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TEMPORAL WINDOW OF VULNERABILITY TO REPETITIVE EXPERIMENTAL CONCUSSIVE BRAIN INJURY

OBJECTIVE: Repetitive concussive brain injury (CBI) is associated with cognitive alterations and increased risk of neurodegenerative disease.

METHODS: To evaluate the temporal window during which the concussed brain remains vulnerable to a second concussion, anesthetized mice were subjected to either sham injury or single or repetitive CBI (either 3, 5, or 7 days apart) using a clinically relevant model of CBI. Cognitive, vestibular, and sensorimotor function (balance and coordination) were evaluated, and postmortem histological analyses were performed to detect neuronal degeneration, cytoskeletal proteolysis, and axonal injury.

RESULTS: No cognitive deficits were observed in sham-injured animals or those concussed once. Mice subjected to a second concussion within 3 or 5 days exhibited significantly impaired cognitive function compared with either sham-injured animals ($P < 0.05$) or mice receiving a single concussion ($P < 0.01$). No cognitive deficits were observed when the interconcussion interval was extended to 7 days, suggestive of a transient vulnerability of the brain during the first 5 days after an initial concussion. Although all concussed mice showed transient motor deficits, vestibulomotor dysfunction was more pronounced in the group that sustained two concussions 3 days apart ($P < 0.01$ compared with all other groups). Although scattered degenerating neurons, evidence of cytoskeletal damage, and axonal injury were detected in selective brain regions between 72 hours and 1 week after injury in all animals sustaining a single concussion, the occurrence of a second concussion 3 days later resulted in significantly greater traumatic axonal injury ($P < 0.05$) than that resulting from a single CBI.

CONCLUSION: These data suggest that a single concussion is associated with behavioral dysfunction and subcellular alterations that may contribute to a transiently vulnerable state during which a second concussion within 3 to 5 days can lead to exacerbated and more prolonged axonal damage and greater behavioral dysfunction.

KEY WORDS: Axonal injury, Cognition, Concussion, Microtubule-associated protein-2, Repetitive brain injury

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In the United States, every year, more than 2 million people sustain a traumatic brain injury (TBI), principally as a result of motor vehicle accidents, falls, violence, and sports-related injuries (43). There are approximately 300,000 hospital admissions annually for patients with mild to moderate TBI, and an additional unknown number of mild brain injury cases involving concussion are not reported but may result in long-term disability (43). Concussion is defined clinically as a trauma-induced complex pathophysiological process affecting the brain that typically results in rapid onset of short-lived neurological dysfunction and a graded set of clinical symptoms that may or may not involve loss of consciousness (14, 27, 50) and is commonly associated with contact sports, with an estimated annual incidence of 300,000 cases (29), 63,000 of which occur among high school athletes (49). Recent studies suggest that athletes with a history of previous concussions are significantly more likely to have a second concussion within 7 to 10 days of the first injury (20). A published series of studies conducted under the auspices of the American National

Football League offer significant new insights into the incidence and mechanisms of sports-related concussion, including a primary relationship with rotational acceleration and neurological systems, including impaired recall (25%), retrograde amnesia (18%), and prolonged difficulty with information processing (17%). A total of 92% of concussed players returned to practice in less than 7 days (44–46).

Laboratory studies have suggested that concussive brain injury (CBI) may be characterized by transient functional impairments and may be associated with specific pathogenic events, including ionic dyshomeostasis, excitatory amino acid release, and alterations in regional cerebral blood flow and metabolism (23, 63). Any of these events may lead to a state of enhanced vulnerability in which a secondary insult or a second traumatic event may exacerbate the damage (17, 23, 26, 63). Although clinical symptoms of concussions such as confusion, amnesia, headache, disorientation, attention deficits, speech alteration, and lack of coordination are often transient, mild CBI may also result in a more prolonged "postconcussive syndrome" characterized by persistent alterations in cognition, emotional functioning, and behavior, which can affect interpersonal relationships, school, and work (3, 9, 30, 34, 35, 37, 38).

Repetitive CBI in the setting of contact sports is believed to be associated with at least two devastating complications: 1) a serious condition in which an increase in brain vulnerability to a second impact may be followed by vasoparalysis, brain swelling, subdural hematoma, increased intracranial pressure, and occasionally death (3, 5, 7, 8) and/or 2) chronic cognitive impairments as seen in boxers and hockey and football players, associated with accelerated and/or increased neurodegeneration in specific brain regions (1, 3, 5, 7, 9, 12, 19, 30, 37, 38, 51, 53). Specific guidelines have recently been developed to determine when athletes who have sustained CBI can be safely reintroduced into their sport, based on the severity of the concussion and the number of concussions experienced by the individual (6, 50). However, reproducible laboratory evidence is lacking regarding the duration of the vulnerability of the brain after a single concussion (19). In the present study, vulnerability to injury was defined as enhanced neurobehavioral susceptibility to the effects of a second concussion.

Animal models of TBI, designed to recapitulate many aspects of human head injury, have been used during the past two decades to elucidate the secondary processes leading to acute and delayed neuronal death and morbidity/mortality after brain injury (31). Our laboratory has recently documented the cumulative effects of repetitive CBI using an experimental model of concussion in the mouse, in which animals remain unconscious and lose their righting reflexes for 2 minutes or less. In a previous study, two concussions, 24 hours apart, were associated with no overt histological damage but long-term microscopic evidence of axonal injury and alterations in motor tasks requiring the coordinated integration of sensorimotor function (32). In the present study, we evaluated the effects of varying the interval between two mild concussions on cognitive function, motor function, and histological damage to investigate the vulnerability of the mildly injured brain to a second concussive insult.

MATERIALS AND METHODS

Experimental Animals

All procedures were conducted in strict accordance with the National Institutes of Health publication *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Before beginning any procedures, the mice (male, C57BL/6, 6–8 wk old) were housed for at least 1 week in their home cages, with a 12-hour light-dark cycle and ad libitum access to food and water. A total of 131 mice were used.

Surgical Procedure

Mice (n = 131) were anesthetized with inhalation anesthesia via a nose cone using 2% isoflurane and placed in a modified stereotactic frame. An eye lubricant ointment (Duratears Naturale; Alcon Laboratories, Fort Worth, TX) was applied to protect the corneal membrane during surgery. A midline scalp incision was made, and the skull was exposed. After disconnection from the nose cone and immediately after a positive response to a tail pinch had been observed, one group of mice (n = 111) was subjected to CBI using a modification of the controlled cortical impact device as described below. Sham-injured mice (n = 20) were subjected to the same procedures without receiving the injury. A subset of animals subjected to CBI received a second concussive injury at either 3 (n = 53), 5 (n = 12), or 7 (n = 12) days. Subsets of the 3-day group were used for different behavioral and histological evaluations. To control for the effects of repeated doses of anesthesia, equal numbers of mice received the same number of exposures to isoflurane anesthesia at either 3, 5, or 7 days. Mice subjected to a single CBI were also subjected to repeat anesthesia and sham surgery to control for these variables.

To produce a concussive injury that better recapitulates the features of a diffuse injury, one of the authors (DFM) designed a replacement for the standard impactor tip (32) with a less rigid, larger-diameter (9 mm) silicone impactor. The use of a larger, more compliant tip provided a method to distribute impact energy over a larger surface of the brain while simultaneously deforming more of the brain, although it made comparisons with earlier studies more difficult. The impactor used to produce CBI was a silicone-covered impactor driven by a pneumatic piston, which was rigidly mounted at an angle of 20 degrees from the vertical plane and driven perpendicularly onto the exposed left parietal bone between bregma and lambda. A metric template was used to locate the impact site directly between lambda and bregma to ensure uniformity of the injury site between animals. The zero point was obtained by lowering the impactor tip until it touched the left parietal bone, midway between bregma and lambda. The impounder was driven at 4.8 to 5.0 m/s to a depth of 3 mm farther than

the zero point, causing a nonpenetrating concussive blow to the head with no skull fracture. A linear velocity displacement transducer (Model ATC-101; Schaevitz, Pennsauken, NJ) was used to produce an analog signal for verification of the impact parameters. Immediately after the CBI, the righting reflex was evaluated by measuring the time required for mice to switch to their normal posture over three consecutive attempts when placed in the supine position (10). The scalp incision was then closed with a 4-0 silk suture. During surgery and recovery, the mice were placed on a heating pad, and core body temperature was maintained at 37°C. We have previously shown that this injury paradigm is not associated with any hemodynamic alterations (32).

Assessment of Cognitive Function

The cognitive function after sham injury, single CBI, and repetitive CBI was evaluated by use of the Morris water maze (MWM). The MWM paradigm is routinely used as a sensitive measure of posttraumatic spatial learning and memory in rodents (32, 56, 59). The MWM is a white circular pool (1 m in diameter) filled with water (18–20°C) made opaque by use of nontoxic, water-soluble white paint. To examine postinjury visuospatial learning, the animals were tested by use of eight trials per day for 3 consecutive days, for a total of 24 trials. After being placed at one of four sites in the pool, the mice used external cues to learn how to locate a submerged platform placed 0.5 cm under the surface of the water. In the majority of the mice (n = 80), learning was assessed starting on the day after the second surgery/injury. In one subgroup of mice (n = 16) subjected to two concussive brain injuries 3 days apart (the interval producing the greatest cognitive alteration), learning was evaluated beginning at 7 days after the second injury to evaluate how long the induced deficit persisted after repetitive brain injury.

Assessment of Motor Function

One day after the cognitive evaluation, animals were tested using the rotarod motor function test, a reliable indicator of deficits resulting from abnormalities in pathways responsible for integrated vestibulomotor and sensorimotor function (22). Motor function was evaluated by measuring the latency during which the animals remained on a 36-mm-diameter rod covered by a rubber surface. After one acclimatization session on the rotarod apparatus, mice received four trials at 5-minute intervals. The latency (in seconds) during which the animals remained on the rod, rotating with an initial velocity of 1 cm/s and an acceleration of 1.75 rpm/s, was measured. Each trial was terminated when the animal fell completely off the rod (onto a well-padded bed) or gripped the rod and spun around one complete revolution. The highest and the lowest latencies were eliminated, and the average of the two remaining latencies was used for statistical analyses according to previously published studies (32, 52).

Histological Analysis

To evaluate the acute histological sequelae of single and repetitive CBI, mice receiving a single CBI were killed at 3 days (n = 14) and 7 days (n = 6) after injury, whereas mice receiving repetitive CBI with a 3-day interval (n = 14) were killed at 3 days after injury. Anesthetized mice (sodium pentobarbital intraperitoneally, 65 mg/kg) were perfused transcardially with heparinized saline, followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The brains were postfixed overnight at 4°C, cryoprotected with 30% sucrose, and snap-frozen. Serial coronal sections (40 μm) were cut along the entire brain.

Neuronal Degeneration

Fluoro-Jade (Histochem, Jefferson, AR), an acidic dye that exhibits a marked affinity for both the degenerating neuronal cell body and its processes (57), has been shown to be an effective indicator of neuronal degeneration after TBI (55). Four sections per animal were selected between bregma −1.1 and −2.0 mm (at every 200 μm), mounted onto gelatin-coated slides, and allowed to dry. The rostrocaudal coordinates from which the sections were chosen correspond to the region of the skull affected by the silicon tip. Sections were then hydrated through a series of graded ethanol solutions, immersed in 0.06% potassium permanganate for 5 minutes, incubated in 0.001% Fluoro-Jade solution for 30 minutes, rinsed, and coverslipped with DPX mounting medium (Sigma-Aldrich, St. Louis, MO).

Semiquantitative analysis of Fluoro-Jade staining was performed in specific regions of both hemispheres, including retrosplenial cortex, sensorimotor cortex, piriform cortex, hippocampus, thalamus, and hypothalamus at ×20 magnification as previously described (24). For each region, a score of 1 was given for the presence of one positive neuron, and a score of 2 was given for the presence of two or more positive neurons. Regional scores were summed for every section, and section scores were averaged to obtain a single score for each animal.

Traumatic Axonal Injury

Immunostaining for β-amyloid precursor protein (APP) has been shown to be a sensitive marker of traumatic axonal injury (58). Immunolabeling for β-APP was performed on sections randomly chosen from bregma −1.1 to −2.0 mm. After incubation in 0.5% H_2O_2/methanol for 30 minutes to quench endogenous peroxidases, antigen retrieval was performed by immersion in 0.1 mol/L citric acid/sodium citrate buffer (pH 6.0) and repeating 10 alternating cycles of microwaving on medium power for 1 minute and cooling for 3 minutes on ice to maintain a temperature of approximately 45°C. Sections were then allowed to equilibrate to room temperature, rinsed four times for 5 minutes in 0.1 mol/L Tris buffer solution (TBS), blocked in 5% normal horse serum (NHS)/TBS-T (0.1% Triton X-100) for 30 minutes, and incubated overnight (16–20 h) at 4°C in a 1:1500 dilution of rabbit anti-C-terminal APP (Zymed, San Francisco, CA) in 5% NHS/TBS-T. Sections were

rinsed and incubated for 60 minutes in biotinylated goat anti-rabbit immunoglobulin G (1:250; Jackson, West Grove, PA), followed by 60 minutes in avidin-biotin complex (1:1000 in TBS; Vector, Burlingame, CA). Reaction product was visualized by use of 3,3'-diaminobenzidine (Sigma).

Semiquantitative analysis of axonal injury was performed in the median subcortical white matter (the central part of the corpus callosum between the beginning of the two hippocampi) and then bilaterally in the temporoparietal cortex, subcortical white matter, hippocampi, thalami, hypothalamus, and median eminence (×20 magnification). For each region, a score of 1 was given for the presence of one β-APP-positive axon per field, and a score of 2 was given for the presence of two or more β-APP-positive axons. For every section, a score was obtained by summing all region scores. A score for each animal was obtained by averaging the scores of all the sections from that animal. This method was adopted from a previously published study and allows for a spatial quantification of β-APP accumulation in well-defined anatomic regions with a clear response, simplicity of scoring, reproducibility, and sensitivity (24).

Cytoskeletal Proteolysis

Reductions in immunoreactivity for microtubule-associated protein-2 (MAP-2) have been shown to be a sensitive indicator of cytoskeletal damage after TBI (48, 54). Four sections per animal, chosen between bregma −1.1 and −2.0 mm, were incubated for 30 minutes in 0.5% H_2O_2/methanol/TBS, rinsed in TBS, blocked in 5% NHS/TBS-T, and incubated overnight (16–20 h) in mouse anti-MAP-2 (1:500 clone AP20; Chemicon, Temecula, CA). Sections were then rinsed and incubated in biotinylated donkey anti-mouse immunoglobulin G (Jackson; 1:1000), rinsed, incubated for 1 hour in an avidin-biotin complex (Vector; 1:1000), and reacted by use of 3,3'-diaminobenzidine (Sigma).

Semiquantitative analysis of cytoskeletal damage was performed in the retrosplenial cortex, sensorimotor cortex, piriform cortex, hippocampus, thalamus, and hypothalamus in both hemispheres at ×20 magnification. For each region, a score of 1 was given for partial loss of staining, and a score of 2 was given for a complete loss of staining (in at least a part of the region). For every section, a score was obtained by summing all region scores. A score for each animal was obtained by averaging the scores of all the sections from that animal.

Brain Water Content

We have previously shown that repetitive CBI occurring with a 24-hour interval is associated with an increase in blood-brain barrier permeability, leading to the development of regional cerebral edema (32). Both clinical and experimental studies suggest that edema contributes significantly to an increase in brain volume after brain injury, and it has been shown after experimental TBI that edema is maximal at 48 hours after injury (25, 36). Mice (sham, n = 6; CBI, n = 8) were killed 72 hours after the first injury (to verify whether, at the moment in which the brain was maximally vulnerable to a second concussion, there was an increase in brain water content) or 48 hours after the second injury, when we were expecting the maximal increase in edema (repetitive CBI, 3-day interval, n = 8) for analysis of brain water content. After anesthesia with sodium pentobarbital (65 mg/kg intraperitoneally), mice were decapitated, and their brains were rapidly removed. A coronal slice (4 mm) was removed from the occipitoparietal level, and the two hemispheres were divided on a cold plate. The fresh tissue was weighed on aluminum foil, dried for 24 hours at 100°C, and then reweighed. Brain water content was defined (4, 60) as % of water = [(wet weight − dry weight)/wet weight] × 100.

Statistical Analysis

Righting reflex times, learning latencies, latencies measured during the rotarod test, and water content are presented as mean ± standard error of the mean, and the comparisons among groups were performed by use of an analysis of variance (ANOVA) followed by Newman-Keuls post hoc tests. Groups of sham-injured mice were pooled for statistical analysis. Histological damage scores are nonparametric data and were compared by use of a Kruskal-Wallis ANOVA followed by Dunnett's *t* test. In all the comparisons, a probability value less than 0.05 was considered statistically significant.

RESULTS

Duration of Unconsciousness/Seizures

The return of righting reflex (duration of unconsciousness) after either the initial CBI (128 ± 7 s) or a second repetitive CBI (150 ± 11 s) was significantly longer than that of the sham-injured mice (6 ± 0.3 s, $P < 0.001$), although the duration of unconsciousness after a second concussive injury was not significantly different from that observed after a single CBI. Moreover, the interinjury interval was found to have no effect on the duration of unconsciousness (ANOVA, $P = 0.3$). Among 111 mice subjected to single CBI, brief apnea (<30 s) occurred in only 3 mice, whereas seizures were observed in 32% of mice. In the group subjected to repetitive CBI, the incidence of seizures was doubled (62%) for those animals in which seizures were observed after the initial concussion (33%). Two mice died immediately after the second injury, and a subdural hematoma was observed in each of these animals.

Cognitive Function

All animals were able to swim in the MWM without notable physical impairment and demonstrated an ability to learn the visuospatial task, as reflected by decreasing latencies to find the platform over the 3-day period. Cognitive function was evaluated beginning on the day after the injury. The task consisted of eight trials per day for 3 consecutive days for a total of 24 trials, and the cognitive score of each animal was determined by averaging the 24 trials over the 3-day testing

period. Mice subjected to repetitive CBI with 3- and 5-day intervals showed consistent behavioral impairment. The average latency to locate the platform in the MWM after a single CBI (26 ± 1.6 s) was similar to the average latency of sham-injured mice (28 ± 0.9 s), indicating that a single CBI was not associated with cognitive impairment (*Fig. 1A*). Although the mean latency of mice subjected to repetitive CBI with a 7-day interval between concussions (30 ± 1.5 s) was not different from the latency of the mice subjected to single CBI (*Fig. 1A*), the mean latency of the mice subjected to repetitive CBI with a 3-day interval between concussions was significantly longer (36 ± 2 s) than the latency of either the sham group or the single CBI group ($P < 0.05$ and $P < 0.01$, respectively) (*Fig. 1A*). The mean latency of the mice subjected to repetitive CBI with a 5-day interval (34 ± 3 s) was also significantly longer than the latency of the mice subjected to single CBI ($P < 0.05$).

An additional subgroup of mice was subjected to repetitive CBI with a 3-day interval between the two concussions and tested at 7 days after the second injury to investigate the duration of the cognitive deficit. At 1 week after the second concussion, the average latency of these mice (31 ± 1.6 s) remained significantly longer than the latency of the sham-injured and the single mild CBI groups ($P < 0.05$), indicating that the cognitive deficit induced by repetitive CBI with a 3-day interval between concussions persisted for at least 1 week (*Fig. 1B*).

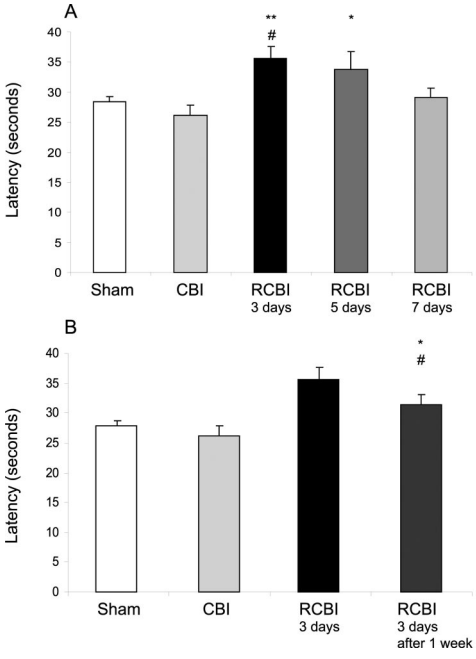

FIGURE 1. *Bar graphs showing effect of single and repetitive concussion on learning performance of mice in the MWM. Testing began at 1 day after the surgery/injury.* A, *animals subjected to repetitive CBI (RCBI) with an interval of 3 or 5 days between the two concussions exhibited significant learning impairment compared with sham-injured mice and mice receiving a single CBI. However, when the interval between the two injuries was 7 days, mice subjected to repetitive CBI showed no significant deficit.* B, *mice subjected to RCBI with an interval of 3 days when tested beginning 7 days after the second concussion performed significantly worse than sham and single CBI groups. #, P < 0.05 compared with sham; *, P < 0.05 compared with single CBI; **, P < 0.01 compared with single CBI. Data are presented as mean ± standard error of the mean.*

Neurological Motor Function

On the day after cognitive testing, all mice were evaluated for neurological motor deficits using the rotarod test of vestibulomotor function. All groups of brain-injured mice exhibited shorter latencies (poorer performance) than the sham-injured mice (12.4 ± 0.5 s). Animals subjected to repetitive CBI with a 3-day interval between concussions showed significantly greater motor deficits (latency to fall off rod = 7.9 ± 0.2 s) than the mice subjected to a single CBI (latency to fall off rod = 10.1 ± 0.4 s, $P < 0.01$). Mice subjected to repetitive CBI with a 5-day interval (latency = 10.6 ± 0.4 s) or 7-day interval (latency = 10.1 ± 0.3 s) between concussions had motor function scores that were similar to those of mice subjected to single CBI, indicating that with respect to vestibulomotor function, the concussed brain is maximally vulnerable to a second insult within the first 3 days (*Fig. 2A*). When animals subjected to repetitive CBI (3-day interval) were tested at 10 days after the second injury, the repetitive CBI-induced neurological motor deficit was equivalent to that exhibited after a single CBI (*Fig. 2B*).

Neuronal Degeneration

Focal lesions such as hematomas or contusions were not visible during gross observation of the brain surface or during microscopic evaluation of brain coronal sections. Degenerating (Fluoro-Jade-positive) neurons were not observed in any of the sham animals. In contrast, extensive Fluoro-Jade-positive neurons were observed in 13 of 14 animals killed within a week after single or repetitive CBI. In the repetitive CBI group, Fluoro-Jade staining was performed only in the 3-day interval group, i.e., the one showing the greatest behavioral impairment. Degenerating neurons were most abundant in the ipsilateral cortex (*Fig. 3*) and less so in the hypothalamus, hippocampus, and thalamus. No differences were found when comparing the semiquantitative scores for Fluoro-Jade in the single and repetitive CBI groups (data not shown), indicating that even a mild single CBI can lead to a scattered neuronal degeneration in several key brain regions.

Traumatic Axonal Injury

None of the sham-injured animals showed signs of pathological accumulation of β-APP in axons. Animals subjected to both single and repetitive CBI exhibited APP-positive axons in the corpus callosum, hippocampus, thalamus, hypothalamus, and median eminence of both hemispheres at 3 and 7 days after injury (*Fig. 4A*). Semiquantitative analysis revealed that animals subjected to repetitive CBI with a 3-day interval between concussions had significantly more axonal injury (in terms of more regions of the brain containing β-APP-positive

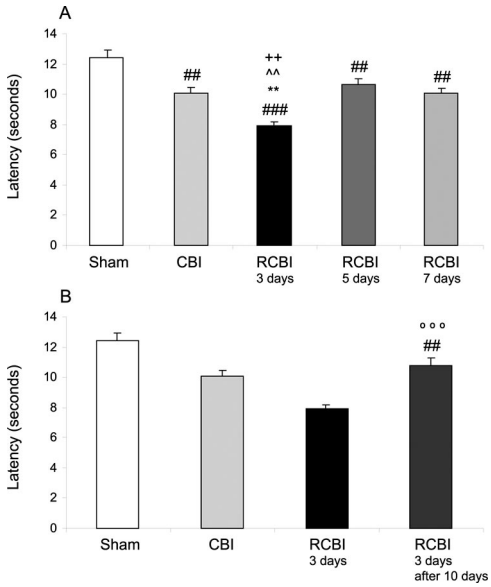

FIGURE 2. *Bar graphs showing effects of single and repetitive concussion on rotarod performances of mice.* A, *sham-injured mice showed longer latencies (better performance) compared with all the injured groups. When evaluated at 4 days after their final injury, mice receiving the repetitive CBI (RCBI) with an interval of 3 days exhibited impaired motor function compared with mice receiving single CBI or RCBI with 5- or 7-day intervals.* B, *when the mice subjected to repetitive CBI with an interval of 3 days were tested on day 10 after injury, their performance was worse than the performance of the sham-injured mice but was equivalent to that of mice subjected to a single CBI.* ##, $P < 0.01$ *compared with sham;* ###, $P < 0.001$ *compared with sham;* **, $P < 0.01$ *compared with CBI;* ^^, $P < 0.01$ *compared with RCBI 5 days;* ++, $P < 0.01$ *compared with RCBI 7 days;* °°°, $P < 0.001$ *compared with repetitive CBI 3 days. Data are presented as mean ± standard error of the mean.*

axons and in terms of more β-APP-positive axons in the same region of the brain) than animals receiving a single concussion when evaluated at 3 or 7 ($P < 0.05$) days after injury (*Fig. 4B*). These data suggest that mild CBI leads to diffuse axonal abnormalities and furthermore, that this damage may be exacerbated if the occurrence of the second concussion is within 3 days.

Microtubule-Associated Protein-2

No regional loss of MAP-2 immunoreactivity was detected in animals subjected to sham injury. Animals subjected either to single or repetitive CBI (3-day interval) showed loss of MAP-2 immunostaining in the retrosplenial, somatosensory, and piriform cortices, as well as in the hypothalamus, during the first week after injury that was consistent in magnitude and location among all brain-injured animals. The regional loss of MAP-2 immunolabeling occurred in the absence of overt cell loss evaluated by use of the Nissl staining method (*Fig. 5*). No differences were observed in the semiquantitative evaluation of MAP-2 loss between the single and the repetitive CBI groups (data not shown), suggesting that repetitive CBI does not exacerbate somadendritic cytoskeletal damage observed after single mild concussion.

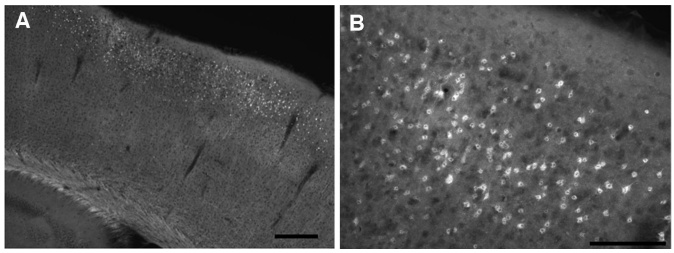

FIGURE 3. *Photomicrographs showing cortical neuronal degeneration detected by use of Fluoro-Jade staining. Neither single nor repetitive CBI was associated with focal lesions such as cerebral contusion, but scattered degenerating neurons were observed in several areas of the brain at 72 hours after repetitive CBI (3-d interval between injuries).* Scale bar = 250 μm *in* A, 100 μm *in* B.

Brain Water Content

The brain water content of the sham-injured animals (n = 6) was 78 ± 0.1% in the right hemisphere and 78 ± 0.2% in the left hemisphere. These values were not different from those values from mice subjected to single CBI (n = 8) and killed at 3 days after injury (77.9 ± 0.1% in the right hemisphere and 78.1 ± 0.1% in the left hemisphere), indicating that increased brain water content was not present during the time in which the mice showed the greatest vulnerability to the repetitive CBI. In addition, similar values were observed in mice receiving the repetitive CBI with a 3-day interval (n = 8) and were analyzed at 48 hours after the second injury (right hemisphere = 77.8 ± 0.1% and left hemisphere = 78.1 ± 0.1%), indicating that neither single CBI nor repetitive CBI with an interconcussion interval of 3 days was associated with increased regional cerebral edema.

DISCUSSION

The results of the present study demonstrate that after a single CBI, a state of transient vulnerability exists during which the occurrence of a second concussion within 3 to 5 days of the first leads to prolonged cognitive deficits, an exacerbation of neuromotor impairment, and increased axonal damage (for animals injured at a 3-d interval). Functionally, this vulnerability seems to be transient, and by 1 week after the first concussion, the occurrence of a second concussion does not produce additional cognitive and/or neurological motor alterations. Surprisingly, a single concussive injury was associated with profound histopathological changes, including neuronal degeneration, cytoskeletal proteolysis, and axonal injury. Perhaps more importantly, the extent of traumatic axonal injury seemed to be profoundly exacerbated by a second concussion if it occurred within 3 days of the first. Although these data are striking, care must be taken to extrapolate the temporal course of these changes from mice directly to humans.

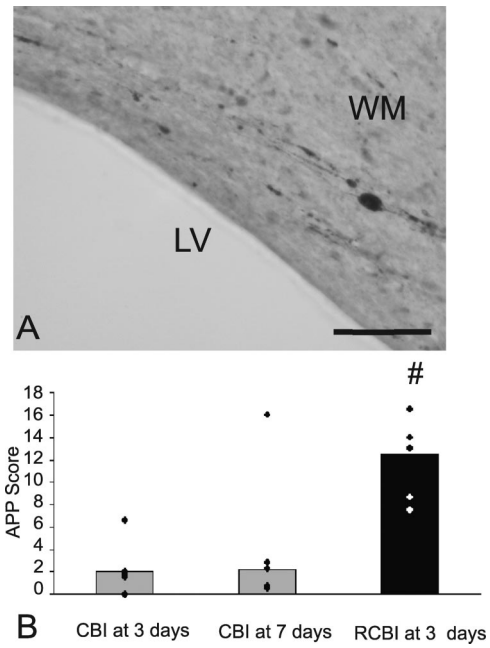

FIGURE 4. A, *photomicrograph showing that mild CBI resulted in axonal injury that was observed bilaterally in several areas of the brain. Swollen β-APP-positive axons in the subcortical white matter* (WM) *in the proximity of the lateral ventricle* (LV) *in a single CBI mouse.* Scale bar = *50 μm.* B, *bar graph showing semiquantitative analysis of axonal injury revealed that animals subjected to repetitive CBI (with a 3-d interval) showed more β-APP-positive axons in more brain regions at 3 days after the second concussion than animals subjected to single CBI evaluated at either 3 or 7 days after injury.* #, $P < 0.05$ *compared with CBI mice killed at 3 and 7 days after injury.* Bars *represent median scores.*

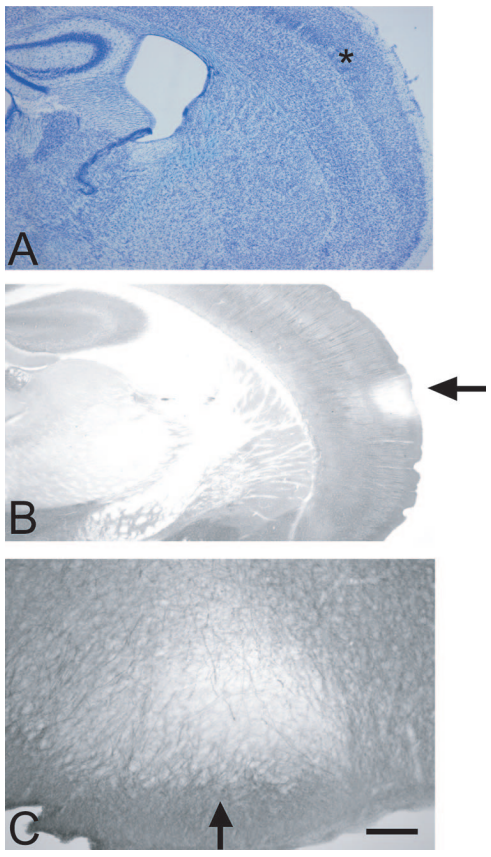

FIGURE 5. *Photomicrographs showing alterations in MAP-2 immunostaining after mild CBI. In the absence of cerebral contusion* (A, *, *Nissl staining), the same cortical region in an adjacent section shows a regional loss of MAP-2 immunostaining ipsilateral to the impact in a single CBI mouse at 72 hours after injury* (B, arrow). C, *MAP-2 loss in the piriform cortex in the cerebral hemisphere contralateral to the impact in a mouse subjected to repetitive CBI killed at 72 hours after injury (3-d interval).* Scale bar = *100 μm.*

In our study, seizures were observed in 32% of the mice after a single concussion, and the occurrence of seizures after an isolated concussion was a significant predisposing factor for further seizures associated with a second concussion. Occurrence of seizures did not affect the sensorimotor/vestibular function of the brain-injured animals (data not shown); however, animals subjected to repetitive CBI exhibiting two seizures had significantly impaired learning latencies (36 ± 1.7 s) compared with those exhibiting a single seizure (30 ± 0.9 s, $P < 0.05$). This cognitive impairment might be explained by additional damage as a result of excitotoxicity involving the hippocampus (64). In humans, concussive convulsions are generally believed to be nonepileptic phenomena that are immediate sequelae of a CBI (1, 14, 40). Electrophysiological recording has shown reproducible abnormalities in the postconcussive state in brain function (16). The pathophysiological mechanism of postconcussive seizures might involve a transient spreading depression with loss of cortical inhibition and disinhibition of brainstem activity (40). McCrory and Berkovic (41) reported that tonic posturing was noted in 25 of 102 concussed football players (associated clonic movements were observed in six athletes). Righting movements (defined as semipurposeful righting or attempts to return to an upright position in the setting of an athlete knocked unconscious) were observed in 40 of 102 players, and gait instability was observed in more than half. Previous studies of concussive seizures suggested that they are not associated with structural abnormalities, long-term neurological sequelae, or increased incidence of early or late posttraumatic epilepsy (42, 47, 61). Our studies suggest that the experimental subject who experiences seizures after a concussion is more likely to express more profound and prolonged seizures if a second concussion is experienced within 3 to 5 days of the first. Because our work was conducted with mice, it remains to be determined whether the same period of vulnerability exists for humans.

Animals subjected to a single CBI performed identically to sham (uninjured) animals in several learning tests, suggesting that a single concussion was not associated with any acute detectable impairment in learning ability. However, the occurrence of a second concussion 3 days after the first CBI did produce a significant learning deficit that persisted when the

mice were tested on days 7 through 9 after injury. This cognitive deficit was also observed when the interval between the two concussions was extended to 5 days but not 7 days, suggesting that the concussed brain remains vulnerable to a second concussion from 3 to 5 days with respect to a significant and prolonged worsening of cognitive function. This cognitive deficit was not caused by the vestibulomotor dysfunction observed, because swim speed and other motor function parameters assessed in the MWM were not adversely affected by injury. Using a weight-drop model of TBI in mice, DeFord et al. (15) showed that four mild impacts to the brain, produced at intervals of 24 hours, led to learning deficits at 3 days after the last impact compared with mice receiving a single injury, suggesting that there is a cumulative effect of repetitive injuries on learning ability. We have previously reported that mice subjected to repetitive CBI (interconcussion interval of 24 h) did not show evidence of a memory impairment at 1 week after injury or a learning deficit at 4 weeks after injury compared with uninjured controls or mice receiving a single injury (32). Our present finding of prolonged cognitive dysfunction with repetitive CBI at 3- or 5-day intervals may reflect differences in injury conditions (impactor material and shape), anesthesia, intervals between the two concussions, or cognitive function evaluation times.

Previous work has shown that the rotarod test of integrated sensorimotor/vestibular function is altered for up to 56 days after two mild brain injuries occurring 24 hours apart in mice (32). Balance testing in concussed athletes has shown similar impairments after injury, although these seem to resolve by 3 days after injury (21, 39), and the mechanism(s) underlying this dysfunction is unknown (1). In the present study, mice receiving a single CBI showed motor deficits that were significantly exacerbated by a second injury occurring 3 days after the first. Two concussions, occurring at intervals of 5 or 7 days, did not produce greater motor deficits than a single CBI. These data suggest that a critically vulnerable period for exacerbation of vestibulomotor deficits exists for up to 3 days after a single CBI in the mouse model.

Recent studies suggest that concussion can be associated with normal magnetic resonance neuroimaging findings and that the clinical picture is more likely to be related to functional rather than structural changes in the brain (1). However, newer magnetic resonance imaging modalities that have greater sensitivity for structured abnormalities have not been explored extensively in the study of concussion. Several studies using magnetic resonance imaging or postmortem analysis of patients revealed that mild TBI may be associated with long-term axonal injury (2, 11). Repetitive concussion in mice (with an interconcussion interval of 24 h) has been associated with axonal injury in the thalamus ipsilateral to the impact up to 4 weeks after injury (32). In the present study, traumatic axonal injury was observed in multiple brain regions as early as 3 and 7 days after a single CBI. After a second concussion sustained 3 days after the first, greater traumatic axonal injury was observed as early as 3 days after concussion bilaterally in the subcortical white matter, fimbria, hippocampus, and hypothalamus and in the median eminence. It has been hypothesized that the "postconcussive" cognitive disturbances observed in humans after CBI might be a result of axonal injury in areas of the brain associated with cognition (18, 28, 33). Interestingly, we observed bilateral axonal injury in the hippocampi of animals showing a learning deficit in the MWM only after the second concussion (3-day interconcussion interval) after CBI in the mouse model. Other indicators of subcellular pathological changes, including cytoskeletal damage (the loss of MAP-2 immunostaining), suggest that CBI is associated not only with axonal damage but also with injury to the soma and dendrites. Further work is in progress to address whether this loss of MAP-2 immunostaining during the first week after injury might be reversible. Although the use of Fluoro-Jade staining to specifically label degenerating neurons has recently been challenged (13), scattered neuronal degeneration was observed during the first week primarily in the cortex and hypothalamus after both single and repetitive CBI, suggesting that even an isolated concussion may initiate secondary processes leading to neuronal degeneration. On the basis of our experience with experimental models of mild TBI, we hypothesize that these acute degenerative, histopathological changes may persist during the weeks and months after repetitive CBI, and further work is certainly warranted to ascertain this possibility.

Two concussive injuries created 24 hours apart have previously been associated with an increase of blood-brain barrier permeability in the injured cortex and ipsilateral hippocampus (32). In the present study, no differences in regional brain water content were detected between uninjured control mice and mice subjected to either single or repetitive CBI, suggesting that increases in brain swelling were unlikely to be responsible for the observed behavioral deficits and histological damage.

In the field of contact sports, the cumulative behavioral effects of repetitive mild brain injury are well known (12, 38, 51), and clinical guidelines have been developed to try to establish the safe interval between a concussion and the subsequent return to play (1, 6, 50). To the best of our knowledge, ours is the first study to suggest that the temporal window of vulnerability of the injured brain to a second injury occurs within the first week in a mouse model of mild TBI. Although significant attempts have been made over the past decade to strengthen the clinical fidelity of experimental TBI models, including the one used here, the progression of postinjury pathophysiological sequelae may differ between mice and humans, and correlative human studies are certainly warranted before definitive clinical conclusions can be drawn. Although 7 days seems to be an acceptable interval for behavioral recovery from a mild TBI, one limitation of our study is that we did not compare the long-term histological sequelae of single and repetitive brain injury occurring at different time points. The consensus for the management and reintroduction of athletes who have sustained a concussion into sports activity relies on current guidelines suggesting that they should be asymptomatic and perform normally on neuropsychological

tests at rest and after exertion. However, it has been reported that some athletes may be allowed to return to contact sports while they remain symptomatic (62). Our data suggest that functional criteria alone are not sufficient to assess the potentially damaging cumulative effects of a second injury. Further work must be performed to better clarify the vulnerability of the brain after CBI to a second insult to translate our experimental findings into clinical practice and develop novel and more meaningful guidelines based on an increased understanding of the vulnerability of the brain to mild TBI.

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COMMENTS

This study by Longhi et al. from the McIntosh group represents state-of-the-art exploration of the long-term effects of repeated concussions on recovery of function. Although these studies were performed in a mouse model of traumatic brain injury, the authors' extensive work with this model has proven it highly reproducible and clinically relevant. Their results demonstrate that the injured brain may be highly vulnerable to a second concussive injury during the first 5 days after the initial concussion. The additional damage included more pronounced vestibulomotor deficits as well as significantly increased axonal damage near the impact sites. Given the heightened awareness of repeated concussions in sports, the authors' findings provide a keen insight into the recovery process after concussion and a physiologic basis for the conventional wisdom of increased vulnerability after a brain injury. Furthermore, these studies should be extended to include the influence of injury severity and subject age on the overall window of vulnerability and the extent of additional deficits induced by repeated concussions.

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This article provides useful further data regarding the periods of brain vulnerability to repeat concussion in mice. The study augments prior results, especially from the laboratory of David Hovda (1) and reports by Chris Giza (2). Those studies enlightened our understanding of the specific physiological and metabolic patterns observed after concussion in mice. They allowed us to understand the energy crisis that develops shortly after concussion as a result of hyperglycolysis with decreased blood flow, ionic cellular K^+ efflux, Ca^{2+} influx, and excitatory neurotransmitter glutamate release, all requiring glycolysis-generated adenosine triphosphate to reestablish homeostasis. Hovda et al. documented the increased susceptibility to repeat concussion in the days after an initial concussion.

The authors provide further insight into the specific time course of heightened susceptibility to a second concussive brain injury. They determined that the mouse concussed brain is highly susceptible to exponentially greater neuronal and axonal damage from a second concussive injury within 3 days (more so than within 5 d) and was back to baseline by 7 days.

In addition to the obvious species difference, it is not known what level of concussion in humans best correlates with the concussive injury in mice, and the time course may differ. However, this reproducible animal work demonstrating heightened susceptibility to a second concussive injury cannot be ignored. As we do not have a metabolic marker to demon-

strate when homeostasis has been returned after a human cerebral concussion, most clinicians use normal neurological examination results, absence of postconcussion symptoms, return to baseline on neuropsychological tests, and normal images for that purpose.

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Macrophage engulfing an infectious yeast cell (photograph courtesy of Biology Media).