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Long-Lasting Suppression of Acoustic Startle Response after Mild Traumatic Brain Injury

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Abstract

Acoustic startle response (ASR) is a defensive reflex that is largely ignored unless greatly exaggerated. ASR is suppressed after moderate and severe traumatic brain injury (TBI), but the effect of mild TBI (mTBI) on ASR has not been investigated. Because the neural circuitry for ASR resides in the pons in all mammals, ASR may be a good measure of brainstem function after mTBI. The present study assessed ASR in Sprague-Dawley rats after mTBI using lateral fluid percussion and compared these effects to those on spatial working memory. mTBI caused a profound, long-lasting suppression of ASR. Both probability of emitting a startle and startle amplitude were diminished. ASR suppression was observed as soon as 1 day after injury and remained suppressed for the duration of the study (21 days after injury). No indication of recovery was observed. mTBI also impaired spatial working memory. In contrast to the suppression of ASR, working memory impairment was transient; memory was impaired 1 and 7 days after injury, but recovered by 21 days. The long-lasting suppression of ASR suggests long-term dysfunction of brainstem neural circuits at a time when forebrain neural circuits responsible for spatial working memory have recovered. These results have important implications for return-to-activity decisions because recovery of cognitive impairments plays an important role in these decisions.

Key words: concussion; lateral fluid percussion; memory; pons; spatial learning

Introduction

Mild traumatic brain injury (mTBI), otherwise known as concussion, is a major health concern and accounts for a large proportion of brain injuries. In the absence of biomarkers or pathology upon structural brain imaging, mTBI is diagnosed on functional disturbances (altered or loss of consciousness [LOC], reduced Glascow Coma Scale scores, and post-trauma amnesia). Complaints span three domains: somatic (e.g., headaches, dizziness, nausea, sleep disturbances, and sensory disturbances); psychological/behavioral (e.g., anxiety, fatigue, interpersonal problems, and depression); and cognitive (e.g., memory loss, problems concentrating and maintaining attention, disorientation, fog, LOC, and impaired information processing).[1]

Until recently, mTBI was generally considered a temporary problem for most, with complaints resolving in a matter of hours to days. The transient nature of the complaints might suggest minimal or no brain injury, but such assumptions are being seriously reconsidered in light of the cytological, axonal, and molecular disturbances that occur after mTBI.[2,3] With the number of incidences of brain insults far outweighing the incidence of substantial post-concussive symptoms (PCS), two possibilities exist. The first possibility is that concussions are generally benign, having mostly subtle and transient sequelae. Further, a small percentage of the population can develop severe or persistent sequelae owing to injury dynamics, site of injury, and severity of injury. The second possibility is that concussions cause persistent sequelae in most everyone, but these effects are not appreciated because our sensitivity is inadequate. Insensitivity may be owing to effects remaining below the level of awareness or not being sufficiently disruptive to everyday life. Although disruption may not arise to the level that one complains, unreported sequelae would be a sign that injury-induced brain changes have not completely resolved.

The effect of mTBI on the acoustic startle response (ASR) has received little attention. ASR is a highly conserved, defensive response to a loud auditory stimulus that can involve whole-body movement or more subtle movement (i.e., eyeblink),[4] utilizes a simple trisynaptic reflex in the brainstem and spinal cord,[5] and indicates integrity of sensorimotor processes, nonassociative learning, and psychological state of a subject (i.e., exaggerated in

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anxious states and fear).[6,7] In humans, moderate-to-severe TBI suppressed ASR in one study,[8] but not in another.[9] The variable results may be owing to differences in injury parameters, such as severity, dynamics, location, and time after injury that are difficult to control in human studies. Further, changes in affective states of individuals with TBI were the focus of these studies, not whether or not ASR was altered after TBI. In rats, effects of TBI on the ASR have been investigated using controlled cortical impact (CCI) and fluid percussion injury (FPI). ASR was suppressed after injuries that cause gross damage of the brain.[10–12] Startle to a tactile stimulus was also suppressed, suggesting a dysfunction of the startle circuit, rather than a specific sensory impairment.[12] Suppression was quite persistent, evident for weeks after injury. A similar suppression of ASR was observed after mTBI with lateral fluid percussion (LFP), but ASR was only monitored up to 1 week.[13] A suppressed acoustic startle after mTBI could potentially be used as an objective sign of concussion because ASR is highly reproducible and translational to humans.

The neural circuit essential for ASR has been identified and is localized to the pons and spinal cord.[5,14] Auditory information enters the brainstem at the level of the cochlear nucleus. This information is relayed to the caudal pontine reticular nucleus (PnC). From the PnC, signals descend to the spinal cord through the reticulospinal tract. In this circuit, the PnC is a key contributor to sensorimotor integration and plasticity of ASR.[15,16] Though previous studies described ASR suppression after TBI, the integrity of the essential circuit was not investigated.

The aim of the present study was to determine whether ASR is altered after mTBI. Three experiments were conducted. Experiment 1 assessed the effect of mTBI on ASR 24 h after injury and evaluated cell degeneration and cell loss of brain areas important for ASR and spatial working memory. Experiment 2 evaluated the chronic effects of mTBI on ASR for 21 days. Finally, experiment 3 evaluated the effects of mTBI on spatial working memory, because working memory impairment represents a symptom that has been well studied and is often reported after mTBI.[17–19]

Methods

Subjects

Male Sprague-Dawley rats (approximately 3 months of age) were used in this study. Rats were housed individually in a room with a 12-h light/dark cycle. Food and water was available *ad libitum*. All procedures were conducted in accord with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the institutional animal care and use committee of the Veterans Affairs Medical Center (East Orange, NJ).

Surgery

Rats were anesthetized with a ketamine/xylazine mixture (60 mg/7 mg per kg, intraperitoneally) and placed in a stereotaxic instrument. A craniectomy (4 mm diameter, centered at 3 mm posterior to bregma and 3.5 mm lateral to midline) was made unilaterally in either the left or right parietal bone plate; left and right locations were counterbalanced across animals. A Luer-Lok connector was glued to the skull surrounding the craniectomy. A plastic cylinder (cut from a 12-mL syringe) surrounded the craniectomy to protect the Luer-Lok connector. Dental cement fixed the plastic cylinder and Luer-Lok connector to stainless steel screws inserted in the skull. A small piece of Kimwipe was inserted into the connector to keep the dura clean of debris.

Fluid percussion

To control for individual differences in ASR reactivity, rats were matched for ASR amplitude at 102 decibels (dB), then randomly assigned to sham and mTBI groups. Fluid percussion injury was produced as described previously.[20,21] One day after surgery, rats were lightly anesthetized with isoflurane. The Kimwipe was removed from the Luer-Lok connector, the connector was filled with saline, and the Luer-Lok was connected to the fluid percussion device. Rats in the sham group underwent all procedures experienced by rats in the injury group, except the fluid percussion impact. Rats in the mTBI group received a fluid percussion impact by a device that utilized a computer-controlled voice coil.[22,23] In this device, a voice coil controlled the movement of a piston in a Plexiglas cylinder, analogous to a plunger in a syringe. At the opposite end of the cylinder was a Luer-Lok connector that could be mated to the Luer-Lok connector on the rat's skull. The cylinder was filled with water so that movement of the voice coil/piston transmitted pressure waves through the water-filled cylinder to the dura mater of the rat. A pressure sensor was located in the cylinder in contact with the water to measure pressure changes associated with piston movement. The pressure wave inducing mTBI was recorded. Presence of hyperextension of the limbs and tails as well as duration of apnea were recorded. In addition, recovery of the righting reflex was assessed. All rats were placed supine after injury, and the righting latency was defined as the time to right themselves on all four limbs spontaneously, not induced by tail or foot pinch. After recovery, rats were placed back in their home cage and moved to the housing room. Peak pressure, peak latency, apnea duration, and righting latency are shown for the three studies in Table 1.

Acoustic startle response

ASR was assessed before injury and then 1, 7, and 21 days after injury using procedures previously described.[24] Rats were placed in holders located on startle sensor platforms (Coulbourn Instruments, Langhorne, PA). A 15-min test session consisted of the presentation of 24 white noise bursts (100 ms with a 5-ms rise/fall time). Acoustic stimuli with three different intensities (82-, 92- or 102-dB) were presented in pseudorandom order in which each intensity was presented in each block of three trials (eight repetitions of each intensity). The interstimulus interval varied between 15 and 25 sec. Startle responses were analyzed offline. The test chamber was wiped with a soap solution between the testing of each rat.

A startle response was scored if movement exceeded a response threshold during a 250-ms window starting at the onset of the stimulus. If movement did not exceed the threshold, no startle response was scored for that trial. A baseline period consisting of 250 ms preceding stimulus onset was used to determine the response threshold. Response threshold was equal to the sum of the maximum amplitude during the baseline period and 4× standard deviation of the baseline activity. Two measures of ASR were calculated at each stimulus intensity: sensitivity and amplitude. Sensitivity is the probability of a startle response. Amplitude is the

Table 1. Peak Pressure and Acute Signs for Rats Receiving Lateral Fluid Percussion Injury[a]

	Peak pressure (psi)	*Peak latency (ms)*	*Apnea (sec)*	*Righting reflex (sec)*
Experiment 1	18.9 ± 1.3	19.9 ± 0.9	12.9 ± 3.3	519 ± 93
Experiment 2	23.2 ± 1.5	19.5 ± 0.8	11.0 ± 1.3	510 ± 33
Experiment 3	19.5 ± 1.4	19.7 ± 0.9	13.0 ± 5.5	554 ± 34

[a]Mean ± standard error of the mean.

magnitude of the startle response. Trials in which movement did not reach threshold (i.e., no startle response) are not included in the calculation of startle amplitude. However, for rats in which no response was evident for all eight trials of an intensity in a session, a true zero was recorded for that stimulus intensity.

Working memory

Working memory was assessed in a water maze before injury and then 1, 7, and 21 days after injury using a procedure similar to that described in Kobori and colleagues.[19] Briefly, rats were trained to find an escape platform (10×10 cm) located below the water surface in a pool (1.5 m diameter). A session consisted of 12 trials (one session per day). Each trial had two phases (sample phase followed by choice phase). During the sample phase, the escape platform was placed randomly in the pool. Rats were started from a predetermined location, but never in the quadrant with the escape platform, and allowed 60 sec to search for the platform. If the rat did not find the platform within 60 sec, it was led to the platform. The choice phase commenced after a variable retention interval of 0, 15, or 60 sec. The start location and the escape platform location were the same as in the sample phase. The rat was allowed 60 sec to find the escape platform. The rat was put in a holding cage for 10 min before the next trial commenced. For each session, the locations of the start and escape platform were distributed such that each quadrant was used on three trials as a start location and on three trials containing the escape platform. In addition, the three retention intervals were utilized four times in a session. Swim paths were recorded for offline analysis of path efficiency (straight-line distance between start and escape platform location/total distance traveled; a value of 1 indicates the most efficient path) and swim speed.

Pain sensitivity

At 24 h postinjury, rats were tested for pain sensitivity on a hotplate. The surface of the plate was heated to 52°C and maintained at this temperature. After a rat was placed on the plate, latency to lick either of the front two paws was recorded and the rat was removed from the hotplate.

Histology

After behavioral testing, rats were perfused with formalin or 4% paraformaldehyde, and brains were removed and examined for gross brain damage (i.e., cavitation) at the site of the craniectomy. Rats with gross damage were excluded from further analysis. In some rats sacrificed 1 day after injury, brains were further processed for analysis using light microscopy. After postfixation overnight in formalin, brains were transferred to a 30% sucrose solution. Brains were sectioned at 50 μm on a freezing sliding microtome and stained with cresyl violet or Fluoro-Jade C (Millipore, Billerica, MA).[25,26] For the Fluoro-Jade stain, sections were incubated in 70% ethanol for 2 min, washed in distilled water, and then followed by incubation in 0.06% potassium permanganate for 10 min and a wash. Sections were incubated in 0.0001% Fluoro-Jade C with 0.001% acetic acid for 10 min. After a final wash, sections were dried, rinsed in xylene, and cover slipped with DPX. Neurons labeled by Fluoro-Jade C were visualized using a fluorescein isothiocyanate filter set on a Leica DM4000B-M microscope (Leica Microsystems, Wetzlar, Germany). Quantification of neurons stained with Fluoro-Jade C or cresyl violet was accomplished by unbiased stereology using the optical fractionator method (40× objective, Stereo Investigator v.9.10.5; MicroBrightField, Colchester, VT). Areas examined were the cortex below the craniectomy and corresponding contralateral site, both hippocampi because of the importance of this brain region in spatial working memory, and the PnC and the cochlear nucleus because these nuclei are part of the essential neural circuit for ASR.[5] The cortex was examined from midline to the rhinal fissure between the anterior and posterior extents of the craniectomy. A similar cortical region contralateral to the craniectomy was evaluated. Further, between the anterior and posterior extents of the craniectomy, dorsal hippocampus, including CA1, CA3, and dentate gyrus, were investigated bilaterally. For the cortex, hippocampus, and PnC, four sections equally spaced throughout each brain region were counted.

Statistical analysis

Values are expressed as mean ± standard error of the mean. Statistical analysis was performed using SPSS for Windows (version 12.0.1; SPSS, Inc., Chicago, IL) with $\alpha = 0.05$. Effects of mTBI on Fluoro-Jade C staining were assessed in a mixed-design analysis of variance (ANOVA) with injury as a between-subject factor and brain region and side as within-subject factors. Total numbers of giant PnC cells in sham and mTBI groups were compared with an independent-samples *t*-test. Effects of mTBI on ASR sensitivity and amplitude were determined by a mixed-design ANOVA with injury as a between-subjects factor and session (preinjury or various times postinjury) and intensity as within-subject factors. Effects of mTBI on working memory were assessed in a mixed-design ANOVA with injury as a between-subjects factor and time, retention interval, and phase (sample or choice phase) as within-subject factors. Follow-up post-hoc analysis was performed using the *F*-test. When repeated-measures factors failed sphericity tests, Greenhouse-Geisser's correction was performed. When uncorrected and corrected *p* values were less than the level of significance ($\alpha = 0.05$), the uncorrected *p* value was reported. When the corrected *p* value was greater and the uncorrected *p* value less than $\alpha = 0.05$, both uncorrected and corrected *p* values were reported. Hotplate measures were evaluated using a Student's *t*-test. Comparisons of peak impact pressure and acute signs between experiments were performed using a one-way ANOVA with Tukey's honest significant difference test for post-hoc comparisons.

Results

Experiment 1: acute effects of mild traumatic brain injury on acoustic startle response

The effects of mTBI on ASR and pain sensitivity were assessed 24 h after injury. Four rats were removed from the study because they died from surgical complications ($n = 1$) or displayed gross damage of the injury site ($n = 3$). The paired sham rats were likewise removed from the study. Data from the remaining rats ($n = 9$ sham and 9 mTBI) were analyzed. Rats in the mTBI group experienced a fluid percussion impact with peak pressure of 18.9 ± 1.3 psi and a peak latency of 19.9 ± 0.9 ms (Table 1). These rats displayed hyperextension of limbs after impact, apnea lasting 12.9 ± 3.3 sec, and a righting latency of 519 ± 93 sec. In comparison, sham rats had a righting latency of 44 ± 18 sec, owing to recovery from anesthesia, with no apnea or limb hyperextension. All mTBI rats had longer righting latencies than sham rats. None of the sham or mTBI rats displayed behavioral signs of seizures.

Acoustic startle response

mTBI reduced the probability of an ASR. ASR sensitivity after mTBI was decreased in comparison to sham rats and in comparison to their own preinjury levels (Fig. 1A). mTBI reduced ASR sensitivity, as demonstrated by a main effect of injury ($F_{(1,16)} = 15.33$; $p = 0.001$) and an injury × session interaction ($F_{(1,16)} = 6.17$; $p = 0.024$). Neither the injury × intensity nor the injury × intensity × session interactions reached significance.

mTBI suppressed ASR amplitude in comparison to sham rats and compared to their own preinjury amplitude (Fig. 1B). mTBI

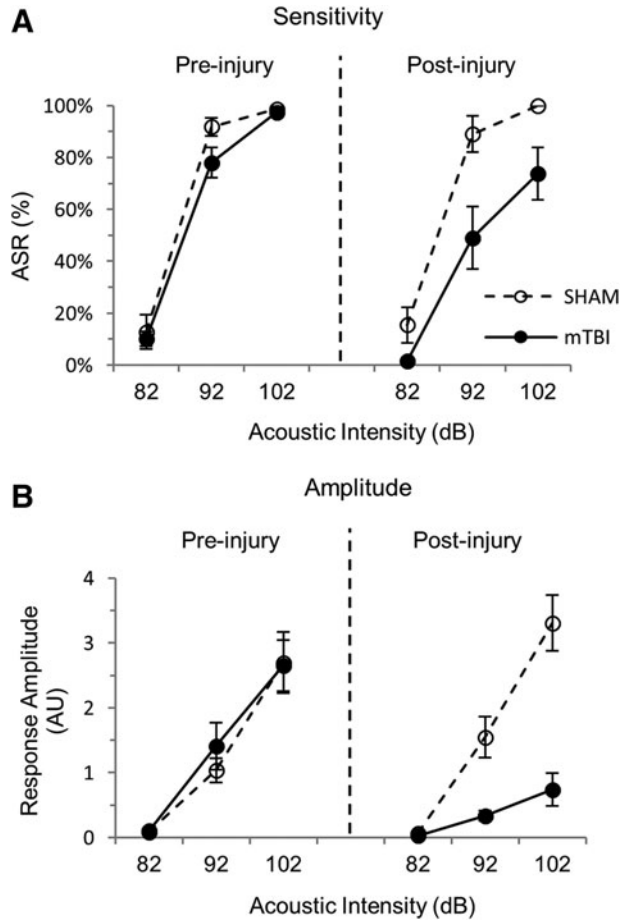

FIG. 1. Acute effects of mTBI at 24 h after injury. Sensitivity (probability of startle, **A**) and amplitude (**B**) of acoustic startle were diminished after lateral fluid percussion injury. ASR, acoustic startle response; mTBI, mild traumatic brain injury; AU, arbitrary units; dB, decibels.

impaired ASR magnitude, demonstrated by significant main effect of injury ($F_{(1,16)} = 5.95$; $p = 0.027$) and interactions involving injury (injury × session ($F_{(1,16)} = 19.04$; $p < 0.001$), injury × intensity ($F_{(2,32)} = 5.47$; $p = 0.009$), and injury × session × intensity ($F_{(2,32)} = 15.86$; $p < 0.001$). Thus, both ASR sensitivity and amplitude were suppressed 24 h after mTBI.

Pain sensitivity

Pain sensitivity was assessed on a hotplate. Mean paw-lick latency was 8.7 ± 1.8 and 11.8 ± 3.2 sec for sham and mTBI groups, respectively. These latencies did not differ between groups ($t_{(16)} = 0.84$).

Histology

The cortex immediately below the craneoctomy showed some disruption of the cortical cell layers in the mTBI group (Fig. 2B). Outside of the immediate area of impact, cortical layers appeared to be intact. In Nissl staining, the hippocampus beneath the site of impact appeared normal, as did the PnC (Fig. 2B).

To quantify cell degeneration after LFP, Fluoro-Jade C staining was performed on 4 sham and 4 mTBI rats at 24 h after the injury procedure. Fluoro-Jade–labeled cells were observed in the cortex and hippocampus, but not PnC or cochlear nucleus; therefore, brainstem sites were not included in subsequent stereological analysis of Fluoro-Jade labeling. Fluoro-Jade–positive cells were more abundant in the cortex and hippocampus of mTBI rats, compared to sham (Table 2). This observation was supported by a main effect of injury ($F_{(1,6)} = 7.55$; $p = 0.033$). Additionally, more degenerating cells were present in the cortex than hippocampus ($F_{(1,6)} = 10.25$; $p = 0.019$). However, the number of degenerating cells did not differ between ipsilateral and contralateral sides and none of the interactions reached significance (all Fs < 3.39).

Because Fluoro-Jade staining was not observed in the PnC and cochlear nucleus, the effect of LFP on PnC was assessed by estimating the total number of PnC giant neurons in sham and mTBI rats (Table 2). These neurons were selected for quantification because they are distinctive, being very large cells, can be easily counted using Nissl stain, and are important for the modulation of ASR[14,15] (Fig. 2, A2 and B2). No differences were observed between sham and mTBI groups in the number of PnC giant neurons ($t_{(6)} = 1.01$; $p = 0.351$).

Experiment 2: chronic effects of mild traumatic brain injury on acoustic startle response

Given the dramatic suppression of ASR in experiment 1, the aim of experiment 2 was to determine the duration of startle suppression after mTBI. Two rats (and their paired sham) were removed from the study because postmortem inspection of the brains showed cavitation at the injury site. Results from the remaining rats ($n = 8$ sham and 8 mTBI) were analyzed. mTBI rats experienced a peak impact of 23.2 ± 1.5 psi with peak latency of 19.5 ± 0.8 ms (Table 1). All rats receiving mTBI displayed hyperextension of limbs in response to the impact, apnea duration of 11.0 ± 1.3 sec, and a righting latency of 510 ± 33 sec. In comparison, sham rats had a righting latency of 55 ± 24 sec with no apnea or limb hyperextension. All mTBI rats had longer righting latencies than sham rats. None of the sham or mTBI rats displayed behavioral signs of seizures.

Acoustic startle response

After injury, ASR was suppressed as early as 24 h and remained suppressed for 21 days after injury. Injury reduced ASR sensitivity (Fig. 3A), as demonstrated by a main effect of injury ($F_{(1,14)} = 15.95$; $p = 0.001$) as well as interactions of injury × session ($F_{(3,42)} = 5.39$; $p = 0.003$) and injury × intensity × session ($F_{(6,84)} = 2.55$; $p = 0.026$). The interaction of injury × intensity was not significant. Reduction of ASR sensitivity did not recover with time after injury, given that analysis of postinjury data showed a main effect of Injury ($F_{(1,14)} = 16.32$; $p = 0.001$) without significant main effect of, or interactions involving, session.

mTBI induced a long-lasting suppression of ASR amplitude (Fig. 3B). mTBI reduced startle amplitude, as demonstrated by significant main effect of injury ($F_{(1,14)} = 5.29$; $p = 0.037$), as well as interactions involving injury (injury × session: $F_{(3,42)} = 5.58$; $p = 0.003$; injury × intensity: $F_{(2,28)} = 4.69$; $p = 0.018$; injury × session × intensity: $F_{(6,84)} = 3.86$; $p = 0.002$). To investigate whether suppression of ASR amplitude recovered with time after injury, analysis of only postinjury data was performed. Suppression of ASR amplitude did not recover. In fact, ASR amplitude becomes more suppressed with time after injury, as demonstrated by significant injury × session ($F_{(2,28)} = 7.32$; $p = 0.003$) and injury × session × intensity ($F_{(4,56)} = 3.08$; $p = 0.023$) interactions.

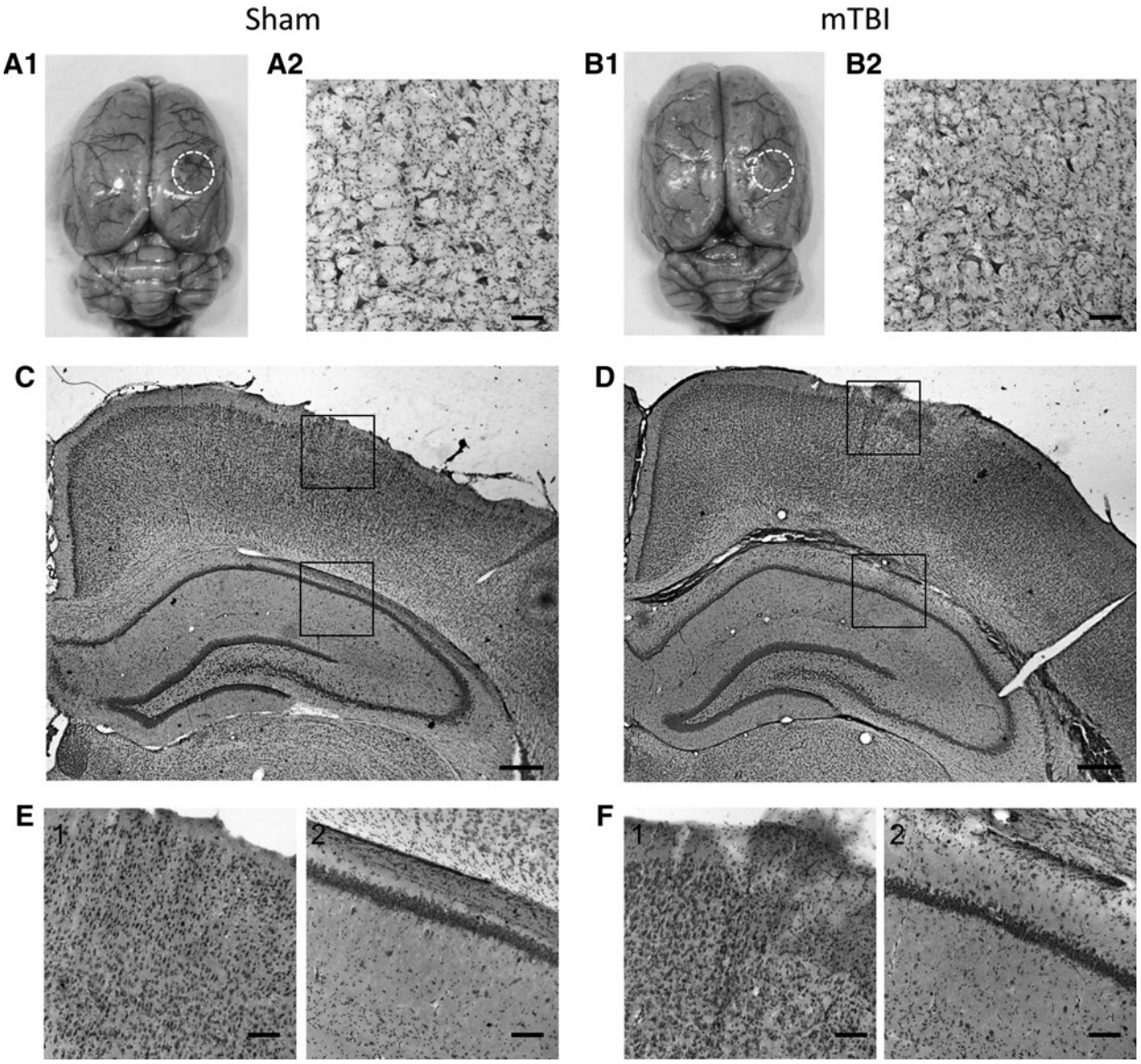

FIG. 2. Histology after mTBI. Gross brain damage at the injury site was not observed at 24 h after mTBI (**A1,** sham; **B1,** mTBI). Photomicrograph of the caudal pontine reticular nucleus (PnC) region with giant neurons (**A2,** sham; **B2,** mTBI). Microscopic examination of the tissue under the craniectomy revealed some disruption of the cortical layers in brains from mTBI rats (**C** and **E1,** sham; **D** and **F1,** mTBI). Photomicrograph of the hippocampus ipsilateral to the injury (**E2,** sham; **F2,** mTBI). White circles in panels A1 and B1 show the approximate location of the craniectomy. Squares in (C) and (D) show the approximate location of images in (**E**) and (**F**). Photomicrographs in all panels are from rats sacrificed 1 day after injury procedure. Scale bars in (C) and (D) are equal to 500 μm; all other scale bars are equal 100 μm. mTBI, mild traumatic brain injury.

Experiment 3: chronic effects of mild traumatic brain injury on spatial working memory

The aim of experiment 3 was to evaluate spatial working memory after a similar brain injury to that producing suppression of ASR. Rats receiving mTBI ($n=8$) experienced a peak impact pressure of 19.5 ± 1.4 psi with a peak latency of 19.7 ± 0.9 ms (Table 1). All rats receiving fluid percussion displayed hyperextension of the limbs in response to the fluid percussion impact and had a mean apnea duration of 13.0 ± 5.5 sec and a righting latency of 554 ± 34 sec. Sham rats ($n=6$) had a mean righting latency of 64 ± 30 sec with no apnea or limb hyperextension. Two mTBI rats had shorter righting latencies than the longest latency displayed by sham rats. However, further analysis revealed that 1 sham rat displayed an abnormally long righting reflex (396 sec); the longest latency for all other sham rats in experiments 1–3 was 216 sec. All mTBI rats had longer righting latencies than 216 sec. Neither sham nor mTBI rats displayed behavioral signs of seizures.

Path efficiency

Rats were assessed for spatial working memory performance before injury and on postinjury days 1, 7, and 21. Path efficiency, as opposed to escape latency or path length, was used as the dependent measure because efficiency is not influenced by differences in distance between start and goal locations. Path efficiency

Table 2. Number of Degenerating Cells in the Cortex and Hippocampus (Fluoro-Jade C) and Total Number of Giant PnC Neurons (Cresyl Violet) in Sham and mTBI Rats 1 Day After Injury Procedure[a]

	Cortex		*Hippocampus*		
	Ispilateral	*Contralateral*	*Ipsilateral*	*Contralateral*	*PnC*
Sham	552 ± 159	744 ± 99	232 ± 110	304 ± 88	13,090 ± 896
mTBI	2522 ± 805	1917 ± 957	954 ± 302	666 ± 123	15,552 ± 2264

[a]Mean ± standard error of the mean.
Fluoro-Jade C was purchased from Millipore (Billerica, MA).
PnC, caudal pontine reticular nucleus; mTBI, mild traumatic brain injury.

on the sample phase increased as training proceeded, but did not differ between sham and injured rats (Fig. 4A). The main effect of session was significant ($F_{(3,36)} = 23.83$; $p < 0.001$), but the main effect of injury and the injury × session interaction failed to reach significance.

As expected, path efficiency increased on the choice phase, compared to the sample phase, demonstrating that rats used information gained in the sample phase to more efficiently locate the escape platform during the choice phase, main effect of phase ($F_{(1,12)} = 112.72$; $p < 0.001$). mTBI rats demonstrated impaired spatial working memory, as exhibited by reduced path efficiency (main effect of injury: $F_{(1,12)} = 18.99$; $p = 0.001$). Importantly, impaired efficiency was observed on the choice phase, but not sample phase, as determined by a significant injury × phase × session interaction ($F_{(3,36)} = 5.70$; $p = 0.003$). Retention interval affected path efficiency, as demonstrated by a main effect of retention interval ($F_{(2,24)} = 5.89$; $p = 0.008$), retention interval × phase interaction ($F_{(2,24)} = 16.32$; $p < 0.001$), and retention interval × phase × session interaction ($F_{(6,72)} = 5.16$; $p < 0.001$; Fig. 4B,C). However, interactions involving injury and retention interval did not reach significance.

The working memory performance recovered with time after injury (Fig. 4A,C). Analysis of postinjury data showed that the main effects of injury ($F_{(1,12)} = 17.28$; $p = 0.001$), session ($F_{(2,24)} = 20.69$; $p < 0.001$), and injury × phase × session interaction ($F_{(2,24)} = 6.72$; $p = 0.005$) were all significant. The injury × session interaction was not significant. Separate analysis of each session was performed to determine whether recovery of working memory was observed. Before injury, performance of sham and mTBI groups was similar, given that neither the main effect of injury nor the injury × phase interaction was significant. One day after injury, mTBI rats were impaired in working memory with significant main effect of injury ($F_{(1,12)} = 26.54$; $p < 0.001$) and injury × phase interaction ($F_{(1,12)} = 51.56$; $p < 0.001$). Subsequent analysis demonstrated that the impaired efficiency in mTBI rats was observed on the choice, not sample, phase (sample phase: $t_{(12)} = 0.2$; choice phase: $t_{(12)} = 7.04$; $p < 0.001$). Seven days after injury, working memory impairments were observed, given that both main effect of injury ($F_{(1,12)} = 12.91$; $p = 0.004$) and injury × phase interaction ($F_{(1,12)} = 22.12$; $p = 0.001$) were significant. Impairments were the result of differences in efficiency on the choice phase (sample phase: $t_{(12)} = 0.64$; choice phase: $t_{(12)} = 4.80$; $p < 0.001$). In contrast to postinjury days 1 and 7, differences in path efficiency between sham and mTBI rats were not observed 21 days after injury. Neither main effect of injury nor the injury × phase interaction was significant, demonstrating recovery from spatial working memory impairments.

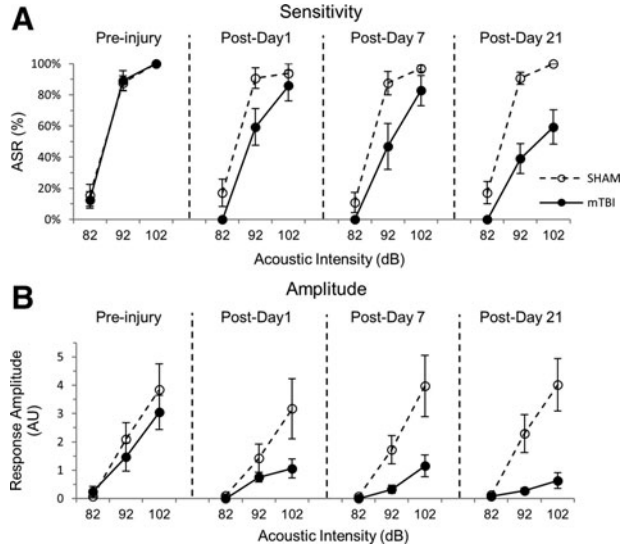

FIG. 3. Chronic effects of mTBI on ASR. mTBI produced a suppression of ASR sensitivity (**A**) and amplitude (**B**) starting 1 day after injury and lasting for 21 days. ASR suppression did not show signs of recovery. ASR, acoustic startle response; AU, arbitrary units; dB, decibels; mTBI, mild traumatic brain injury.

Swim speed

Swim speed between sham and injured rats was not different. Neither main effect of injury nor interactions involving injury were significant. Swim speed for both groups increased as a function of session and phase, main effect of session ($F_{(3,36)} = 5.77$; $p = 0.002$) and phase ($F_{(1,12)} = 20.84$; $p = 0.001$).

Comparison of injury and acute signs between experiments

Comparisons of peak impact pressure, apnea duration, and righting time were made across the three experiments to assess the extent to which injuries were similar between experiments. Peak impact pressure differed across experiments ($F_{(2,26)} = 3.74$; $p = 0.039$). However, post-hoc analysis revealed no significant pairwise differences (Exp 1 vs. 2: $p = 0.063$; Exp 2 vs. 3: $p = 0.06$; Exp 1 vs. 3: $p = 0.999$). Neither apnea time nor righting latency was different between mTBI groups from the three experiments.

ASR sensitivity and amplitude 24 h after injury was not different between experiments 1 and 2. ASR sensitivity was suppressed by mTBI, as demonstrated by a significant main effect of injury ($F_{(1,30)} = 15.48$; $p < 0.001$) and significant injury × session interaction ($F_{(1,30)} = 9.07$; $p = 0.005$). Neither main effect of experiment nor interactions involving experiment were significant. mTBI also

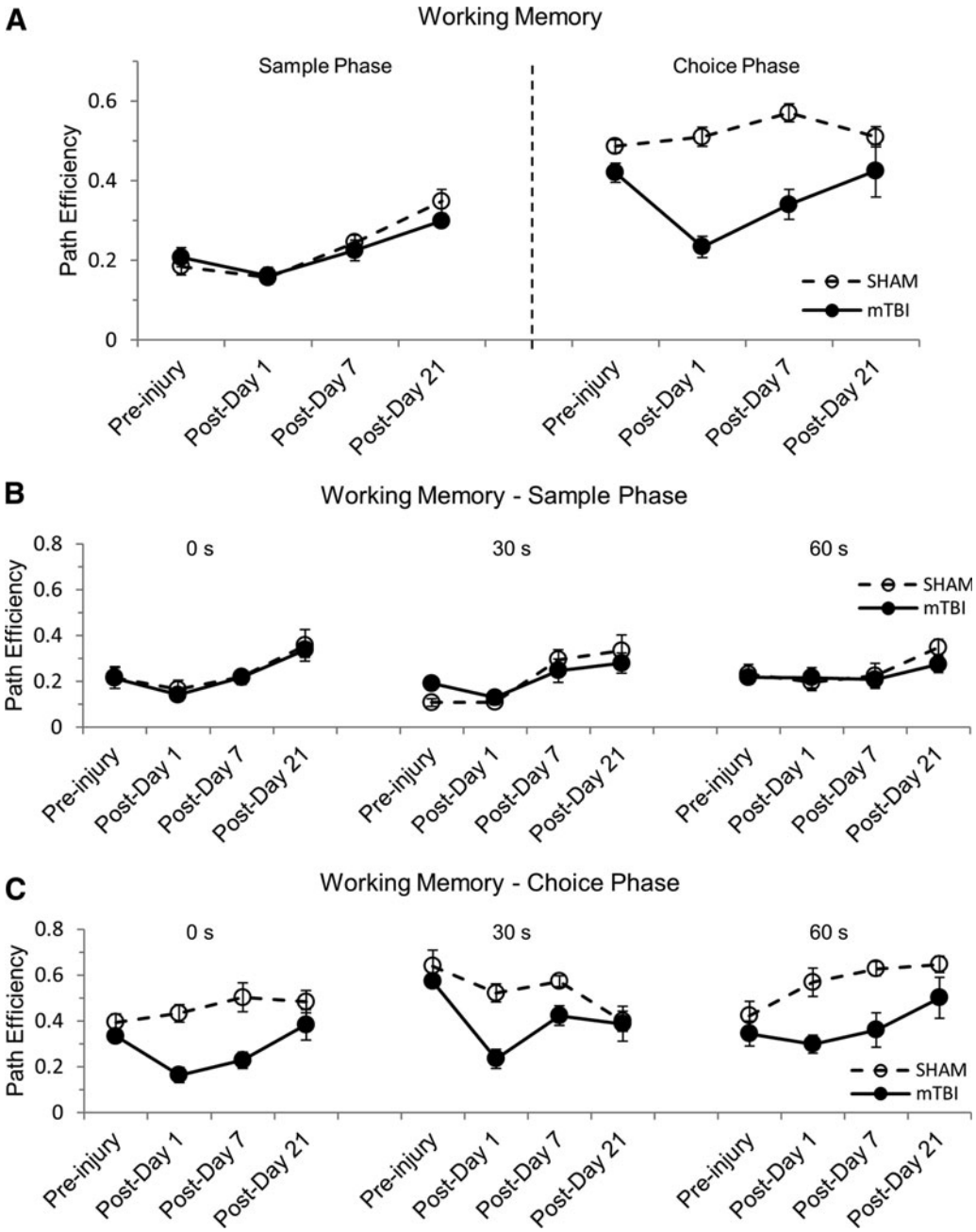

FIG. 4. Effects of mTBI on spatial working memory. Sham and mTBI groups did not differ in finding the hidden platform location during the sample phase (**A** and **B**). With training, rats became more efficient in finding the platform during the sample phase, but this learning was not different between treatment groups. Efficiency in finding the hidden platform during the choice phase was impaired by mTBI, demonstrating an impairment of working memory (A and **C**). Working memory was impaired by mTBI at 1 and 7 days after injury, but recovered by day 21. Retention interval did not alter path efficiency on the sample phase (B). However, path efficiency was affected by retention interval in the choice phase (C). Importantly, injury did not interact with retention interval in either sample or choice phases. mTBI, mild traumatic brain injury.

suppressed ASR amplitude 24 h after injury, as evidenced by a significant main effect of injury ($F_{(1,30)}=5.59$; $p=0.025$), injury × session ($F_{(1,30)}=11.95$; $p=0.02$), injury × intensity ($F_{(2,60)}=5.51$; $p=0.006$), and injury × session × intensity ($F_{(2,60)}=9.56$; $p<0.001$) interactions. Similar to ASR sensitivity, neither main effect of experiment nor interactions involving experiment were significant.

In summary, peak impact pressure was significantly different between experiments 1–3, and differences approached significance between experiment 2 and the other experiments. No differences were observed between experiment 1 and 3. Despite these small, but significant, differences in peak pressure, acute signs of injury were not different. Neither apnea time nor righting time assessed immediately after impact was different between any of the three experiments. Moreover, injury-induced suppression of ASR sensitivity and amplitude were not different between experiment 1 and 2. Taken together, these comparisons suggest similar severity of

injury between experiments, even with a small difference in peak impact pressure.

Discussion

In the present study, the effects of mTBI on ASR and spatial working memory were investigated. mTBI caused a long-lasting suppression of ASR that lasted at least 21 days after injury. In contrast, a similar injury produced a transient impairment of spatial working memory that recovered by 21 days. These findings suggest that brain regions involved in ASR (i.e., PnC and cochlear nucleus) may be more affected by mTBI than brain areas involved in spatial working memory (i.e., hippocampus). These results have important implications regarding return-to-activity decisions after concussion.

The present study focused on the effects of mTBI. The most commonly reported measure to define mild severity after LFP is peak pressure. A wide range of peak pressures (14.7–38.9 psi) has been considered mild severity in the literature.[27–30] Because peak pressure may not reliably translate to injury severity, we also evaluated duration of apnea and righting reflex, although these measures are not often reported. In studies that described injury-induced apnea, apnea duration for mTBI ranged from 7 to 17 sec, whereas moderate injuries resulted in apnea durations of approximately 30 sec.[28,30] Some investigators have suggested that injury severity is best assessed through righting reflex.[28] Righting latency, though, is highly dependent on the manner in which they are assessed. In some studies, righting is elicited by providing sensory stimulation (i.e., foot pinch). In other studies, spontaneous righting reflex without sensory stimulation is assessed. For the present study, spontaneous righting latencies are most relevant and these range from 4.0 to 11.5 min for mild injuries[29,30] and from 15 to 30 min for moderate-to-severe injuries.[29] By all of these measures, the brain injury in the present study would be considered mild severity (Table 2).

To make the study more directly translatable to mTBI in humans, we also constrained our injuries to those that did not result in gross brain damage, such as cavitation. This exclusion criterion is consistent with the clinical distinction between mild and moderate TBI. For most animal studies, exclusion of gross brain damage is not used and the designation of mTBI is dictated exclusively by peak pressure amplitude. Even in rats without gross brain damage, degenerating cells, as determined by Fluoro-Jade C staining, were more prevalent in mTBI rats than in sham rats. Whereas some studies report no Fluoro-Jade staining in tissue from sham rats, our estimates of degenerating hippocampal and cortical cells in sham rats compare well with other studies[31]; discrepancies may be owing to staining protocols. Still, degenerating cells in the hippocampus and cortex from mTBI rats were nearly 3–5 times more than in sham rats. More degenerating cells were found in the cortex than hippocampus of mTBI rats, although it must be noted that the amount of degenerating cells was small, compared to the total number of neurons in each area. Interestingly, cell degeneration did not differ between the ipsi- and contralateral sides; this result may be owing to a lack of statistical power given that the mean values are slightly higher in the ipsilateral side, compared to the contralateral side. Comparison of cell counts between studies is often difficult because of the lack of standards in reporting cell counts. Some studies report actual cell counts of a sampling of tissue; other studies provide estimates of total cell number. Stereology is a procedure that estimates the total number of cells in a region using unbiased sampling procedures.[32] The present study used stereology to estimate the number of degenerating cells (Fluoro-Jade positive) in the cortex and hippocampus after mTBI. Our estimates of degenerating hippocampal cells are similar to estimates obtained from stereology after mTBI using LFP[33] or to estimates extrapolated from actual Fluoro-Jade cell counts after CCI mTBI.[34] In the cortex, our estimates of degenerating cells were much less than those reported after slightly higher impact pressures (24 psi) using LFP.[35] Thus, mTBI in the present study agrees with results from previous descriptions of mTBI using neurodegeneration in the cortex and hippocampus and acute injury signs.

Although neurodegeneration was observed in the cortex and hippocampus in the present study, Fluoro-Jade–labeled cells were not present in the PnC or cochlear nucleus, suggesting the lack of neuronal degeneration in the brainstem. To confirm this conclusion, Nissl-stained PnC giant neurons were counted, and the number of these neurons did not differ between sham and mTBI rats. Therefore, mTBI produced by LFP does not lead to neuronal degeneration of the essential ASR neural circuit.

ASR is a defensive response to loud, sudden auditory stimuli; this response is well conserved throughout the animal kingdom.[4] The main finding of this study was a long-lasting suppression of ASR resulting from mTBI by LFP. Suppression of ASR sensitivity and startle amplitude were first observed 24 h after injury and lasted for the duration of our study (21 days after injury). A few studies have examined ASR after mild-to-severe TBI (i.e., presence of gross anatomical damage). mTBI produced by LFP (36 psi peak pressure) suppressed ASR for at least 7 days, the longest time investigated.[13] The present study extended these findings to show that a milder impact ($\sim$20 psi) suppressed ASR for at least 21 days. Moderate TBI using midline fluid percussion in rats also produced a dramatic, long-lasting suppression of ASR.[12] Suppression of tactile startle was also noted, but was suppressed to a lesser degree than ASR. Suppression of tactile startle and ASR suggests involvement of common output pathways.[36] LFP in rats that resulted in gross damage of the ipsilateral cortex and hippocampus suppressed ASR for 3 days postinjury, the longest time investigated.[10] Finally, modest suppression of ASR was observed in mice after a severe cortical contusion injury, but the duration of suppression was not investigated.[11]

To our knowledge, ASR has not been investigated in humans after mTBI. An exaggerated ASR is often noticed by individuals and can be a source of concern. In contrast, suppressed startle does not merit much attention, and if ASR is suppressed in concussed humans, it is likely to remain unnoticed. Still, suppressed ASR would reflect a dysfunction of the brain that should be monitored and factored into return-to-activity decisions.

The essential neural circuit for ASR is a trisynaptic pathway in the pons and spinal cord[5,14]; this circuit is well conserved through mammalian species, including humans.[37] Auditory information is relayed to the cochlear nucleus by the eighth cranial nerve (VIII). Information is transmitted from the cochlear nucleus to the PnC. From the PnC, signals descend to the spinal cord motor neurons through the reticulospinal tract. Subsequent to an impact, the brainstem experiences some of the greatest shearing forces in the brain, based on models of concussions experienced by NFL players,[38] but the dysfunction of brainstem circuits after mTBI is poorly understood. Suppressed ASR after mTBI in our study suggests that a primary impact to the cortex can influence the function of brainstem circuits. However, mTBI leading to a persistent ASR suppression did not cause cell degeneration in the cochlear nucleus or PnC, as assessed by Fluoro-Jade C and Nissl stain. It is possible that cell death occurs at later times after injury, but delayed degeneration would not explain ASR suppression at 24 h postinjury.

Preliminary results from our lab show increased proinflammatory cytokine messenger RNA in the PnC at 24 h and subsequent times after injury (unpublished observations), suggesting that inflammatory processes may underlie suppression of ASR. It is interesting to note that some common symptoms of PCS, such as headaches, sleep disturbance, feeling of being in a fog, attention impairments, and lightheadedness, could have roots in brainstem dysfunction owing to the fact that nuclei critical for baroreception, regulation of respiration and blood pressure, emesis, and sensing blood oxygen reside in this brain region. The brainstem also contains important nuclei of major neurotransmitters. Serotonergic and noradrenergic nuclei are located in the pons and project widely to the forebrain; dysfunction of these nuclei has the potential to impair cognition and emotion.[39,40]

mTBI resulting from LFP also impaired spatial working memory. In contrast to ASR, impairment of spatial working memory was transient. Working memory impairments were observed as early as 1 day after injury, remained impaired at 7 days, but recovered by 21 days. This timeline for recovery is similar to previous studies using the FPI model. Midline fluid percussion injury without gross brain damage impaired spatial working memory in a radial maze at 5 days with recovery by 15 days postinjury.[18] LFP mTBI also impaired spatial reference learning 7 days after injury that recovered by 8 weeks, whereas the effects of moderate-to-severe TBI lasted up to 8 weeks.[41] Using a similar behavior paradigm as the present study, moderate TBI after midline fluid percussion slowed the latency to find the platform on both sample and choice phases to a similar degree.[42] Uninjured rats are more efficient in finding the platform during the choice phase because of information gained on the sample phase. In contrast, injured rats had similar escape latencies on sample and choice phases, suggesting a working memory impairment. However, this interpretation is complicated by the finding that rats experiencing moderate TBI were slower than sham rats in finding the platform during the sample phase, indicating possible impairment in searching strategy, swimming ability, or sensory problems. In the present study, such complications are minimized because mTBI did not impair the ability of rats to find the platform on the sample phase. In fact, both sham and mTBI rats improved their efficiency in locating the platform during sample trials with continued training, and this improvement was not different between the groups. Impairment was only observed on the choice phase in the present study, providing evidence for a more selective dysfunction of working memory after mTBI.

Of particular importance for this study is the differential recovery of spatial working memory and ASR after mTBI. Whereas working memory recovered by 21 days after injury, ASR suppression remained for the duration of the study (21 days postinjury). The difference in recovery suggests that dysfunction of brainstem neural circuits responsible for suppressed ASR may outlast dysfunction of forebrain circuits responsible for working memory. However, the persistent ASR suppression occurs in spite of no observed cellular degeneration in the PnC or cochlear nucleus, and the transient spatial memory impairment is present despite cell degeneration in the hippocampus as early as 24 h after injury. Therefore, neural circuits involved in spatial working memory are able to compensate for the loss of hippocampal neurons after mTBI. It is possible that the differential recovery is owing to a small, but significant, difference in peak pressure. Experiments 1 and 3 had similar peak pressure readings, whereas experiment 2 had slightly increased values compared to the other experiments. Despite the higher peak pressure, acute signs (hyperextension of limbs as well as latency of righting reflex and apnea) observed in the mTBI group of experiment 2 was similar to acute signs for mTBI rats in experiments 1 and 3. Moreover, suppression of ASR at 24 h after injury was not different between experiments 1 and 2. Therefore, comparable acute measures of mTBI suggest that the injury severity was similar in all three experiments.

In summary, a long-lasting suppression of ASR was observed after mTBI produced by LFP. These injuries were not accompanied by gross brain damage, consistent with clinical definitions of mTBI. The suppression of ASR outlasted spatial working memory impairments, implicating different recovery rates for neural circuits involved in ASR, compared to those responsible for spatial working memory. If humans demonstrate a similar finding after concussion, ASR suppression may provide a good objective marker to assess brainstem dysfunction after mTBI. Importantly, our results also suggest that recovery of brainstem function should be considered in return to activity decisions.

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Author Disclosure Statement

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