Regional Neurodegeneration and Gliosis Are Amplified by Mild Traumatic Brain Injury Repeated at 24-Hour Intervals

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Abstract
Most traumatic brain injuries (TBIs) that occur every year are classified as “mild.” Individuals involved in high-risk activities may sustain multiple mild TBIs. We evaluated the acute physiologic and histopathologic consequences of mild TBI in a mouse model, comparing sham injury, single impact, or 5 impacts at a 24- or 48-hour interinjury interval. A single closed skull impact resulted in bilateral gliosis in the hippocampus and entorhinal cortex that was proportional to impact depth. Midline impact, at a depth just above the threshold to induce transient unconsciousness, produced occasional axonal injury and degenerating neurons accompanied by astrogliosis in the entorhinal cortex and cerebellum. Mild TBI repeated every 24 hours resulted in bilateral hemorrhagic lesions in the entorhinal cortex along with significantly increased neurodegeneration and astrogliosis that were not observed with standard imaging techniques (4–6). Mild TBI presents with a wide variety of signs and symptoms. These may include symptoms such as dizziness, headache, and/or nausea and signs such as loss of consciousness, memory loss, agitation, and/or motor deficits (4–6). For many patients, rest over time helps alleviate symptoms of mild head injury within hours to a few days. In 10% to 15% of individuals, however, symptoms will remain for much longer (6).

Emphasis specifically in sport-related TBI has been put on alleviating symptoms before an athlete should return to play. Yet it is unclear whether cellular responses in the brain subside during that time or if the brain remains in a state of heightened vulnerability to subsequent trauma. Individuals then return to play with a higher risk of sustaining another mild TBI, especially in high-contact sports such as boxing and football (4–6). Furthermore, the neurologic and pathophysiologic sequelae of additional TBIs may be more severe than that initiated by the first TBI if the brain has not fully recovered.

Animal models are important tools for elucidating the molecular events triggered by mild TBI, investigating the cellular basis for continued vulnerability, and correlating brain pathology with neurobehavioral dysfunction. Models allow for a controlled reproducible impact and enable the systematic variation of experimental parameters to explore the effects of repeated mild TBIs.

The study of TBI is informed by a variety of well-established animal models including weight drop (7, 8), fluid percussion injury (9, 10), impact acceleration (11, 12), and controlled cortical impact (13). These models have been used to describe patterns of gliosis, axonal injury, and/or neuron death, as well as deficits in memory and motor coordination after a single “moderate to severe” injury. Many of these models have been modified to examine mild TBI and the effects of repeated mild TBIs.

INTRODUCTION
Approximately 1.7 to 3.8 million people in the United States sustain a traumatic brain injury (TBI) every year (1, 2), with the vast majority categorized as “mild” and occurring during sport-related activities (1, 3). A mild TBI is caused by a traumatic or biomechanical force that results in altered brain function without neuropathologic complications, as can be observed with standard imaging techniques (4–6). Mild TBI presents with a wide variety of signs and symptoms. These may include symptoms such as dizziness, headache, and/or nausea and signs such as loss of consciousness, memory loss, agitation, and/or motor deficits (4–6). For many patients, rest over time helps alleviate symptoms of mild head injury within hours to a few days. In 10% to 15% of individuals, however, symptoms will remain for much longer (6).
the pathologic alterations. We then characterized histologic and physiologic responses to a single mild TBI as a function of injury severity and compared the response of a single mild TBI with those of mild TBIs repeated at either a 24- or 48-hour interval. We provide corroborated that axonal injury and astrogliosis are features of a single mild closed head injury (CHI) but demonstrate an enhanced vulnerability of the brain to mild TBI repeated at a 24-hour interval, resulting in more extensive neurodegeneration and neuroinflammation, including injury within the entorhinal cortex, cerebellum, and brainstem.

MATERIALS AND METHODS

Animals

Two- to 3-month-old male C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) for all experiments. Upon arrival, mice were group housed under a controlled 14:10 light:dark cycle and provided food and water ad libitum. Animal husbandry and all surgical procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee and followed the federal guidelines set by the Institute of Laboratory Animal Resources (US) and Committee on the Care and Use of Laboratory Animals.

Closed Head Injury

After induction of anesthesia in a chamber using 3% isoflurane/3% oxygen for 1 to 2 minutes, mice were transferred into a stereotaxic frame with nonrupture Zygomar ear cups (Kopf Instruments, Tujunga, CA) where anesthesia was maintained using 2.5% isoflurane/2.5% oxygen delivered via a nose cone. The surgical area of the scalp was cleaned with a Betadine solution. After injection of 0.2 mL of 1:200,000 epi- nephrine and 0.5% bupivacaine (Henry Schein Animal Health, Dublin, OH) in sterile normal saline for local analgesia, the scalp was resected. Mice then received a mild CHI using a pneumatically controlled cortical impact device (TBI-0310 Impactor; Precision Systems and Instrumentation, Fairfax Station, VA) with a custom-made, 5-mm-diameter, pliant, silicone tip with a hardness of 55 Shore A. The tip was aligned along the midline suture between the bregma and lambda sutures. The device was programmed to impact at an intended depth (0.5–3.0 mm) at a 3.5-m/s velocity with a 500-millisecond dwell time. Sham-injured mice received anesthesia and underwent the same surgical procedure without receiving an impact. The total duration of anesthesia was controlled for all animals to be 10 minutes.

To assess loss of consciousness caused by injury versus anesthesia most accurately, mice were immediately removed from the stereotaxic device after impact and placed onto a heating pad on their backs. Apnea duration and time to right spontaneously to a prone position (righting reflex) were assessed. After righting, mice were briefly reanesthetized to suture their scalps and returned to the heating pad to recover. Mice were subcutaneously injected with 1 mL of sterile normal saline to increase hydration, encouraging a normal eating response after injury and maintenance of healthy weight. For repeated sham and CHI injuries, sutures were removed, the scalp was resected, and the above injury and assessment procedures were then performed.

For initial characterization of the impact depth on histologic and physiologic responses, a single impact was delivered with a prescribed depth of 0.5, 1.0, 1.5, 2.0, 2.5, or 3.0 mm (n = 2–3 per impact depth). Apnea and righting reflex data were analyzed separately by 1-way analysis of variance (ANOVA) followed by post-hoc Newman-Keuls tests using GraphPad Prism 6 software (La Jolla, CA). Mice from these groups were killed 48 hours after injury for analysis of astrocytosis and neuron degeneration.

For repeated CHI experiments, mice were randomized into 3 major groups: 2.0 mm repeated CHI; 2.0 mm single CHI; and sham CHI (Fig. 1). The repeated CHI group received 5 impacts at either a 24-hour interval between injuries (rCHI-24h; n = 9) or a 48-hour interval (rCHI-48h; n = 8). These intervals were selected based on multiple studies that suggest that the interval of vulnerability for repeated mild TBIs in rodents may be 24 to 48 hours (18, 21, 22, 26, 28, 30). Five impacts were used based on previous studies of repeated mild TBIs (22, 23, 26). The rCHI mice were killed 24 hours after their final impact to assess the acute consequences of repeated CHI at varying interinjury intervals. The single CHI group was divided into 3 subgroups to parallel the survival times of the rCHI groups relative to the first and last injuries: 24 hours (single 24h; n = 6), 5 days (single 5d; n = 5), and 9 days (single 9d; n = 5). Sham control mice for the rCHI-24h (n = 4) and rCHI-48h groups (n = 3) were pooled for analysis (n = 7), except in Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A637. One mouse assigned to the rCHI-24h group and one assigned to the rCHI-48h group died before receiving 5 CHIs. Therefore, the final group sizes were 8 for rCHI-24h and 7 for rCHI-48h. Apnea and righting reflex data were analyzed by repeated-measures 1-way ANOVA (injury group × number of CHIs) followed by post-hoc Newman-Keuls tests using GraphPad Prism 6 software.

Tissue Processing

Mice were killed by intraperitoneal injection of Fatal Plus (130 mg/kg; Henry-Schein Animal Health) before transcardial perfusion with cold heparinized sterile saline followed with 10% neutral buffered formalin (Fisher Scientific, Atlanta, GA). Mice were decapitated and heads placed into formalin for 24 hours. Brains were then removed from the skull and allowed to postfix for an additional 24 hours. After
this period, the brains were placed into 30% sucrose in 1x Tris-buffered saline (TBS) for 24 to 48 hours to cryoprotect the tissue. The tissue was frozen in -25°C to -35°C isopentane before being cut into 40-μm-thick coronal sections using a sliding microtome (Dolby-Jamison, Pottstown, PA). Tissue sections were stored at -20°C in cryoprotectant (30% glycerol, 30% ethylene glycol in 1x TBS).

**Histology**

For initial assessment of cell loss, a series of 9 sections spaced at 400-μm intervals were Nissl stained with 2.5% cresyl violet. Degenerating neurons were examined with Fluoro-Jade C (FJC), as previously described (31). For each animal, 4 sections at 400-μm intervals within the caudal hippocampus and entorhinal cortex (from ~2.2 to ~3.4 mm bregma) and 6 to 8 sections at 200-μm intervals within the cerebellum and the brainstem were selected. Sections were initially treated with diaminobenzidine (Vector Laboratories, Burlingame, CA) for 5 minutes, washed in ddH2O, and mounted onto slides. The diaminobenzidine treatment was used to quench endogenous peroxidases, eliminating any nonspecific fluorescence that may occur with hemorrhage. The tissue was dehydrated by placing slides on a warmer (45°C) for 30 minutes and then leaving them at room temperature overnight. On the following day, slides were immersed sequentially in 1% NaOH in 80% ethanol, 70% ethanol, and ddH2O before being placed into 0.06% potassium permanganate solution for 10 minutes. The tissue was washed with ddH2O before incubating in 0.0001% FJC solution (Millipore, Temecula, CA) in 0.1% acetic acid for 10 minutes. The tissue was rinsed with ddH2O, air-dried, coverslipped with Cytoseal 60 (Thermo Scientific, Hanover Park, IL), and protected from light.

**Immunohistochemistry**

Free-floating tissue sections were used for immunohistochemical analysis. To label tissue for accumulation of amyloid precursor protein (cAPP) and hyperphosphorylated tau (PHF-1), antigen retrieval was performed using 10 mmol/L citric acid in a 65°C water bath for 15 minutes. Tissue was then allowed to cool at room temperature for 20 minutes before being washed with 1x TBS. For all tissues, to quench endogenous peroxidases, tissue was treated with 3% H2O2 in 50/50 methanol/ddH2O for 30 minutes, rocking at room temperature. Tissue was subsequently washed and blocked for 1 hour with 5% normal horse serum in 1x TBS/0.1% Triton X-100 before incubation in primary antibody overnight at 4°C. The following antibodies were used: anti-amyloid precursor protein (cAPP, rabbit polyclonal, 1:2000; Invitrogen/Life Technologies, Carlsbad, CA), anti-tau pSer396/404 (PHF-1, mouse monoclonal, 1:500; Peter Davies; The Feinstein Institute for Medical Research, Great Neck, NY), anti–gli fibrillary acidic protein (GFAP) mouse monoclonal, 1:300; Sigma-Aldrich, St. Louis, MO), anti–ionized calcium–binding adaptor molecule-1 ([Iba-1] rabbit polyclonal, 1:1000; Wako, Richmond, VA), and anti-MHC II (I-A/I-E, rat monoclonal, 1:1000; eBiosciences, San Diego, CA). On the following day, anti-GFAP, -cAPP, and -PHF-1–labeled tissue sections were incubated in appropriate biotin-conjugated secondary for 1 hour.

The tissue was washed before incubating in the avidin-biotin complex (Vector Laboratories) for 1 hour and then treated with diaminobenzidine as directed by the manufacturer. Iba-1– and I-A/I-E–labeled tissues were incubated in appropriate secondary antibodies conjugated with Alexa-488 and Cy-3, respectively.

**Quantification of Histology and Immunohistochemistry**

The FJC-stained sections were viewed at 40× magnification using an Olympus AX80 microscope with a TRITC filter. The FJC-positive cells in bilateral entorhinal cortices were counted by an individual blinded to the injury conditions. Counts were summed for each animal for statistical analysis. Cerebellum and brainstem sections labeled with FJC were examined qualitatively.

The cAPP-positive axons in 4 sections per animal spaced at 200-μm intervals (~bregma level, ~2.2 to ~3.4 mm) were viewed under bright field using an Olympus AX80 microscope with a 40× lens. Axonal swellings and bulbs were counted by a blinded examiner. Counts were summed for each animal for statistical analysis.

Glial fibrillary acidic protein immunoreactivity was viewed using an Olympus BX51 microscope (20×) with an ASI XY automated stage. Twenty-four-bit RGB images of the entorhinal cortices and hippocampi were captured using a 12bit Q Imaging camera. The calibrated Stage-Pro module of Image Pro Plus (Media Cybernetics, MD) was used to assemble the images into a montage. The entorhinal cortex was analyzed in 4 sections per animal taken at 200-μm intervals (bregma ~2.8 to ~3.4 mm). Astrocytosis in the hippocampus was analyzed in 3 sections per animal taken at 200-μm intervals (bregma ~2.8 to ~3.2 mm). To quantify GFAP immunoreactivity, the area of interest was outlined using the anatomic features as described in *The Mouse Brain in Stereotaxic Coordinates, 4th Edition* (Paxinos and Franklin). The hippocampus was outlined along the dorsal edge by following the alveus of the hippocampus and then above the thalamus on the ventral side of the hippocampus. To outline the entorhinal cortex, the rhinal fissure was used as a starting point. A line was drawn ventrally along the periphery of the entorhinal cortex, approximately 1.5 mm from the rhinal fissure. From there, the line was extended horizontally to the lateral ventricle before continuing dorsally along the edge of the lateral ventricle until horizontal to the rhinal fissure. The line was continued horizontally to the rhinal fissure to connect to the point of origin. The range was set at 0 to 210 for each color, and the minimum object area was set at 5 pixels to capture anti-GFAP–labeled astrocytes and eliminate background noise. The number of GFAP-immunoreactive astrocytes was quantified, and the mean integrated optical density (IOD) was determined by dividing the total IOD by the number of astrocytes within a given area of interest. A higher IOD corresponded to a greater GFAP immunoreactivity. Data collected were averaged for each region (entorhinal cortex, hippocampus) for each animal for statistical analysis. In the cerebellum and brainstem, GFAP immunoreactivity was qualitatively assessed in a series of 6 to 8 sections selected at 200-μm intervals.
FIGURE 2. Acute physiologic and histopathologic consequences of single impact as a function of depth. Righting reflex (A) and apnea (B). *p < 0.05 compared with all other groups. Bars represent mean ± SE. (C–O) Fluoro-Jade C (FJC) staining after sham (C), 1.0-mm (G), 2.0-mm (K), and 3.0-mm (O)–impact depths. FJC-positive cells were evident in the dentate gyrus 48 hours after a 3.0-mm-impact depth. Glial fibrillary acidic protein (GFAP)–labeled astrocytes in the hippocampus and entorhinal cortex after sham (D–F), 1.0-mm (H–J), 2.0-mm (L–N), and 3.0-mm (P–R)–impact depths. Mild astrocytosis was evident in the caudal hippocampus and entorhinal cortex after a 2.0-mm impact. A 3.0-mm impact increased astrocytosis in the entire hippocampus and motor cortex. Scale bars = (C, D, G, H, K, L, O, P) 500 μm; (D, O, P inserts) 100 μm; (E, F, I, J, M, N, Q, R) 1 mm.
Impact Depth Characterization

Iba-1 immunoreactivity was viewed using a fluorescein isothiocyanate filter on an Olympus BX51 microscope (20×) with an ASI XY automated stage and 8-bit gray-scale images were taken and montaged, as previously described. The percentage of Iba-1 immunoreactivity in the entorhinal cortex and hippocampus was analyzed by first setting the display range at 0 to 255 to outline the hippocampus or entorhinal cortex as previously described. A blinded examiner then chose the range that selected immunoreactive microglia for each tiled image. The percent of the total area, which contained Iba-1-labeled microglia, was measured and averaged for the entorhinal cortex across 8 montages per animal and for the hippocampus across 6 montages per animal. Tissue labeled with Iba-1 was colabeled with I-A/I-E (MHC Class II). I-A/I-E immunolabeling was evaluated qualitatively.

A 1-way ANOVA followed by post-hoc Newman-Keuls tests where appropriate was performed using the GraphPad Prism 6 software to determine the statistical significance among groups for each marker within each region.

RESULTS

Impact Depth Characterization

An initial cohort of mice was subjected to CHI of differing impact depths to characterize the CHI model and to determine the injury parameters that resulted in a mild TBI as evaluated by neuronal degeneration, gliosis, and acute postconcussive physiologic responses. Described impact depths on the midline suture of the mouse skull of 0.5, 1.0, and 1.5 mm did not prolong suppression of the righting reflex after impact (Fig. 2A) but produced a graded increase in apnea with increased injury severity (Fig. 2B) when compared with anesthesia alone in sham control animals. Mice injured with a prescribed depth of 2.0 mm had a significantly increased righting reflex suppression (p < 0.05) and apnea duration (p < 0.05) versus sham control mice and mice receiving CHI at all other smaller impact depths. Impact depths of 2.0 mm did not result in skull fracture. In contrast, impact depths of 2.5 mm and 3.0 mm produced skull fractures or prolonged apnea that resulted in death in 6 of 10 animals tested.

At 48 hours after CHI, FJC-positive cells were observed in only the 3.0-mm impact group in the dentate gyrus (Fig. 2O), suggesting that a single CHI of less than 3.0-mm depth did not produce notable neurodegeneration in the cerebrum at 48 hours after impact. Glial fibrillary acidic protein immunohistochemistry was used to visualize astroglial changes throughout the cerebrum. Impact depths of 0.5 mm (data not shown), 1.0 mm (Fig. 2H–J), 1.5 mm (data not shown), and 2.0 mm (Fig. 2L–N) caused only slight increases in GFAP immunoreactivity in the hippocampus and entorhinal cortex. In contrast, hippocampal and cortical gliosis was more evident after CHI at a 2.5-mm (data not shown) or 3.0-mm impact depth (Fig. 2P–R), particularly in the dentate gyrus and hilus where neuronal degeneration was also occurring. Based on the initial characterization of impact depths in the range of 0.5 to 3.0 mm, a depth of 2.0 mm was selected for subsequent experiments evaluating repeated CHI based on the presence of acute physiologic responses (increased righting reflex and apnea times) and mild gliosis indicative of tissue responses in the absence of notable neuronal death. The cerebella and brainstems of this cohort of animals were not available for assessment.

Acute Physiologic Responses Decrease With Repeated CHI

To evaluate the effects of repeated CHI on acute physiologic responses and determine if apnea and righting reflex depend on the interinjury interval, responses were compared among mice from sham, rCHI-24h, and rCHI-48h groups. The amount of time to elicit a righting reflex response was dependent on both the injury group (p < 0.0005) and the number of CHI (p < 0.0001), with a significant interaction between these factors (p < 0.05) (Fig. 3A). Suppression of the righting reflex in both rCHI groups was significantly longer than in the sham group (p < 0.001 for rCHI-24h; p < 0.005 for rCHI-48h). Across time, return of the righting reflex response in the rCHI-24h group was significantly more delayed versus the rCHI-48h group (p < 0.05). Post-hoc testing did not reveal a significant difference in righting reflex between the 2 rCHI groups for any specific CHI (first through fifth). Within the

FIGURE 3. Righting reflex (A) and apnea (B) after sham injury, repeated closed head injury at 24-hour intervals (rCHI-24h), and repeated closed head injury at 48-hour intervals (rCHI-48h). $p < 0.05$ versus sham; $% p < 0.05$ versus rCHI-48h; # $p < 0.05$ versus CHI 1 of same group. Bars, mean ± SE.
rCHI-24h group, righting reflex suppression was prolonged significantly after the first and second CHI (p < 0.05 vs sham). Reflex suppression was significantly reduced after the fourth and fifth impacts versus the rCHI-24h group’s response to the initial impact. The rCHI-48h group also exhibited a significant increase in the time to restore righting reflex after the first CHI but not after subsequent CHIs.

Apnea duration varied as a function of injury (p < 0.0001) and the number of CHI (p < 0.005) with a significant interaction between these factors (p < 0.05) (Fig. 3B). Both the rCHI-24h and rCHI-48h groups had significant apnea after the first impact versus the sham group (p < 0.0001 and p < 0.001, respectively), but apnea duration decreased significantly when the second impact was imparted 48 hours as opposed to 24 hours after the first (p < 0.05). Both repeated mild TBI groups showed a trend toward shorter apnea with successive impacts. As compared with apnea after the first impact, apnea was significantly reduced after the third, fourth, and fifth impacts for the rCHI-24h group and the second, fourth, and fifth impacts for the rCHI-48h group, suggestive of a compensatory mechanism at play with multiple mild TBIs.

Mild TBI Repeated at 24-Hour but Not 48-Hour Intervals Exacerbates Acute Neuron Death in the Entorhinal Cortex

Several histologic assessments were used to evaluate the effects of repeated CHI on the brain and whether the time interval between impacts influenced these responses. Nissl staining did not reveal any overt pathologic changes at 1, 5, or 9 days after a single CHI (Fig. 4A). However, 5 of 8 mice in the rCHI-24h group exhibited hemorrhagic lesions in the entorhinal cortex at 24 hours after the fifth concussion (Fig. 4C). The incidence of hemorrhagic lesions decreased to 1 of 7 in the rCHI-48h group, and the lesions were smaller versus those observed in mice injured at a 24-hour interval (Fig. 4E).

In the entorhinal cortex, a single impact of 2.0 mm resulted in a small number of FJC-positive cells 24 hours after the injury (Fig. 4B), indicating neuronal degeneration; however, numbers of degenerating neurons were not significantly different from sham (Fig. 4G). In the rCHI-24h group, FJC-positive cells were located around the region of hemorrhage (Fig. 4C) as well as in the entorhinal cortex in tissue sections that did not contain a lesion. Mild TBI repeated at a 24-hour interval, but not a 48-hour interval, resulted in a significant increase in numbers of FJC-positive cells within the entorhinal cortex versus sham injury (p < 0.05) (Fig. 4G). Neither single nor repeated CHI at a 2.0-mm impact depth resulted in FJC staining in the hippocampus.

In the cerebellum, qualitative assessment of FJC staining revealed a small amount of bilateral neuronal degeneration within the Purkinje cell and molecular layers of the most distal aspect of the lateral cerebellar folia 24 hours after a single CHI (Fig. 5A) that was primarily restricted to neuronal process staining in the molecular layer by 9 days after a single CHI (not shown). In contrast, somas of degenerating neurons were rarely labeled in mice that received CHI at 24-hour intervals. With an interinjury interval of 48 hours, some rCHI mice exhibited FJC labeling of a small number of neurons and processes within the

![FIGURE 4. Neuronal degeneration in the entorhinal cortex after single and repeated closed head injury (CHI).](http://jnen.oxfordjournals.org/) Nissl staining of mice killed 24 hours after a single CHI (single 24h; A), 5 CHIs repeated with a 24-hour interinjury interval (rCHI-24h; C), and 5 CHIs repeated with a 48-hour interinjury interval (rCHI-48h; E). Nissl staining shows hemorrhagic lesions in the entorhinal cortex of the rCHI-24h group. (B, D, F) Fluoro-Jade C (FJC) staining in the entorhinal cortex of mice in the single 24h (B), rCHI-24h (D), and rCHI-48h (F) groups. Scale bars = (A, C, E) 500 μm; (B, D, F) 250 μm. (G) The number of FJC-positive cells was increased in the entorhinal cortex of the rCHI-24h group versus sham. Bars represent mean ± SE. $ p < 0.05$ versus sham; $^\wedge p < 0.05$ versus Single 9 days.
FIGURE 5. Neuronal degeneration in the cerebellum and brainstem after a single and repeated closed head injury (CHI). Fluoro-Jade C (FJC) staining in mice killed 24 hours after a single CHI (single 24h) indicated degenerating neurons within the Purkinje cell (PC) layer and some axonal injury in the white matter (WM) tract of the cerebellum (A), with very little injury to the brainstem (B). Five CHIs repeated at a 24-hour interinjury interval (rCHI-24h) exacerbated axonal degeneration throughout the WM of the cerebellum (C) and in the ventrolateral aspects of the brainstem (D). When the interval between repeated CHIs was lengthened to 48 hours (rCHI-48h), a decrease in axonal degeneration was observed in the cerebellum (E) and brainstem (F) versus the rCHI-24h group.

^degenerating neurons; ML, molecular layer; GC, granular cell layer. Scale bar = 100 μm.
lateral cerebellar folia, similar to that observed in the single CHI groups.

Mild TBIs Produce Axonal Injury

Although FJC staining did not reveal notable axonal injury in the cortex, cerebral white matter, or hippocampus, it provided clear evidence of axon damage within the cerebellum and brainstem at 24 hours after repeated mild TBIs. Mild axonal injury was observed in the lateral cerebellar white matter tracts (Fig. 5A) but not in the brainstem (Fig. 5B) with a single CHI. Axonal injury was more extensive and more diffusely distributed in the rCHI-24h group, with axonal injury in lateral (Fig. 5C) and central white matter tracts within the cerebellum and scattered throughout the brainstem, with most concentrated ventrolaterally (Fig. 5D). Repeated CHI at a 48-hour interval resulted in a similar distribution of axonal injury as with the 24-hour interval, but the amount of axonal injury appeared to be less (Fig. 5E, F).

Within the cerebrum, axonal injury was detected by the accumulation of cAPP in axonal swellings and small bulbs in the entorhinal cortex 24 hours after a single CHI (Fig. 6A, D). The number of cAPP-positive axons/bulbs diminished with longer survival durations (5 days, 9 days), indicating an acute but not protracted wave of axonal injury (Fig. 6G). When CHI was repeated at 24-hour intervals, the number of labeled axons increased versus a single CHI, but this was not statistically significant (Fig. 6G). The incidence of axonal injury was also similar between the rCHI-24h group (5 of 8 with >1 swelling per section) and the single 24h group (3 of 6). Despite these similarities, cAPP-labeled bulbs appeared much larger in the rCHI-24h group (Fig. 6B, E) than in single CHI groups (Fig. 6A, D). In addition, in the rCHI-24h group, the clusters of bulbs were located adjacent to the external capsule (Fig. 6B, E), whereas axonal injury in the single 24h group was more centrally located within the entorhinal cortex. When CHI was repeated at 48-hour intervals, few mice exhibited axonal injury (2 of 7). Although the mean number of cAPP labeled axons was similar to the single 24h group, this was caused by one animal with an unusually high number of swellings.

Astrocytosis Is Exacerbated by Repeated CHI at 24-Hour but Not 48-Hour Intervals

Glial fibrillary acidic protein-positive astrocytes were present in the entorhinal cortex in sham-injured animals, but their cell bodies and processes did not appear swollen (Fig. 7A, D). Acutely after a single CHI, some astrocytes in a small area of the entorhinal cortex appeared more swollen versus those in sham mice. Although the number of GFAP-positive astrocytes at 24 hours was not increased versus sham injury, numbers increased progressively for 9 days after a single mild TBI (p < 0.05 vs single 24h; Fig. 7G). A single mild TBI also resulted in a delayed increase in the intensity of GFAP immunoreactivity per astrocyte at 9 days after injury (p < 0.05 vs sham; Fig. 7G). When CHI was repeated at 24-hour intervals, astrocytes across the entire entorhinal cortex region appeared reactive with swollen cell bodies and processes (Fig. 7B, E).

![FIGURE 6. Axonal injury after repeated closed head injury (CHI). Immunohistochemical labeling of amyloid precursor protein (cAPP) at 24 hours after a single CHI (single 24h; A, D), 5 CHIs repeated at 24-hour intervals (rCHI-24h; B, E), and 5 CHIs repeated at 48-hour intervals (rCHI-48h; C, F). cAPP-labeled axons; *external capsule; +rhinal fissure. Scale bar = 250 μm. (G) The number of immunolabeled axonal swellings was not significantly different across groups. Bars represent mean ± SE.](image-url)
FIGURE 7. Astrocytosis in the entorhinal cortex and hippocampus after sham and repeated closed head injuries (CHIs). Immunohistochemical labeling for glial fibrillary acidic protein (GFAP) after sham (A, D), 5 CHIs repeated at 24-hour intervals (rCHI-24h; B, E), or 48-hour intervals (rCHI-48h, C, F). Scale bars = (A–C) 500 μm; (D–F) 250 μm. Immunoreactive astrocytes were counted in the entorhinal cortex (representative area of interest outlined in black) (G) and hippocampus (H). The mean integrated optical density of GFAP per astrocyte was quantified in the entorhinal cortex (G) and hippocampus (H). Bars represent mean ± SE. * p < 0.05 versus all other groups; $ p < 0.05 versus sham; ! p < 0.05 versus single 24h; # p < 0.05 versus Single 5d and Single 9d.
GFAP-positive astrocytes in the entorhinal cortex was significantly increased in rCHI-24h mice compared with mice evaluated 24 hours after a single CHI (p < 0.05; Fig. 7G) and appeared to be increased compared with the single CHI 5d survival group, although this did not reach statistical significance. The intensity of GFAP immunoreactivity per astrocyte was also increased in the entorhinal cortex of the rCHI-24h group versus the single CHI groups and sham injury (p < 0.05; Fig. 7G). When the interinjury interval was lengthened to 48 hours, the regional extent of reactive astrogliosis was similar to that observed 9 days after a single CHI, but some cell bodies and processes appeared swollen as in the rCHI-24h group (Fig. 7C, F). Numbers of GFAP-positive astrocytes in the entorhinal cortex of the rCHI-48h group increased compared with 24 hours after a single CHI but were not different from 9 days after a single CHI (Fig. 7G). Gial fibrillary acidic protein immunoreactivity for a given astrocyte in the rCHI-48h group did not change compared with sham and a single CHI, suggesting that a longer interval between injuries may protect against cumulative impacts (Fig. 7G). These data point to an additive effect of CHI repeated at a 24-hour but not a 48-hour interval on astrogliosis in the entorhinal cortex.

In the hippocampus, both the number and GFAP immunoreactivity per astrocyte were higher at 24 hours after a single CHI than at 5 days and 9 days, consistent with acute transient gliosis (Fig. 7H). The response 24 hours after a single CHI was equivalent to sham, however, suggesting possible anesthesia-induced gliosis in sham-injured mice that had received 5 anesthesia exposures. Increased hippocampal astrogliosis has been previously reported with repeated anesthesia (22), but the degree of anesthesia-induced astrogliosis was not influenced by the interval between anesthesia bouts (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A637). Mild TBI repeated at a 24-hour interval did not result in a greater number of GFAP-positive astrocytes compared to sham or single CHI (Fig. 7H). GFAP immunoreactivity per astrocyte was significantly increased in the rCHI-24h group versus sham, but not the single 24h group (Fig. 7H). When CHI was repeated at 48-hour interinjury intervals, astrocytes appeared less swollen than in the rCHI-24h group and neither the number of GFAP-positive astrocytes nor GFAP expression per astrocyte was significantly different compared with sham or the single 24h group (Fig. 7H).

Qualitative analysis of astrogliosis within the cerebellum and brainstem revealed patterns among injury groups similar to those described for the entorhinal cortex. A single CHI induced mild gliosis in the molecular layer of the most distal aspect of the lateral cerebellar folia in regions similar to those exhibiting neuron degeneration with FJC. Very little astrocyte reactivity was noted in the brainstem. When repeated at 24-hour intervals, 5 CHIs caused an increased response of astrocytes bilaterally within the cerebellum, most notably in the lateral folia (Fig. 8B). Astrocyte processes extending across the molecular layer became thickened, and cell bodies within the granular cell layer hypertrophied (Fig. 8E). Repeated CHI at 24-hour intervals also resulted in increased GFAP immunostaining in the gray and white matter of the brainstem (Fig. 8H, K). When the interval was extended to 48 hours, 5 CHIs induced a milder astrogliotic response within the lateral cerebellum (Fig. 8C, F) and brainstem (Fig. 6I, L) compared with the rCHI-24h group.

**Microglia Are Activated by CHI Repeated at 24-Hour but Not 48-Hour Intervals**

In an uninjured mouse, Iba-1 mostly labeled small uniform microglia cell bodies and very thin processes. Microgliosis was observed as morphologic changes to Iba-1–labeled cells such as swelling of the cell bodies and enlarged Iba-1–positive processes. Microgliosis, measured via the percent area occupied by Iba-1 immunolabeling, was not increased at 24 hours, 5 days, or 9 days after a single CHI compared with sham injury in either the entorhinal cortex or the hippocampus (Fig. 9E). However, after rCHI at 24-hour intervals, microglial cell bodies and processes in the entorhinal cortex appeared swollen (Fig. 9A, C). The percent area of Iba-1–labeled microglia was increased in the entorhinal cortex (p < 0.0001 vs all other groups; Fig. 9E) but not in the hippocampus (p > 0.05). The rCHI repeated at 48-hour intervals did not induce significant microgliosis (Fig. 9B, D) in the entorhinal cortex or hippocampus when compared with sham injury or a single CHI (p > 0.05; Fig. 9E).

I-A/I-E (MHC II) has been used as a marker for activated microglia (32), but mild CHI did not induce microglial expression of MHC II after injury, as evidenced by the lack of coexpression with Iba-1 (Fig. 9I). Instead, in areas of hemorrhage, I-A/I-E appeared to label macrophages that had infiltrated the brain because of blood-brain barrier breakdown (Fig. 9F, G). I-A/I-E–labeled cells also lined the ventricles in all groups (data not shown).

**Repeated CHI Does Not Induce Acute Tau Hyperphosphorylation**

Immunohistochemistry with PHF-1 did not reveal qualitative increases in hyperphosphorylated tau in the cortex or hippocampus in any injury group versus sham controls (data not shown).

**DISCUSSION**

Many mild TBIs that occur every year can be attributed to activities such as boxing and football where the risks of repeated concussions are increased. In this study, we developed a mouse model of mild TBI by using a cushioned midline impact to the closed skull. This model induced injury primarily to the entorhinal cortex and cerebellum, with additional pathology in the brainstem with repeated impacts. By varying the programmed depth of impact, a 2.0-mm depth of impact was selected as an optimal injury severity, inducing acute physiologic consequences and mild gliosis without skull fracture or overt cortical or hippocampal cell death. This is representative of the human condition in which loss of consciousness may occur after impact, but imaging analysis of the brain is typically unremarkable. We then demonstrated that 5 mild TBIs, at either 24-hour or 48-hour interinjury intervals, resulted in apnea and righting reflex suppression that diminished with successive impacts. Despite the diminishing response, 5 CHIs repeated at a 24-hour interinjury interval amplified gliosis and increased neuronal degeneration and axonal injury compared with a single CHI. When the interinjury interval between multiple head injuries was increased from
FIGURE 8. Astrocytosis in the cerebellum and brainstem after repeated closed head injury (CHI). Immunohistochemical labeling of glial fibrillary acidic protein (GFAP) after sham CHI indicates a baseline level of immunostaining in the Purkinje cell layer of the cerebellum (A, D) and in the brainstem (G, J). When 5 CHIs were repeated at 24-hour intervals (rCHI-24h), reactive astrocytosis was observed throughout the cerebellum, with the greatest hypertrophy occurring in the molecular and granular layers of the lateral cerebellar folia (B, E). After 5 CHIs repeated at 48-hour intervals (rCHI-48h), reactive astrocytosis was not as robust and was focused in the lateral cerebellar folia (C, F). Astrogliosis was more pronounced in the brainstem after rCHI at 24-hour intervals (H, K) than at 48-hour intervals (I, L). Scale bars = (A–C, G–I, J–L) 500 μm; (D–F) 250 μm.
24 hours to 48 hours, the resultant histopathologic consequences were similar to a single CHI.

Transient loss of consciousness of up to 30 minutes in duration occurs in human cases of mild TBI (33). In mice, the righting reflex has been used for more than 50 years to indicate loss of consciousness (34). Although smaller impact depths did not significantly alter the righting reflex response, a 2.0-mm-depth single CHI produced a loss of consciousness of approximately 10 minutes, within the range representing mild TBI in rodent models (20). Although this reflex suppression is of longer duration than in some previous reports (2–5 minutes), most of these studies used a toe/tail pinch response or other painful stimulus to assess consciousness (15, 27, 30), which may result in a more rapid return of responsiveness (35). Although anesthesia suppresses the righting reflex, we maintained a consistent duration of inhalant anesthesia throughout each surgery.

Apnea duration increased in a graded fashion as a function of impact depth. Although apnea is not a typically noted feature of mild TBI in humans, apneic responses are common in rodent mild TBI models using midline impact to the skull (12, 22, 36) and are substantially longer than apneic responses reported with lateral injury (15, 30). Whereas lateral impact may direct tissue deformation across midline as well as downward through deeper brain structures, midline impact likely directs tissue deformation toward the brainstem, transiently affecting neurons involved in respiration (37). In midline models of more severe TBI, animals require intubation to aid in respiratory efforts (12). Although it is difficult to separate the potential effects of apnea from those of the mechanical insult, brief apnea in our model is unlikely to contribute significantly to the pathologic alterations. Repeated apnea during obstructive sleep apnea has been implicated in memory dysfunction in humans and animal models but only in severe cases involving upward of 100 episodes of apnea every night, with each episode lasting seconds to minutes (38).

During a series of 5 concussions, loss of consciousness and apnea diminished with subsequent CHI to near sham control levels. Diminution of the righting reflex response and apnea occurred at a faster rate when the interinjury interval was lengthened from 24 hours to 48 hours, suggesting that an adaptive or protective mechanism was initiated by 48 hours but not 24 hours after CHI. Our findings corroborate a study by Mouzon et al (22) in which apnea duration diminished with repeated midline mild TBI in mice, but they contrast with other studies demonstrating increased or sustained apnea duration or righting reflex responses across repeated mild TBIs (14, 15, 22, 30, 39). Differences in the impact device, impact tip characteristics, severity of injury, location of injury, or anesthesia may contribute to these disparate observations. Further investigation is needed to understand the evolution and underlying mechanisms of righting reflex suppression and apnea responses with repeated mild TBIs, but our data raise the interesting question as to whether people adapt to repeated concussions in terms of their acute physiologic responses. That is, in response to an equivalent concussive insult, would athletes such as boxers who receive many blows to the head during the course of their careers exhibit different symptoms from individuals with no prior concussions? If symptomatology decreases with multiple concussions, the severity and potential consequences of any given head injury could be underappreciated.

Historically, models of mild TBI produced some degree of neuron degeneration that is observed early after injury (20),

![Figure 9](http://jnen.oxfordjournals.org/)

**FIGURE 9.** Microgliosis after repeated closed head injuries (rCHIs). Immunohistochemical labeling of ionized calcium-binding adaptor molecule 1 (Iba-1) after 5 CHIs repeated at 24-hour intervals (rCHI-24h; A, C) and 48-hour intervals (rCHI-48h; B, D). (E) The percent area of Iba-1 immunolabeling was quantified within the entorhinal cortex and hippocampus. Bars represent mean ± SE. *p < 0.05 versus all other groups. (F-I) Infiltrating macrophages labeled with I-A/I-E in the hemorrhagic lesion (F, G) do not colabel with Iba-1–expressing microglia (H, I). Scale bars = (A, B, F) 500 μm; (C, D) 250 μm; (G-I) 125 μm.
suggesting a transient wave of cell death (36). With the growing interest in repeated concussions, injury parameters have been modified to reduce or eliminate cell death as a feature of a single (26, 29) or repeated mild TBIs (26, 28, 40). However, many of these studies have used relatively insensitive measures such as Nissl staining to assess cell death. In our model, after a single 2.0-mm midline CHI, no overt cell loss was observable with Nissl staining, but a small amount of acute neuron degeneration in the entorhinal cortex and Purkinje cell layer of the cerebellum was detectable with FJC. When CHI was repeated at a 24-hour interinjury interval, the number of FJC-labeled neuron cell bodies increased and a hemorrhagic lesion developed in the entorhinal cortex. Worsening of cell loss, cortical thinning, or the development of a lesion with repeated as compared with a single TBI has been reported (15, 22, 29), but these observations were qualitative or semiquantitative and did not use neuron-specific markers for acute degeneration. Longhi et al (27) noted that acute cortical neurodegeneration was qualitatively equivalent after one and two CHIs at a 3-day interval. We examined shorter intervals and provide quantitative data to demonstrate that lengthening the interinjury interval from 24 hours to 48 hours prevents exacerbation of neuronal death in the entorhinal cortex, indicating that the initial cellular dysfunction was reversible within 48 hours after injury. To our knowledge, this is the first report quantifying the effects of repeated mild TBIs and time interval variations on the accumulation of degenerating neurons. Hence, we provide a model in which multiple injuries may convert sublethal neuronal damage to neuronal degeneration, an important platform for investigating both neuronal and axonal vulnerability to mild TBI. Interestingly, increased acute cell death was not observed in the cerebellum, which may point to unique damage mechanisms or to accelerated degeneration that was not captured using FJC staining. Further evaluation of long-term neuron damage, including stereologic cell counts, is necessary to understand the degree of neuron death in the entorhinal cortex and cerebellum.

Axonal injury is a common feature of mild diffuse TBI, which is typically exacerbated with repeated injuries (16, 18, 29, 30, 39). In the present study, a single CHI produced mild axonal injury in the entorhinal cortex and the cerebellum, a distribution unique from other mild TBI models that most commonly report subcortical white matter tract damage (18, 27, 29, 30, 36). It is not clear why midline impact did not result in subcortical white matter axonal injury as detected by APP immunostaining or FJC staining, but future studies will use alternative detection techniques such as silver staining that may be more sensitive or detect alternative phenotypes of axonal injury (28). Traumatic brain injury repeated at a 24-hour interval did not significantly increase the number of APP-positive axons in the entorhinal cortex, but axonal swellings appeared larger, which may point to a functional distinction. Although the size of axonal swellings has been reported to correlate with survival time (41), larger swellings in the rCHI-24h group cannot be solely attributed to longer survival because equivalent swellings were not observed in single CHI groups with matched survival times. In contrast, rCHI at a 24-hour interval resulted in marked enhancement of cerebellar axonal injury and the appearance of diffuse axonal damage in the brainstem. The development of axonal injury after repeated but not a single mild TBI has been reported in the brainstem (16, 22) and the thalamus (29) in other midline CHI models and could indicate that one mild TBI triggers axonal dysfunction that only evolves into APP-positive pathology with repeated injury. When mild TBI were repeated at 48-hour interinjury intervals, axonal injury in the entorhinal cortex was similar to that in mice examined 9 days after a single CHI, and degenerating axons in the cerebellum and brainstem were observed less frequently than in the rCHI-24h group. These data suggest that longer intervals between mild TBI protect against exacerbation of axonal injury, as has been shown by Fujita et al (16). Combined, our data support the hypothesis that repeated mild TBIs within a certain time frame can induce and/or accelerate axonal injury.

Although the cellular mechanisms for transient neuronal vulnerability to repeated mild TBIs are not well understood, dynamic changes in cell membrane permeability, glutamate release, calcium regulation, and mitochondrial function may play roles. Decreased cerebral glucose metabolism has also been proposed as the underlying cause of vulnerability to a second TBI (42). The interplay between neurons and reactive glia after trauma further complicates identification of causative cellular mechanisms.

Upon a traumatic event, astrocytes become reactive and initiate protective repair mechanisms to restrict tissue damage by repairing any blood-brain barrier breakdown (43) and aid in synaptogenesis (44). A single CHI of 2.0 mm or less resulted in very mild progressive astrogliosis, as in other rodent models of single CHI (22, 45). When mild TBIs were repeated at 24-hour interinjury intervals, astrocytosis was enhanced in the entorhinal cortex, cerebellum, and brainstem, corroborating previous reports of increased astroglial reactivity with repeated compared with single mild TBI (21, 22). What remains unclear, however, is whether exacerbated astrocytosis is neuroprotective or detrimental to the surrounding neurons. Astrocytes are responsible for the reuptake of glutamate from the synaptic cleft but, after a contusion injury, glutamate transporters are downregulated, enhancing excitotoxicity within the synaptic cleft (46). Future studies will examine the persistence of the glial response after repeated CHI and its relationship to initial and ongoing neuronal damage.

Similar to astrocytosis, microglial reactivity has been noted in rodents after single and repeated mild TBIs primarily in the cortex below the site of impact, corpus callosum, and hippocampus acutely after injury (15, 22, 28) and as long after injury as 2 months (15) and 12 months (47). In this study, microglial reactivity was not evident during a 9-day period after a single CHI or acutely after rCHI at a 48-hour interval; however, rCHI at a 24-hour interval significantly increased entorhinal cortex microgliosis. In contrast, Mouzon et al (22) demonstrated that 5 mild TBIs at 48-hour intervals increased microgliosis in the cortex and corpus callosum within 24 hours after the final injury. However, the injury paradigm used by Mouzon et al induced microgliosis after a single mild TBI, whereas our model produced microgliosis only with repeated concussions, suggesting that our single CHI may represent a milder initial injury. Microgliosis activation was observed in the entorhinal cortex in concert with axonal injury and neuronal
degeneration. On activation, microglia release many cytokines and chemokines that signal the onset of an inflammatory cascade. Depending on the levels of these various signaling molecules after trauma, microglia can develop phenotypes promoting either cell death or tissue repair (48). Further investigation into microglial cytokine/chemokine expression between repeated insults and chronically after repeated mild TBIs is needed to understand whether microglia aid or antagonize neuronal repair after repeated traumatic insults.

The combination of neuroglial responses in the hippocampus, entorhinal cortex, cerebellum and brainstem in our CHI model is distinctive; most mild TBI models create cellular damage in the sensory and motor cortices near the impact site, the corpus callosum and/or the hippocampus. The entorhinal cortex, in conjunction with the hippocampus, is involved in the memory circuit. Bilateral electrolytic lesions of the entorhinal cortex have shown to induce deficits in memory and learning (49). Thus, in our model of repeated mild TBIs, bilateral entorhinal cortex neurodegeneration coupled with hippocampal gliosis may facilitate the investigation of posttraumatic memory deficits. Bilateral damage of the cerebellum and brainstem axonal injury may have functional implications for complex motor coordination and balance. Thus, future work should determine whether repeated CHIs with this model result in behavioral dysfunction. The present study was limited to the evaluation of acute physiologic and histopathologic changes. Because repeated mild TBIs are believed to be a risk factor for or cause of neurodegenerative diseases such as Alzheimer disease and chronic traumatic encephalopathy (50), it will be important to examine long-term histologic consequences of repeated mild TBIs in this model.

In conclusion, as compared with a single mild TBI, mild TBIs repeated at a 24-hour interval resulted in greater neuron degeneration, axonal injury, astrogliosis, and microgliosis throughout caudal regions of the mouse brain. These increased tissue responses were not simply caused by the greater number of impacts because 5 concussions induced at 48-hour intervals did not increase neuron death or gliosis as compared with a single injury. Additional studies are needed to identify cellular and molecular factors contributing to the transient vulnerability to repeated mild TBIs. Enhanced neuropathologic changes associated with repeated head injuries were evident despite diminishing acute physiologic responses to successive impacts. These data support the need to supplement symptomatic analysis of mild TBI with assessment of brain pathology through the use of imaging, biomarkers, or other technologies.

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