze was 109 cm in diameter and the platform was 11 cm in diameter (21). Hidden platform testing was performed on 4 days with 3 trials per day lasting a maximum of 60 seconds per trial. At the end of day 4, a single 30-second probe trial was performed with the platform removed. On the following day, visible platform testing was performed, again with 3 trials per mouse, with the platform above the surface of the water, marked with a pole, and placed in a different location of the pool.

Histopathology

Mice were killed by deep isoflurane anesthesia and cardiac perfusion with ice-cold phosphate-buffered saline with 0.3% heparin. Brains were removed and examined for subdural or subarachnoid hemorrhage. Hemorrhages that usually underlay the impact site were observed in fewer than 5% of mice, and these brains were not used for analysis. The remaining brains were fixed in 4% paraformaldehyde for 24 hours and equilibrated in 30% sucrose for 3 days. Coronal slices were made every 50 μm using a freezing sliding microtome from the most anterior portion of the corpus callosum to the most posterior portion of the hippocampus.

Gross contusions, tissue loss, and selective cell loss were assessed using cresyl violet staining. Amyloid-precursor protein immunohistochemistry was performed as previously described (22, 23). The 3′-diaminobenzidine (DAB) chromogen is also very sensitive to blood, and the presence of
DAB-positive red blood cells was used to assess for intraparenchymal hemorrhage.

Silver staining was performed using the FD NeuroSilver Kit II (FD NeuroTechnologies, Ellicott City, MD) according to the manufacturer’s instructions with the following modification. The protocol was made more sensitive using 2-minute incubations instead of 4-minute incubations in step 6 and constant vigorous agitation from steps 5 to 7. This also resulted in slightly increased background. Brain sections were stained in batches such that each batch had brain sections from each postinjury time point or experimental group. Thus, there were no systematic biases introduced by variability in staining intensity from batch to batch.

Iba1 immunohistochemistry was performed using a rabbit polyclonal anti-Iba1 antibody (Wako Chemicals USA, Richmond, VA). Free-floating sections were blocked with 3% normal goat serum in Tris-buffered saline (TBS) containing 0.25% Triton X (TBS-X) for 30 minutes to eliminate nonspecific binding. Sections were incubated overnight in TBS-X solution containing anti-Iba1 antibody (0.5 μg/ml) and 1% normal goat serum. Sections were washed with TBS 3 times for 5 minutes each between applications of antibody solutions. Bound antigen-antibody complexes were detected with biotinylated goat anti-rabbit secondary antibody. Horseradish peroxidase method (ABC Elite Kit, PK6100; Vector Laboratories, Burlingame, CA) and DAB were used for visualization.

Quantitative Analyses of Histopathology

Densitometric analysis of silver staining was performed on 4 sections per mouse separated by 400 μm for ipsilateral cortex and ipsilateral thalamus. Three sections per mouse were used for ipsilateral dentate gyrus, midline corpus callosum plus ipsilateral external capsule, and contralateral hippocampal CA1. These sections spanned approximately bregma −1.1 to −2.3 mm (24). The ipsilateral cortical region quantified was defined as dorsal to a horizontal line extending from the dorsal-most portion of the thalamus and lateral to a vertical line extending through the tip of the cingulum. Images were acquired and digitized using a Nanozoomer HT system (Hamamatsu Photonics, Hamamatsu City, Japan). ImageJ (National Institutes of Health, Bethesda, MD) was used for quantitation of the staining on these digitized images. Silver staining was expressed in arbitrary units, ranging from 0 (minimum) to 255 (maximum). Mice with group sizes between 5 and 8 were killed at each time point for histologic analyses. There were no systematic biases introduced by variability in staining intensity from batch to batch.

Electron Microscopy

Separate mice were injured or underwent sham procedures for electron microscopic analysis. They were perfused for 5 minutes with 2% glutaraldehyde plus 2% paraformaldehyde in 0.1 mol/L sodium cacodylate 2 mmol/L calcium chloride buffer (pH 7.4). Brains were removed, sectioned into 1-mm-thick coronal slabs and further fixed in the same solution for 24 hours. After dehydration, the slabs were embedded in the cortex and thalamus, a grid of 400 × 400 μm and a counting frame of 80 × 80 μm were used. For the corpus callosum, dentate gyrus, and CA1, a grid of 180 × 180 μm and a counting frame of 80 × 80 μm were used.

The optical fractionator method was also used to count cresyl violet-stained cells in the polymorphic region of the dentate gyrus, excluding the densely packed granule cells and CA3 cells (25) in 4 sections per mouse. A grid of 100 × 100 μm and a counting frame of 50 × 50 μm were used. Gunderson coefficients of error were less than 0.1.

FIGURE 1. Closed-skull traumatic brain injury in mice using an electromagnetic controlled stereotaxic impact device. Top: A mouse in the stereotaxic frame with the stereotaxic electromagnetic impactor aligned above the skull. Bottom: A mouse in the stereotaxic frame with exposed skull and the rubber tip touching the surface of the skull.
in plastic, and ultrathin sections were prepared for electron microscopy as described (22).

**Statistical Methods**

All analyses were performed using Statistica 6.0 (StatSoft, Tulsa, OK). Repeated-measures analysis of variance (ANOVA) was used to compare hidden platform Morris water maze performance between groups. One-way ANOVAs with Tukey post hoc pairwise comparisons were used to compare Morris water maze probe performance, visible platform performance, and quantitative histologic parameters between groups. Dunnett post hoc tests were used to compare quantitative histologic parameters at each time point after injury to control (Sham-Sham) groups. Although both the silver staining intensity and Iba1-immunoreactive microglial counts were approximately normally distributed, the correlation between these variables did not satisfy the assumptions required for Pearson correlation; the residuals after linear regression were not normally distributed. Therefore, nonparametric Spearman correlations were used to test for correlations between silver staining intensity and Iba1-immunoreactive microglial counts. \( p < 0.05 \) was considered significant. Error bars represent SEM.

**FIGURE 2.** Repetitive closed-skull traumatic brain injury (TBI) caused behavioral impairments in the Morris water maze. Male C57BL/6J mice at 2 to 3 months of age were randomly assigned to receive 2 impacts (TBI-TBI), 1 impact and 1 sham (TBI-Sham) or 2 sham procedures (Sham-Sham) on days 0 and 1. Mice were tested in the Morris water maze 2 to 5 days later in a blinded fashion. (A) Time to hidden platform as a function of day of training. TBI-TBI mice performed worse than either Sham-Sham (**, \( p = 0.006 \)) or TBI-Sham (*, \( p = 0.039 \)) mice. (B) Mean time to hidden platform across all 4 days of hidden platform training (**, \( p = 0.006 \); *, \( p = 0.039 \)). (C) Mean distance to hidden platform across all 4 days of hidden platform training. No difference between groups. (D) Mean swimming velocity across all 4 days of hidden platform training. The TBI-TBI mice swam more slowly than Sham-Sham mice (*, \( p = 0.024 \)). (E) Probe trial. After hidden platform training on day 5, a 30-second probe trial was performed. TBI-TBI mice performed at no better than chance and worse than Sham-Sham mice (\( p = 0.012 \)). (F) Mean time to visible platform. On day 6, a single day of visible platform training was performed. TBI-TBI mice performed worse than Sham-Sham mice (*, \( p = 0.013 \)). (G) Mean distance to visible platform. No differences between groups. (H) Mean swimming velocity to visible platform. The TBI-TBI mice swam more slowly than Sham-Sham mice (*, \( p = 0.02 \)).

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RESULTS

A Stereotaxic Electromagnetic Impact Model of Repetitive Closed-Skull TBI

We adopted the repetitive impact injury model of Laurer et al (13) to a stereotaxic format with an electromagnetic impact device (Fig. 1). Using a rubber tip and an impact depth of 3.3 mm below the surface of the exposed skull, mild injuries were consistently produced. Smaller impact depths did not cause significant behavioral impairments and larger impact depths increased the incidence of skull fractures (data not shown).

The TBI-TBI mice had significant impairments in the Morris water maze test (Fig. 2). Impacts were performed on days 0 and 1; testing was performed on days 3 to 6. In hidden platform testing, there were significant main effects of injury status (F = 6.57, p = 0.0055, repeated-measures ANOVA) and day of training (F = 6.72, p = 0.00048) but no interaction between injury status and day of training (p = 0.78) (Figs. 2A–D). In prespecified post hoc testing, the TBI-TBI mice had 71% longer mean latencies over all 4 days to find the hidden platform versus Sham-Sham mice (42.1 ± 3.5 vs 24.6 ± 3.7 seconds) (Figs. 2A, B; p = 0.006, Tukey post hoc test). Similarly, TBI-TBI mice had longer latencies than TBI-Sham mice (29.2 ± 3.5 seconds, p = 0.039). There was no difference between Sham-Sham and TBI-sham mice (p = 0.63). Mean distance to the hidden platform was 32% longer (116 ± 12 vs 88 ± 8 cm) in TBI-TBI versus Sham-Sham mice, but this was not statistically significant in this experiment (Fig. 2C; p = 0.13, Tukey post hoc test). Swim speed was 20% slower in the TBI-TBI versus Sham-Sham mice (Fig. 2D; p = 0.024, Tukey post hoc test). This likely accounts for the larger magnitude change in latency measurements compared with distance measurements.

Probe test performance was markedly impaired in the TBI-TBI mice (Fig. 2E). These mice spent no more time in the target quadrant than the 25% that would be expected by chance (mean, 19.2%; 95% confidence interval, 4.0%–34.5%). In
contrast, TBI-Sham mice spent more time in the target quadrant than would be expected by chance (mean, 36.7%, 95% confidence interval, 26.3%–47.1%) as did the Sham-Sham mice (mean, 43.6%; 95% confidence interval, 32.5%–54.7%). The difference between TBI-TBI and Sham-Sham mice was significant ($p = 0.012$, Tukey post hoc test).

Visible platform performance was also impaired in the TBI-TBI mice (Figs. 2F–H), with significantly longer latencies (Fig. 2F; $p = 0.013$), a trend toward longer distances (Fig. 2G; $p = 0.08$), and significantly slower velocities (Fig. 2H; $p = 0.02$) versus the Sham-sham mice. There were no differences in visible platform performance between TBI-Sham and Sham-Sham mice.

The scatter plots of the Morris water maze performance for individual mice suggested that some aspects of the data were not normally distributed. The sample sizes were modest, so direct testing for normalcy (e.g. Shapiro–Wilks) was not performed. However, using nonparametric Mann–Whitney U tests to compare the TBI-TBI and Sham-Sham mice resulted in similar conclusions. Specifically, time to the hidden platform was longer (Fig. 2B; $p = 0.02$), hidden platform swim speed was slower (Fig. 2D; $p = 0.034$), probe test target quadrant occupancy was lower (Fig. 2E; $p = 0.016$), time to the visible platform was longer (Fig. 2F; $p = 0.007$), distance to the visible platform was greater (Fig. 2G; $p = 0.012$), and visible platform swim speed was slower (Fig. 2H; $p = 0.012$).

In a second experiment performed by an independent investigator, Morris water maze performance deficits after 2 impacts were very similar to those in the first experiment, indicating reproducibility (Fig. 3). Notably, in the second experiment, the mean distances to reach the hidden platform were statistically significantly longer in the TBI-TBI mice than the Sham-Sham mice ($p < 0.001$). There were some differences in absolute performance between experiments, as has been reported by others (26).

In summary, the behavioral performance of TBI-TBI mice was worse than that of either TBI-Sham (Figs. 2A, B) or Sham-Sham mice (Figs. 2A, B, and 3). Differences between TBI-TBI and TBI-Sham mice were not always statistically significant, but this was likely due to the relative insensitivity of some of the behavioral measures. These results indicate impairments in spatial learning and memory as well as procedural and motor dysfunction in the TBI-TBI mice. The longer times to reach the hidden platform are likely due to both spatial learning and motor impairments, whereas the probe test is a relatively pure indicator of spatial memory impairment. The reduced swim speeds suggest a motor impairment. The visible platform deficits could be related to motor impairments, visual dysfunction, or other motivational factors.

**Few Gross or Histopathological Abnormalities in TBI-TBI Mice**

The brains of mice killed at 7 days after the first of 2 impacts, 1 impact plus 1 sham, or 2 sham procedures were typically indistinguishable on gross pathological examination. Cresyl violet staining revealed no contusions (Figs. 4A–C).
cortical cell loss underlying the site of impact (Figs. 4D–F), no ipsilateral dentate gyrus cell loss (Figs. 4G–I), no corpus callosum abnormalities (Figs. 4J–L), and no ipsilateral thalamic cell loss (Figs. 4M–O). No intraparenchymal hemorrhages were detected microscopically. We performed stereological counts of cresyl violet–stained cells in the polymorphic region of the dentate gyrus, excluding the densely packed granule cells and CA3 cells (25). This revealed no difference between TBI-TBI and Sham-Sham mice (8,316 ± 2,275 vs 8,884 ± 1,239 cells, p = 0.53, Mann-Whitney U test). Thus, the behavioral impairments and absence of obvious structural injury indicate that this model shares essential features with human repetitive mild TBI.

APP Immunohistochemistry

There were subtle abnormalities on APP immunohistochemistry in serial brain sections from mice subjected to 2 impacts at 7 days after injury. These included a few punctate immunoreactive swellings in the ipsilateral cortex and external capsule and increased perinuclear cytoplasmic staining of cortical neurons (Fig. 5A). TBI-Sham and Sham-Sham mice did not show these abnormalities (Figs. 5B, C). The corpus callosum was entirely unremarkable in the TBI-TBI mice (Fig. 5D). As a positive control, immunohistochemistry on brain sections from a mouse subjected to controlled cortical impact TBI (23) revealed extensive APP-immunoreactive axonal varicosities (Fig. 5E). There was no difference in APP immunoreactivity between TBI-TBI and TBI-Sham mice at 24 and 72 hours after the second injury/procedure (not shown). Therefore, although APP immunohistochemistry can detect injured axons in many contexts, it revealed only minor abnormalities after mild, repetitive closed-skull TBI in mice. This is consistent with previous findings (13, 17).

Silver Staining and Electron Microscopy

Silver staining revealed extensive abnormalities in the brains of TBI-TBI mice (Fig. 6). Specifically, at day 7, there was prominent argyrophilia in the ipsilateral superficial cortex underlying the impact site (Figs. 6A, D), ipsilateral thalamus (Figs. 6A, G), bilateral corpus callosum (Figs. 6A, J), and bilateral external capsule (Figs. 6A, D, P). Notable, but less consistent changes were observed in the ipsilateral dentate gyrus (Figs. 6A, M) and contralateral CA1 stratum oriens and stratum radiatum (Figs. 6A, P). Most of the argyrophilia was distributed in a pattern that suggested axonal and dendritic localizations, but there were also some cortical neurons underlying the impact site with somatic silver staining as well (not shown).

There were generally minimal argyrophilic abnormalities in the brains of TBI-Sham mice (Figs. 6B, E, H, K, N, Q), but a minority of these mice (2/8) had abnormalities that were similar to those in TBI-TBI mice. No abnormalities were seen in the brains of Sham-Sham mice (Figs. 6C, F, I, L, O, R).

Electron microscopic examination of several brain regions identified based on abnormal silver staining revealed axonal pathology in the TBI-TBI mice (Fig. 7). Four mice were examined: 2 were killed at 4 days after the second impact and 2 were killed at day 7 after the second impact. There were consistent cytoskeletal abnormalities (Figs. 7A, B, D, F), axoplasmic collapse (Figs. 7B, D, H), aggregation of intraxoplasmic electron dense material (Figs. 7A, B, E, F, G), and organelle compaction (Fig. 7H) in myelinated axons in these mice. The dystrophic axons were scattered among many normal-appearing axons (Figs. 7A–H). Images from the ipsilateral cortex, external capsule, lateral geniculate nucleus of the thalamus, stria medullaris, and contralateral external capsule all showed similar abnormalities. We did not detect injury to unmyelinated axons, nor did we observe demyelination or microvascular pathologies. We observed very few massively dilated axons, consistent with the relatively subtle abnormalities found on APP immunohistochemistry. This contrasts with the ultrastructural findings in pericontusional white matter after controlled cortical impact, characterized by massively dilated axons and secondary demyelination (22). We interpret these observations to indicate that repeated closed-skull TBI primarily causes a relatively isolated cytoskeletal disruption in large axons, which then leads to axoplasmic collapse and partial impairment of axonal transport.

Brains from 2 control animals killed 4 days after the second of 2 sham procedures that were prepared identically for electron microscopy revealed no abnormalities (Figs. 7I–J). Both large myelinated and small unmyelinated axons appeared normal with intact axoplasm and no cytoskeletal rearrangements. This indicates that the ultrastructural changes observed after 2 impacts were not likely to have been artifacts due to tissue preparation.

Microglial Activation in Regions With Injured Axons

In all regions examined, cells with the ultrastructural characteristics of activated microglia (27) were observed near injured axons (Fig. 8). These cells were often located near capillaries and had dense heterochromatin near the nuclear envelope, granular cytoplasm, and extended cytoplasmic processes. In some cases, these cytoplasmic processes were in direct contact with dystrophic axons, suggesting that they are a microglial response to axonal injury (Fig. 8B).

Iba1 immunohistochemistry was performed on sections adjacent to those used for silver staining in several brain

FIGURE 6. Silver staining reveals extensive, multifocal argyrophilic abnormalities in 2 closed-skull impacts (TBI-TBI) mice 7 days after the first impact (A, D, G, J, M, P) in comparison to TBI-Sham (B, E, H, K, M, Q), and Sham-Sham (C, F, I, L, O, R) mice. (A–C) Low-power views of coronal sections at approximately bregma −2.18 mm (24). White boxes indicate areas of interest, shown at higher magnification in lower panels. (D–F) Ipsilateral cortex and external capsule underlying the impact site. Silver staining is prominent in scattered cortical regions and the external capsule in TBI-TBI mice. (G–I) Ipsilateral thalamus. There is intense silver staining in the lateral and dorsomedial portions in TBI-TBI mice. (J–L) Corpus callosum. Prominent silver staining bilaterally in TBI-TBI mice. (M–O) Ipsilateral dentate gyrus. More subtle but clearly abnormal silver staining is seen in the neuropil in TBI-TBI mice. (P–R) Contralateral hippocampal CA1 and external capsule. There is more prominent silver staining in the external capsule, CA1 stratum oriens and stratum radiatum in TBI-TBI mice.
regions. There were marked increases in Iba1-immunoreactivity after 2 impacts in the same regions with abnormalities on silver staining (Fig. 9A). The TBI-Sham and Sham-Sham mice had less Iba1 immunoreactivity (Figs. 9B, C).

After 2 impacts, the Iba1-immunoreactive cells often had the characteristic uniform distribution (Fig. 10A) and morphologies (Figs. 10C–E) of activated microglia, including hypertrophic (Fig. 10C), bushy (Fig. 10D), and amoeboid (Fig. 10E) appearances with thick, densely labeled processes and large cell bodies. Iba1-immunoreactive cells with resting microglial morphologies (i.e. thin, highly ramified processes and a small cell body; Fig. 10B) were seen in all experimental groups, independent of injury.

**Quantitative Analysis of the Time Course of Silver Staining Abnormalities and Iba1-Immunoreactive Activated Microglial Cell Counts**

We next explored the relationship between the silver staining abnormalities and the Iba1-immunoreactive activated microglia. To do this, we killed separate groups of 5 to 8 mice at 2, 4, 7, 14, 28, and 49 days after repetitive closed-skull injury. We assessed adjacent sections from the same mice with silver staining and Iba1 immunohistochemistry. Densitometric methods were used to quantify the extent of silver staining. This revealed that the silver staining abnormalities were most prominent and persistent in the corpus callosum, with high levels of staining as late as 49 days after injury (Fig. 11E). The staining was statistically significantly elevated in the corpus callosum at 4, 7, 14, 28, and 49 days. At peak, the silver staining was 5-fold greater than that in Sham-Sham mice. The silver staining was more transient in the ipsilateral cortex (Fig. 11A), ipsilateral thalamus (Fig. 11C), ipsilateral dentate gyrus (Fig. 11G), and contralateral hippocampal CA1 (Fig. 11I), with nearly complete resolution by 28 to 49 days after injury. In all regions examined, densitometric analysis demonstrated statistically significant increases in silver staining above control at 7 days.

The time course of microglial activation was compared with that of the silver staining. Stereological methods were used to quantify the numbers of Iba1-immunoreactive microglia with activated morphologies in each region of interest at the same time points. Iba1-immunoreactive microglial activation was persistent as late as 49 days in the corpus callosum (Fig. 11F). The microglial activation in the corpus callosum was detectable as early as 2 days, statistically significant by 4 days, and undiminished out to 49 days at approximately 5- to 6-fold higher than control levels (Fig. 11F). The staining was statistically significantly elevated to 6-fold higher than control at 7 days.

Thus, the time course of microglial activation was similar to that of silver staining abnormalities in the corpus callosum (Fig. 11E vs F).
Microglial activation was more transient in the gray matter regions (Figs. 11B, D, H, J). Increases in Iba1-immunoreactive activated microglia were detectible as early as 2 days and were statistically significant in all regions examined by 4 days (Figs. 11B, D, H, J). The peak of the microglial response occurred at 7 days in the cortex (Fig. 11B), 28 days in the thalamus (Fig. 11D), and 4 days in the hippocampus (Figs. 11H, J). In the cortex, the peak was more than 8-fold higher than in control mice (p = 0.00005; Fig. 11B). In the contralateral hippocampal CA1, there was a sharp spike as evidenced by an 18-fold elevation at 4 days (p = 0.0002; Fig. 11J). In the thalamus, the delayed rise reached a 15-fold increase (p = 0.0001; Fig. 11D). In the ipsilateral dentate gyrus, the elevations ranged from 13- to 17-fold (p = 0.02 to 0.003; Fig. 11H). By 49 days after injury, the numbers of Iba1-immunoreactive activated microglia were not statistically different from control in any of the gray matter regions. At days 2 to 7 after injury, Iba1-immunoreactive activated microglia in

**Figure 9.** Iba1 immunohistochemistry indicates multifocal microglial activation after 2 closed-skull impacts (TBI-TBI). (A) Iba1 immunoreactivity in a TBI-TBI mouse 7 days after the first impact is extensive in the ipsilateral cortex, hippocampus, and thalamus, the corpus callosum and the contralateral hippocampus. (B, C) There is markedly less prominent Iba1 immunoreactivity in TBI-Sham and Sham-Sham mice.

**Figure 10.** High-power views of the distribution and morphology of Iba1-immunoreactive cells after 2 closed-skull impacts (TBI-TBI). (A) There are uniformly distributed Iba1-positive microglia in the ipsilateral cortex at 7 days after the first of 2 impacts. (B) Ramified morphology (resting) morphology. (C) Hypertrophic morphology (activated). (D) Bushy morphology (activated). (E) Ameboid morphology (activated). Scale bar = 50 μm in B applies also to C to E.
the cortex were typically uniformly distributed. However, at 14 to 49 days after injury, the activated microglia tended to be clustered together in cortical layers 2 and 5 (not shown). Thus, in the gray matter regions examined, microglial activation became statistically significant at earlier times than silver staining abnormalities (Fig. 11A vs B, C vs D, G vs H, I vs J).

There were both greater silver staining (Fig. 12A) and more Iba1-immunoreactive activated microglia (Fig. 12B) in the TBI-TBI mice than in TBI-Sham and Sham-Sham mice. The distributions of data sets passed Shapiro-Wilk normality tests; therefore, parametric statistics were performed for primary analyses, but the results were not substantially changed when nonparametric Mann-Whitney U tests were performed. There was a moderately strong positive correlation between the extent of silver staining and the number of Iba1-immunoreactive activated microglia in individual mice. Across all 3 experimental groups, the mice with more extensive silver staining also had more numerous Iba1-immunoreactive activated microglia (Fig. 12C; Spearman $r = 0.64, p = 0.0018$). Taken together, these findings indicate that the silver staining and microglial reactivity were closely related but not completely parallel processes.

**Recovery of Behavioral Performance at 7 Weeks in TBI-TBI Mice**

Morris water maze performance had improved but did not completely return to normal at 7 weeks in the mice subjected to 2 impacts (Fig. 13). Specifically, mean time to reach the hidden platform was longer in the TBI-TBI versus Sham-Sham mice (Figs. 13A, B; p = 0.01). In contrast, distance to the hidden platform (Fig. 13C; p = 0.08), swim speed (Fig. 13D), probe trial performance (Fig. 13E), and visible platform performance (Figs. 13F–H) were not different between groups.

**DISCUSSION**

We found that repetitive closed-skull TBI in mice causes behavioral performance deficits, extensive argyrophilic abnormalities indicative of multifocal axonal injury, ultrastructural evidence of axonal injury, and reactive microgliosis. The use of silver staining, electron microscopy, and immunohistochemistry for microglia revealed these abnormalities in brains that appeared largely uninjured on gross examination and by conventional histology. Even APP immunohistochemistry, which is felt to be a sensitive marker of traumatic axonal injury, revealed only subtle abnormalities in the TBI-TBI mice. This may be because many of the injured axons were not extensively swollen and therefore not readily distinguishable at the light microscopic level. Our findings suggest that standard histologic assessments may not be sufficiently sensitive to detect structural injuries after repetitive mild TBI. However, this study does not represent a comprehensive assessment of the differential sensitivity of the various methods for assessing structural brain injury. The persistence of abnormal silver staining and microglial activation in white matter as late as 7 weeks after TBI indicates that the effects of repetitive mild TBI can be long-lasting.

Several limitations of the current study should be noted. First, the injuries were produced by direct impact with the skull of anesthetized mice. In contrast, many human concussive injuries involve rotational acceleration of the head. Nonetheless, the fundamental responses of the brain to trauma may be similar. Injured axons with activated microglia in close proximity have been described in a pig rotational injury model with features consistent with concussive TBI (28). In a primate model involving controlled rotational acceleration, axonal injury and white matter microglial clusters were observed in more severe injuries, but not in those that caused “concussion,” defined as unconsciousness for less than 15 minutes (29). However, the effects of repeated injury were not assessed, and sensitive methods for the detection of axonal injury or microglial activation were not used.

Second, we have not systematically assessed the effects of varying the interval between impacts. Longhi et al (17) found that behavioral impairments were present with 3- and 5-day intervals but not a 7-day interval between impacts. The mechanisms underlying the period of vulnerability after a first impact are of great interest because interventions that shorten this period might improve outcomes in persons at high risk of recurrent injury such as military personnel and athletes. Biochemical and electron microscopic analysis of axonal integrity at early times after initial injury (30) represent an important area for future investigation.

Third, the full time-course of the behavioral deficits in this model has not been determined. It is also possible that a Sham-TBI group could have shown impairments not seen in our TBI-Sham control group. We observed partial normalization of behavioral performance by 7 weeks after 2 impacts. At this time point, the abnormal silver staining and microglial activation in gray matter had largely resolved, whereas these abnormalities were persistent in the corpus callosum. These findings suggest, but do not prove, that both abnormalities in gray matter structures and white matter structure contribute to these behavioral deficits. To assess the consequences of the axonal injury and microglial activation in white matter, additional behavioral or electrophysiological assessments may be required (31). Although white matter is a quantitatively small part of the mouse brain, it is a much larger fraction of the brain’s surface area. Involvement of white matter in axonal injury may be of particular concern, given that white matter tracts constitute 70% of the brain at birth and have been shown to be highly susceptible to injury in newborns. White matter injuries have been described in a pig rotational injury model with features consistent with concussive TBI (28). In a primate model involving controlled rotational acceleration, axonal injury and white matter microglial clusters were observed in more severe injuries, but not in those that caused “concussion,” defined as unconsciousness for less than 15 minutes (29). However, the effects of repeated injury were not assessed, and sensitive methods for the detection of axonal injury or microglial activation were not used.

Finally, we have not fully investigated the possibility of subtle neuronal degeneration in this model. Longhi et al (17) reported Fluo Jade–stained cells and loss of MAP2 immunoreactivity in a similar experimental model. Our preliminary data reveal similar findings (not shown). Indeed, the silver staining abnormalities and Iba1-immunoreactive microglia were present in gray as well as in white matter structures.

The precise origins of the specific spatial distribution of silver staining and microglial activation we observed are not known. Regions of primary injury may be those for which mechanical forces are highest. In addition, regions of secondary injury may occur in response to delayed pathophysiological events. The thalamic injury seemed to involve the lateral geniculate nucleus and ventroposterior nuclei, areas related to the primary visual cortex and somatosensory cortex, respectively.
The primary visual cortex and somatosensory cortex, in turn, were directly beneath the impact site (24). Thus, the thalamic abnormalities could be a secondary response to cortical or subcortical white matter injury. Likewise, the abnormalities in the contralateral hippocampal stratum radiatum and stratum oriens may be related to the connections of this region to the injured ipsilateral dentate gyrus or entorhinal cortex. Alternatively, biomechanical vulnerability of these ipsilateral thalamic and contralateral hippocampal regions could underlie the pattern of abnormalities observed.

There are several possibilities regarding the relationship between the axonal injury and activated microglia. We favor the hypothesis that the Iba1-immunoreactive cells with activated microglial morphology represent a response to primary traumatic axonal injury. It is also possible that the axonal injury is secondary to toxic factors secreted by the activated microglia or that both the axonal injury and activated microglia are responses to some additional unknown pathophysiological factor. These possibilities are not mutually exclusive. Although the microglial activation became statistically significant before the silver staining abnormalities, the descriptions of time courses are not sufficient to resolve this issue. Direct experimental manipulations of microglial responses will be required to address these possibilities.

The prominent and persistent microglial activation after repetitive closed-skull TBI may lead to advances in diagnostic strategies. For example, positron emission tomography or single-photon emission computed tomography ligands that bind to activated microglia may be useful to assess immunologic response to brain injury in a noninvasive fashion. The 5- to 18-fold increases in microglial activation suggest that a high signal-to-noise ratio should be achievable with this approach. Microglial-targeted ligands such as $^{[11]}$C DAA1106, $^{[11]}$C PK11195, $^{[18]}$F FEPPA, or others (38) could be tested in the setting of concussive TBI. Although microglial responses in isolation are unlikely to be specific for TBI, their spatial distribution and time course in relation to the clinical history might be useful for diagnostic and prognostic purposes.

On the therapeutic front, modulation of microglial activation or specific microglial signaling could be tested after experimental TBI. However, it is not known whether persistent microglial activation is beneficial, harmful, or neutral on the whole in this context. We recognize that morphologic evidence of microglial activation may fail to account for functional differences among subsets of microglia (39). Pharmacological and genetic manipulations of specific aspects of microglial signaling (40) after repetitive closed-skull TBI will be required to address this issue and identify targets for candidate therapeutics. It is unlikely that global immune suppression will be a net benefit (41). However, microglial inhibition with minocycline has been shown to improve outcomes in several experimental injury models (42, 43).

FIGURE 11. Quantitative analysis of silver staining intensity and Iba1-immunoreactive activated microglial cell counts. Separate groups of 5 to 8 mice were killed at each time point. Silver staining was quantified using densitometric analysis (arbitrary units, range 0–255); Iba1-positive cells with activated microglial morphology were counted using stereological methods. Dashed lines indicated levels in Sham-Sham mice. (A, B) Ipsilateral cortex. (C, D) Ipsilateral thalamus. (E, F) Corpus callosum. (G, H) Ipsilateral dentate gyrus. (I, J) Contralateral hippocampal stratum oriens and stratum radiatum (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$, Dunnett post hoc test compared with Sham-Sham levels).

FIGURE 12. Relationship between silver staining intensity and Iba1 immunoreactive, activated microglial cell counts. (A) Silver staining intensity in ipsilateral cortex 7 days after 2 closed-skull impacts (TBI-TBI), traumatic brain injury (TBI)–Sham, or Sham-Sham procedures (*, $p = 0.007$; **, $p = 0.0019$, unpaired 2-sided $t$ tests). (B) Iba1 immunoreactive, activated microglial cell counts in the same ipsilateral cortical region from the same mice (*, $p = 0.066$; **, $p = 0.0013$, unpaired 2-sided $t$ tests). (C) Quantitative correlation between silver staining intensity and Iba1-immunoreactive, activated microglial cell counts. Scatter plot of silver staining intensity versus Iba1-immunoreactive activated microglia cell counts in ipsilateral cortex at 7 days. Each symbol represents 1 mouse. Spearman $r = 0.64$, $p = 0.0018$. 

The primary visual cortex and somatosensory cortex, in turn, were directly beneath the impact site (24). Thus, the thalamic abnormalities could be a secondary response to cortical or subcortical white matter injury. Likewise, the abnormalities in the contralateral hippocampal stratum radiatum and stratum oriens may be related to the connections of this region to the injured ipsilateral dentate gyrus or entorhinal cortex. Alternatively, biomechanical vulnerability of these ipsilateral thalamic and contralateral hippocampal regions could underlie the pattern of abnormalities observed.
Microglial activation and traumatic axonal injury have previously been described in relatively mild concussive human TBI with death from other causes. Multifocal clusters of reactive microglia were found in a case of concussion with 13-day survival (44). These were present in the white matter, thalamus, and brainstem. Likewise, APP immunohistochemical evidence for axonal injury has been reported in several cases of mild TBI (45, 46). Our findings using more sensitive methods raise the intriguing hypothesis that axonal injury and microglial activation may be much more widespread and intense than previously believed. Other experimental animal studies using sensitive silver staining support this possibility (47-49). Further characterization of human pathological material from patients who have sustained both single and repetitive concussive brain injuries using the sensitive methods described here may help to address this hypothesis. The axonal pathology and sustained activation of microglia for many days to weeks after injury might underlie some of the persistent symptoms (e.g. fatigue, cognitive impairment, mood instability) that follow mild brain injuries in a subset of patients. Future investigations into the role of sex, age, genetic factors such as ApoE, and post-TBI care will be of great interest.

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REFERENCES


29. Kim HS, Suh YH. Minocycline and neurodegenerative diseases. Behav Brain Res 2009;196:168–79


