Multiple Mild Traumatic Brain Injuries Lead to Visual Dysfunction in a Mouse Model

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Abstract

Visual dysfunction is a common occurrence after traumatic brain injury (TBI). We investigated in this study effects of single or multiple mild TBI on visual function in mice using a closed head injury model that permits unconstrained head movement after impact. Adult mice were briefly anesthetized with isoflurane and given one or three mild TBI with the closed head injury by mechanically engineered rotational acceleration (CHIMERA) device with an interinjury interval of 24 h. Mice were then tested in the Morris water maze, visual cliff, and open field tests from day 19 to day 32 and for visual evoked potential at 5 weeks after the last injury and euthanized. Mice with multiple TBI showed impaired performance in the visible platform water maze test and had increased errors in the visual cliff test. Further, there was a graded difference in visual evoked potential, with the single injury mice showing modest reduction in N1 amplitude whereas the multiple injuries produced significant reduction compared to sham and single injury groups. The optic tract of the injured mice showed increases in glial cell immunostaining. The increase in glial fibrillary acid protein immunostaining reached statistical significance for both injured groups whereas the ionized calcium binding adaptor molecule 1 immunostaining was only significantly increased in the optic tract of repeatedly injured mice. These results indicate that multiple injuries using CHIMERA may result in visual deficits, which can affect certain behavioral performances. The change in vision may be a useful marker when monitoring repeated TBI outcome and screening for protective agents from TBI.

Keywords: behavior; CHIMERA; repeated TBI; VEP; visual acuity

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a very heterogeneous condition where the outcome depends on numerous factors, including the location and severity of injury. Mild TBI is the most common form of TBI. Although the prevalence of repeated TBI is not clear, past TBI is considered a risk factor.¹ Specific populations are at particular risk to suffer from repeated TBI. For example, athletic activities, such as boxing, rugby, soccer, hockey, and martial arts, have resulted in 1.8–3.6 million sports-related concussions in the United States every year.² Repeated TBIs are also common in military personnel, with a survey of U.S. army soldiers who were deployed in Iraq or Afghanistan revealing that 17% of the soldiers had sustained TBI, of whom 59% had sustained multiple TBIs.³ Occurrence of a mild TBI increases the vulnerability of the brain to another TBI,⁴ particularly if the second TBI occurs in the “temporal window of vulnerability.”⁵ Therefore, repeated mild TBI is expected to result in more severe damage as compared to a single mild TBI.

Repeated TBI causes acute inflammation, sustained increase in glial cell immunostaining, and cognitive impairment.^{6,7} Owing to

sustained pathological changes, many cases of repeated TBI may eventually lead to chronic traumatic encephalopathy.⁸ Mild TBI also results in diffuse axonal injury, with the vulnerable axons damaged from acceleration-deceleration forces. The affected axons may have disrupted transport followed by axonal swelling, secondary disconnection, and Wallerian degeneration.⁹ Given that white matter tracts relay information across different brain parts and the peripheral organs, their selective vulnerability to acceleration forces has important implications for the functional deficits that occur after TBI.¹⁰ White matter abnormalities have been detected by diffusion tensor imaging in the brains of individuals after mild TBI who also display functional deficits.^{11–13} Changes in the white matter, including increased microgliosis, astrogliosis, and axonal injury, have been reported in the corpus callosum, optic tract, and corticospinal tract after repeated TBI.^{7,14,15}

Along with other deficits, TBI can also lead to visual dysfunction and visual impairment.^{16–19} Whereas moderate-to-severe TBI can cause structural lesions in brain regions relevant to vision, even mild TBI can cause vision impairment.²⁰ Visual complaints after TBI include photophobia, double vision, blurred vision, visual processing problems, and loss of vision.²⁰ Despite this fact, there

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have been very few pre-clinical studies designed to investigate the effect of TBI on visual function. In the present study, we investigated the effects of repeated mild TBI on vision and behavior in a mouse model.

Methods

Repeated traumatic brain injury model

C57BL/6Ncr mice were purchased from Charles River (Wilmington, MA) and were kept in the animal facility with free access to food and water. Closed head injury by mechanically engineered rotational acceleration (CHIMERA)^{7,14} was used to induce repeated TBI. Mice at 4–5 months of age were anesthetized with isoflurane and strapped supine at an angled position on the CHIMERA apparatus with the head resting flat on the base plate. A computer-controlled, pneumatically driven piston was then fired from below to strike the head of the mouse, delivering 0.55 J of energy. The mouse was immediately removed from the apparatus and placed in the home cage to recover. The whole procedure was completed within 5 min per mouse. This procedure was repeated for 3 successive days for the repeated injury group, producing a three-hit model with 24 h between each injury. The single TBI group was injured on the last day of the repeated TBI group so that both groups had the same time post-injury for further testing. The sham group was anesthetized with isoflurane and strapped to the device but not injured. Half the mice in the sham group were anesthetized once whereas others were anesthetized for 3 consecutive days.

Morris water maze

The water maze (120 cm in diameter) was filled with water (22–24°C) with non-toxic white paint. The visible platform water maze was conducted at 19 days after injury followed by the hidden platform water maze at 24 days after injury. For the visible platform test, a black disk was placed on the platform, and it was marked with a red and white flag to clearly indicate its location. Each mouse was trained for 3 consecutive days with four trials each day. Mice were released into the water and given 60 sec to locate and climb onto a 10-cm² platform. If they were unable to locate the platform within 60 sec, they were gently guided to it. The platform location was changed for each trial.

Open field test

Mice were tested in the open field at 32 days after injury. Mice were individually placed in the acrylic, black open field apparatus (40 cm²) and allowed to freely explore the arena for 5 min. Distances covered during the test, time spent in the center, and the periphery were considered as the relevant parameters.

Visual cliff test

The visual cliff test has been used to assess depth perception and visual acuity.²¹ Mice were tested at 34 days after injury. A flat, transparent acrylic surface was partly lined by a checkerboard pattern with the rest of the surface overhanging around 50 cm above another surface lined with a similar checkerboard pattern to give the illusion of a “cliff.” A small tube (4 cm in diameter) was taped at the junction of the checkerboard covered and the transparent surface. In each trial, the mouse was gently placed on the tube with its head directed along the length of the tube. It was removed as soon as it stepped down, and the side chosen (checkerboard = safe or transparent = cliff) was recorded. Each mouse was given 10 trials.

Visual evoked potential

Flash visual evoked potential (VEP) was used to assess visual function at five weeks after injury using ColorDome Ganzfeld (Diagnosys LLC, Lowell, MA). Mice were injected with ketamine and xylazine intraperitoneally and placed on a heated platform. A reference needle electrode was inserted in the lower lip whereas the ground electrode was inserted in the tail. The test electrode was inserted medially on the head through the scalp such that it was in contact with the skull over the visual cortex. Drops of 1% tropicamide were put in the eyes to dilate the pupils, and the mouse was placed on a heated platform with its head covered by the Ganzfeld dome. The flash stimuli of white light (6500K) were generated by ColorDome Ganzfeld. The flash stimuli had an intensity of 3.0 cd-s/m² with each set including 100 sweeps. Three sets of readings were recorded and averaged. The sample frequency was 1000 Hz with the sweep pre-trigger and post-trigger time at 10 and 250 ms, respectively. Espion software (V6.0.56; Diagnosys LLC, Lowell, MA) was used to generate and record the readings and obtain the data table for N1 amplitude. Readings for all sweeps were manually averaged to obtain the VEP wave. After the test, mice were transferred to the home cage on a heating pad and allowed to regain consciousness.

Immunohistochemistry

Mice were perfused with chilled phosphate-buffered saline (pH 7.4) followed by chilled 4% paraformaldehyde under isoflurane anesthesia at 5 weeks after injury. Brains were removed, fixed in 4% paraformaldehyde for 24 h, and immersed in 30% sucrose. Twenty-five-micrometer sections were cut through the optic tract using a cryostat. Sections were blocked in 5% bovine serum albumin and immersed overnight at 4°C in anti-GFAP (glial fibrillary acid protein) or anti-Iba-1 (ionized calcium binding adaptor molecule 1) antibody, washed, and immersed in fluorescent-tagged secondary antibody before mounting them on slides. Images of immunostained brain sections were captured using an attached digital camera (Hamamatsu Photonics K. K., Hamamatsu City, Japan) and immunofluorescence quantified for the optic tract using Metamorph software (Version 7.10.0.119; Molecular Devices, San Jose, CA).

Silver staining was performed using the FD NeuroSilverTM Kit II (FD NeuroTechnologies, Columbia, MD). Brain sections were immersed in 4% paraformaldehyde at 4°C for 7 days and stained according to the manufacturer's suggested protocol. Sections were then mounted on slides and visualized under brightfield illumination.

Statistical analyses

One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to analyze data of open field activity and the visual cliff test. Repeated-measures two-way ANOVA, followed by Tukey's multiple comparisons, was used for the analysis of the Morris water maze test. The N1 amplitude for VEP was analyzed by the Kruskal-Wallis test with Dunn's correction for multiple comparisons. Results are expressed as mean ± standard error of mean (SEM), and *p* values <0.05 were considered statistically significant.

Results

Morris water maze performance after single and repeated closed head injuries in mice

The visible platform test was performed for 3 consecutive days with four trials per day. There was a significant main effect of time and injury on the latency to platform and the distance to platform (*p* < 0.001) as well as that of average speed (*p* < 0.01; Fig. 1). The

sham and single TBI groups had comparable time to platform as well as speed and distance to platform whereas the repeated TBI group took significantly longer to get to platform than the other two groups (Fig. 1A). The repeated injury group had marginally slower average swim speed (Fig. 1B) and swam a significantly longer distance on all test days ($p < 0.01$; Fig. 1C) than the other two groups. Striking differences were observed on the first day of the test for both the time to platform, which was 2- to 3-fold that of the sham and single injury groups, and the distance, which was 2- to 4-fold that of the sham and single injury groups. Although the performance of the repeated injury group improved for both parameters over the 3 days of testing, the relative difference of this group remained the same or even increased by the third day when compared to the other two groups for the respective parameter. These results indicate that multiple, but not single, mild TBI impair water maze performance.

Open field activity at 32 days after single and multiple TBI in mice

The total distance traveled in the open field and the time in center was analyzed over a 5-min period. The repeated injury group had

increased scores for both parameters whereas no difference was observed between the sham and single injury groups. Sham and single injury mice had mean distance of 12.78 ± 1.36 and 11.83 ± 1.08 m, which was less than the 16.33 ± 0.72 meters traveled by the repeated injury group (Fig. 2A). The distance traveled by the repeated injury group was significantly greater than the single injury group ($p < 0.05$). Similarly, the time in center was comparable between the sham (4.53 ± 0.87) and single injury (9.20 ± 3.32) groups and was increased for the repeated injury group (15.28 ± 2.53), with the increase being statistically significant compared to sham ($p < 0.05$; Fig. 2B). These data indicate that the mice suffering from repeated TBI are hyperactive in the open field, at least in response to the novel environment. The increased exploratory activity along with more time spent in the center of the open field by the repeated TBI group may also be attributable to decreased inhibition/anxiety-like behavior.

Visual cliff test on day 34 after single and repeated TBI in mice

The visual cliff test was used to assess whether mice display a strong preference for the "safe" side after single or multiple TBI

FIG. 1. Multiple TBI in mice impair performance in the Morris water maze test with visible platform. Sham, single TBI, and multiple TBI mice were tested in the visible platform water maze from day 19 to day 21 after injury. Significant increase was observed in the latency (A) and distance (C) to platform of the mice with repeated injuries as well as reduced average swim speed (B) compared to sham and single injury mice. Data are shown as average $\pm$ SEM and analyzed by two-way ANOVA with Tukey's multiple comparisons test; $n = 10$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to respective sham; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ compared to respective single injury. ANOVA, analysis of variance; CHI, closed head injury; rCHI, repeated closed head injury; SEM, standard error of the mean.

FIG. 2. Multiple TBI in mice alter open field activity. Sham, single, and multiple injury mice were tested in the open field at 32 days after injury. Mice with repeated head injuries traveled more in the open field (A) and also spent more time in the center of the open field (B). Data are shown as average $\pm$ SEM and analyzed by one-way ANOVA with Tukey's multiple comparisons test; $n=10$. * $p<0.05$ compared to sham; # $p<0.05$ compared to single injury. ANOVA, analysis of variance; CHI, closed head injury; rCHI, repeated closed head injury; SEM, standard error of the mean.

(Fig. 3A). In the 10 step-down trials, the sham group stepped on the "cliff" side for an average of 1.4 ± 0.37 whereas the single injury group stepped 2.9 ± 0.64 times, displaying a preference for the safe side. On the other hand, the repeated injury group did not prefer either side and, on an average, chose to step down 4.1 ± 1.7 times on the cliff side (Fig. 3B). Analysis by one-way ANOVA showed a significant difference between sham and repeated injury groups with an adjusted p value of 0.0036. These results imply that mice with repeated TBI are unable to distinguish between the safe and cliff sides, which may be a result of decrease in visual acuity.

Visual evoked potential on day 35 after single and repeated TBI in mice

The VEP differed in a graded fashion, with more injuries resulting in greater deviation from the sham (Fig. 4A). The N1

component of the VEP showed the most striking changes in the injured mice. Whereas the average N1 value of the sham mice was 59.3 ± 4.03 , the single injury group had lower value (41.6 ± 5.3) whereas the multiple injury group had a still lower value of 16.0 ± 3.6 that was significantly different from both the sham and the single injury groups. The drastic alteration in VEP pattern in the mice having multiple TBI suggests that there are changes in the visual signaling.

Immunostaining for glial fibrillary acid protein and ionized calcium binding adaptor molecule 1 in the optic tract of mice with single and repeated closed head injury

GFAP and Iba-1 immunostaining signals detected in the optic tract progressively increased from sham to repeated TBI samples,

FIG. 3. Multiple TBI in mice result in increased errors in the visual cliff test. On day 34 after injury, sham, single TBI, and multiple TBI mice were placed on a tube at the interface of "safe" and "cliff" side (A) and allowed to step down. Mice that had multiple injuries did not show a clear preference for either side, unlike the other two groups, which avoided stepping on the "cliff" side (B). Data are shown as average $\pm$ SEM and analyzed by one-way ANOVA with Tukey's multiple comparisons test; $n=10$. * $p<0.05$ compared to sham. ANOVA, analysis of variance; CHI, closed head injury; rCHI, repeated closed head injury; SEM, standard error of the mean.

FIG. 4. Mice with multiple TBI showed impaired visual evoked potential pattern. Visual evoked potential was recorded in sham, single, and multiple TBI mice on day 35 after injury. Single injury mice had slight deviation of the VEP whereas multiple TBI mice had distinctly different VEP compared to the sham (**A**). The N1 amplitude of the multiple injury group was significantly reduced from either of the other two groups (**B**). Data are shown as average $\pm$ SEM and analyzed by Kruskal-Wallis test with Dunn's correction; $n=10$. *** $p<0.001$ compared to sham; # $p<0.05$ compared to single injury group. CHI, closed head injury; rCHI, repeated closed head injury; SEM, standard error of the mean; VEP, visual evoked potential.

FIG. 5. GFAP immunostaining in the optic tract increases after single and multiple mild TBI. Increased GFAP immunostaining was observed in the optic tract of single injury and multiple injury mice at 5 weeks after injury. Data are shown as average $\pm$ SEM and analyzed by one-way ANOVA with Tukey's multiple comparisons test; $n=4-5$. * $p<0.05$ and ** $p<0.01$ compared to sham. ANOVA, analysis of variance; CHI, closed head injury; GFAP, glial fibrillary acid protein; rCHI, repeated closed head injury; SEM, standard error of the mean. The optic tract is indicated by dotted lines.

indicating that these glial cell markers increase in proportion to the injury and are expressed more because of cumulative effects of repeated injuries. Mean fluorescence intensity of GFAP immunostaining was increased from 676 ± 38 in the sham to 1258 ± 102 in the case of single injury and 1577.0 ± 202.5 for the multiple injury group (Fig. 5). The increase in both single and repeated injury groups was statistically significant compared to sham ($p=0.04$ and $p=0.003$, respectively). Similarly, Iba-1 immunofluorescence increased from 400 ± 14 in the sham mice to 546 ± 32 in the single injury and 703 ± 50 in the repeated injury mice (Fig. 6). The increase was statistically significant between the sham and repeated injury groups and between the single injury and repeated injury groups ($p=0.0005$ and $p=0.02$, respectively).

Silver staining of brain sections of mice with repeated TBI

Silver staining of brain sections revealed a conspicuous uptake of the stain by the optic tract region of mice with repeated head injuries whereas there was negligible staining of the optic tracts of

sham mice (Fig. 7). This indicates the presence of degenerating axons in the optic tract as a result of repeated head injuries.

Discussion

Visual impairment is a common result of TBI in human populations.²² However, interest in its occurrence in pre-clinical settings is beginning to be appreciated in recent reports. Given that vision drives our perception and interpretation of the world, it is important to investigate the effect of TBI on this function. To the best of our knowledge, this is the first study that investigates visual impairment in a rotational acceleration model of head injury allowing for unconstrained head movement on impact. In this study, we studied the impact of single versus multiple mild TBI on vision in two ways—by assessing visual acuity using behavior through the visual cliff test (Fig. 3) and water maze (Fig. 1) and electrophysiologically by VEP (Fig. 4).

Some fairly recent publications have documented the effects of TBI on visual function in different animal models.^{23–27} A single blast exposure was found to reduce visual acuity, increase Iba-1 staining in the optic tract, and lead to axonal injury in mice.^{26,27}

FIG. 6. Iba-1 immunostaining in the optic tract increases after multiple TBI. Iba-1 immunostaining increased in the optic tract of multiple injury mice at 5 weeks after injury. Data are shown as average $\pm$ SEM and analyzed by one-way ANOVA with Tukey's multiple comparisons test; $n=4-5$. *** $p<0.001$ compared to sham; # $p<0.05$ compared to single-injury group. ANOVA, analysis of variance; CHI, closed head injury; Iba-1, ionized calcium binding adaptor molecule 1; rCHI, repeated closed head injury; SEM, standard error of the mean. The optic tract is indicated by dotted lines.

FIG. 7. Increased silver staining in the optic tract of mice with repeated TBI. Silver staining is distinctly observed in the optic tract of mice with repeated TBI at 5 weeks after injury. The figure shows representative images from two different sham mice and three different rCHI mice. rCHI, repeated closed head injury.

Blast injury models of TBI are likely to directly injure the ocular tissue and lead to visual deficits. However, no correlation was found between the use of ballistic protective eyewear by war fighters and the extent of closed-eye injury, indicating that the blast wave may penetrate the protective barrier or travel around it.²⁴ It may also indicate that TBI results in vision abnormalities in the absence of direct damage to ocular tissue. The term “cerebral visual impairment” has been used to describe visual disturbances attributable to disorders of the visual pathways behind the optic chiasm and visual perceptual dysfunction because of damage or malfunction of the visual association territories of the brain and their pathways.²⁸ Some studies have used closed head injury models to explore the effect of single²⁹ or multiple TBIs on vision or vision-related regions in the brain.^{15,25,30} Single²⁹ as well as repeated TBI¹⁵ result in increased Iba-1/GFAP immunostaining and degeneration of neurons in the optic tract.

In our study, significant increase in GFAP, but not Iba-1, was observed in the optic tract between sham and single injury mice (Figs. 5 and 6). Studies using the weight-drop model for repeated TBI have focused on the retina and optic nerve,^{15,25,30} with electroretinography as the functional test used to reveal difference in the photopic negative response amplitude attributable to repeated TBI.³⁰ We have not found any significant difference in electroretinography when comparing sham and rCHI mice (Supplementary Fig. S1), which indicates that the changes in VEP are observed because of injury to the optic nerve and/or to the connecting regions such as the optic tract. We have previously observed increased glial immunostaining in the optic tract of repeatedly injured mice at different time points from 1 week to 6 months after injury, which indicates that this feature is both robust and sustained.⁷ We have found impaired VEP to be a chronic feature that lasts for at least 4 months after repeated TBI. Therefore, it is tempting to speculate an association between inflammation in the brain regions that convey visual signals with impaired vision.

However, given that studies have found increase in gliosis in the optic tract without affecting vision,³¹ inflammation in the optic tract may be insufficient to affect visual function. Increased silver staining and presence of axonal degeneration has been reported after single and multiple TBI.¹⁵ We also found increase in silver staining after repeated TBI (Fig. 7), indicating axon degeneration, which, together with increased inflammation, may have caused impairment in parameters related to vision.

Many studies have used behavioral tests to evaluate visual function and acuity. These include the visual cliff test and the visible platform version of the Morris water maze test.^{32–34} The visual cliff test has been rarely used for TBI models. The visible platform water maze has been used in a few studies with Shitaka and colleagues reporting increased mean time to platform and decreased mean velocity in injured mice on day 6 after repeated (two injuries 24 hours apart) concussive injuries with no significant changes in the distance.³⁵ Other studies do not find changes in the visible platform water maze performance attributed to TBI. Velosky and colleagues did not find change in latency to platform in mice that suffered five repeated concussive injuries 24 h apart.³¹ In our study, the multiple TBI group took significantly more time and covered more distance to find the visible platform and also had less swim speed than the sham group whereas the single TBI group did not differ from the sham (Fig. 1). The reduced swim speed of the multiple injury group in the water maze may be an indicator of residual motor deficits attributed to repeated injuries. However, this is improbable given that the mice increased open field exploration after repeated TBI (Fig. 2). Also, according to our previous finding, no changes were observed in the performance of mice on the beam walk beyond the first 3 days after injury.⁷

Although the results presented in this study are coherent, a possibility of potential confounding effect cannot be ruled out. The reduced swim speed of the repeatedly injured mice in the water maze may be attributable to lack of motivation. Also, increase in

impulsivity in mice after repeated injuries may make them prone to more mistakes in the visual cliff test. Given that both motivation and impulsivity are affected by TBI,^{36–38} additional tests, such as functional optometry, can be done to verify the findings presented in this study.

Taken together, the effect of multiple TBI on VEP, visual cliff test, and water maze tests indicated that multiple injuries likely result in some degree of visual impairment. Given that cognitive tests used to evaluate functional endpoints in TBI models, such as the water maze and Barnes maze, have vision as the primary sensory component, a difference in vision attributed to TBI is likely to obfuscate behavioral test results. Thus, there is a need to include cognitive tests in mice that are less dependent on vision. Also, in addition to changes in cellular/biochemical markers in the optic tract, it may also be advisable to include a functional test such as VEP, especially when monitoring temporal progression of injury outcome as well as testing the putative neuroprotective or anti-inflammatory compounds in TBI.

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All animal procedures were done according to NIH Guide for Care and Use of Laboratory Animals. The animal study protocol (LMS-HK-13) was approved by the institutional Animal Care and Use Committee.

Ethics Approval

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Author Disclosure Statement

No competing financial interests exist.

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