Delayed reduction in hippocampal postsynaptic density protein-95 expression temporally correlates with cognitive dysfunction following controlled cortical impact in mice

Laboratory investigation

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Object. Traumatic brain injury (TBI) induces significant neurological damage, including deficits in learning and memory, which contribute to a poor clinical prognosis. Treatment options to limit cognitive decline and promote neurological recovery are lacking, in part due to a poor understanding of the secondary or delayed processes that contribute to brain injury. In the present study, the authors characterized the temporal and spatial changes in the expression of postsynaptic density protein-95 (PSD-95), a key scaffolding protein implicated in excitatory synaptic signaling, after controlled cortical impacts in mice. Neurological injury, as assessed by the open-field activity test and the novel object recognition test, was compared with changes in PSD-95 expression.

Methods. Adult male CD-1 mice were subjected to controlled cortical impacts to simulate moderate TBI in humans. The spatial and temporal expression of PSD-95 was analyzed in the cerebral cortex and hippocampus at various time points following injury and sham operations. Neurological assessments were performed to compare changes in PSD-95 with cognitive deficits.

Results. A significant decrease in PSD-95 expression was observed in the ipsilateral hippocampus beginning on Day 7 postinjury. The loss of PSD-95 corresponded with a concomitant reduction in immunoreactivity for NeuN (neuronal nuclei), a neuron-specific marker. Aside from the contused cortex, a significant loss of PSD-95 immunoreactivity was not observed in the cerebral cortex. The delayed loss of hippocampal PSD-95 directly correlated with the onset of behavioral deficits, suggesting a possible causative role for PSD-95 in behavioral abnormalities following head trauma.

Conclusions. A delayed loss of hippocampal synapses was observed following head trauma in mice. These data may suggest a cellular mechanism to explain the delayed learning and memory deficits in humans after TBI and provide a potential framework for further testing to implicate PSD-95 as a clinically relevant therapeutic target.

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Key Words: • traumatic brain injury • glutamate • synaptic plasticity • hippocampus

Preventative measures, such as helmet and seatbelt laws, stricter enforcement of drunk driving laws, improved safety devices, and educational programs, have been effective in reducing the number and severity of brain injuries. However, despite advances in critical care medicine and the development of the “trauma center,” the medical management of TBI remains limited. Treatment options to maintain cognitive function after these injuries are lacking, at least in part due to poorly understood mechanisms. Neurological injury following a head trauma is complex and likely involves multiple mechanisms and signaling cascades beyond the initial traumatic event. Cellular necrosis within the cerebral cortex occurs immediately after trauma and contributes to increased intracranial pressure and acute patient deaths. However,
delayed cellular loss within the hippocampus correlates with cognitive deficits and long-term prognosis following experimental12,19 or clinical head injury.15,20,26,36,37,39 The temporal delay between the acute trauma and delayed hippocampal injury reflects a clinically feasible window for a treatment strategy to limit secondary damage and promote posttraumatic hippocampal plasticity.

Long-term potentiation, a process that promotes synaptic neurotransmission, may contribute to the processes of learning and memory. Rat brain slices prepared 1 week after lateral fluid percussion injury did not induce or maintain LTP and exhibited attenuated NMDA-induced potentials and glutamate-induced excitatory currents.5,6,9,17,18,23,24,30,33 Notably, PSD-95 controls activity-dependent AMPA receptor incorporation at synapses during LTP in vitro and during experience-driven synaptic strengthening in vivo.8 Together, these data suggest that the loss of hippocampal PSD-95 expression underlies the development of delayed cognitive impairments following TBI.

Herein, we report on the temporal and spatial patterns of PSD-95 expression within the brain following controlled cortical impacts in mice. These changes correlated with delayed hippocampal damage and behavioral deficits. Together, these data provide a framework and rationale for the therapeutic targeting of hippocampal synapses to improve long-term neurological outcome following TBI.

Methods

Controlled Cortical Impact

Animal experiments were reviewed and approved by the Committee on Animal Use for Research and Education at the Medical College of Georgia, in compliance with NIH guidelines. Male CD-1 mice 8–10 weeks old (Charles River) were anesthetized with 8 mg/kg xylazine and 60 mg/kg ketamine and then placed in a stereotactic frame (Amscien Instruments). A 3.5-mm craniotomy was made in the right parietal bone midway between the bregma and lambda with the medial edge 1 mm lateral to the midline. Impacts were delivered at approximately 4.5 m/s with a 20-msec dwell time and 1-mm depression using a 3-mm-diameter convex tip to induce a moderate TBI, as described elsewhere by our group.22 The incision was then surgically stapled, and mice were maintained at 37°C until recovery. Sham-operated controls were subjected to identical surgical procedures but did not receive an impact. Throughout all procedures, body temperature was maintained at 37°C by using a small-animal temperature controller (David Kopf Instruments).

Cresyl Violet Staining

Gross injury was assessed in coronal tissue sections (12 µm) incubated with 0.1% cresyl violet in 100% ethanol (pH 4.0) for 5 minutes. Sections were washed in distilled water followed by successive ethanol washes (70, 95, and 100%). The tissues were then briefly washed 3 times in xylene, and a coverslip was placed. For all treatment groups digital images of ipsilateral cortices were captured on a Zeiss Axiophot microscope using a 2.5 × objective.

Western Blotting

Western blotting was performed as described by our group.7,22,32 Blots were incubated overnight at 4°C with an anti–PSD-95 primary antibody (1:1000, Cell Signaling Technology) or an anti–β-actin primary antibody (1:3000, Santa Cruz Biotechnology) and visualized on a Li-Cor Odyssey near-infrared imaging system using an Alexa Fluor 750 secondary antibody. Densitometric analysis was performed using Quantity One software (Bio-Rad).

Immunohistochemical Analysis

Immunohistochemical analysis was performed as described by our group.10,22 To unmask postsynaptic PSD-95 labeling, sections were digested in pepsin (1 mg/ml in 0.2 N HCl at 37°C) for 5 minutes prior to antigen retrieval, using a published protocol.11 Sections were washed, incubated with 3% normal donkey serum in phosphate-buffered saline containing 0.1% Triton X-100, followed by a 2-hour incubation with an anti–PSD-95 polyclonal antibody (1:100, Cell Signaling Technology) or NeuN (1:100, Millipore) diluted in blocking buffer containing 3% donkey serum, and then incubated with an Alexa Fluor secondary antibody (1:200, Invitrogen). The omission of a primary antibody served as a negative control. At least 6–10 alternate tissue sections per mouse were analyzed per group. Sections were visualized using an LSM510 Meta confocal laser microscope, as described elsewhere by our group.10,22

Assessment of Neurological Injury

To determine the effect of TBI on neurological outcome, an open-field activity test was conducted, as described elsewhere.40 Briefly, mice were placed in a 14 × 14–in black box divided into a 2 × 2–in square grid (49 squares total). Open-field activity, as determined by the number of crosses within a 3-minute trial, was measured by at least 2 investigators who were blinded to the experimental conditions. The 2-trial novel object recognition task was also performed in which a mouse was placed in an enclosed box with 2 identical objects situated within a 4-inch diameter circle and located a set distance apart. The mouse was then removed from the environment for a set amount of time and 1 of the 2 previously used (familiar) objects was replaced with a novel object that was different from the familiar object in shape, texture, and appearance. The mouse's behavior on exposure to the novel object was then recorded. This test is based on the natural tendency of mice to investigate a novel object rather than a familiar one, which reflects the use of learning and recognition memory processes.

Statistical Analysis

The effects of treatments were analyzed using a Student t-test or ANOVA followed by the Student-Newman-Keuls post hoc test. At least 5 animals per group were
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included for all analyses. Results are expressed as the mean ± SEM. A probability value < 0.05 was considered to be statistically significant.

Results

Delayed Reduction in Hippocampal PSD-95 Expression

Western blotting of PSD-95, a scaffolding protein abundantly expressed within excitatory synapses, was performed to determine the effect of TBI on the number of synapses in the hippocampus. In contrast to the pericontusional cortex, which exhibited a dramatic reduction in PSD-95 expression, the hippocampus showed no significant differences in PSD-95 expression for the first 3 days post-TBI (Fig. 1). Conversely, a dramatic reduction in hippocampal PSD-95 staining was noted 1 week after injury (46.6 ± 5.2% of that in sham-operated controls; p < 0.05, compared with controls), a finding in line with the visualization of hippocampal injury at Day 7 using cresyl violet staining.

Spatial information cannot be ascertained from Western blotting; thus, to establish whether the reduction in PSD-95 expression was restricted to individual regions of the hippocampus or whether this effect was widespread, immunolocalization of PSD-95 was performed at various time points following TBI and sham operation. The hippocampus in sham-operated mice did not appear injured and instead showed a characteristic pattern of staining for NeuN, a neuron-specific marker, as well as abundant PSD-95 staining (Fig. 2). Whereas in the TBI

![Western blotting and immunolocalization images](image-url)

**Fig. 1.** Delayed reduction in hippocampal PSD-95 expression following experimental TBI. **A:** Representative gel blots (left) indicating a time-dependent reduction in the expression of PSD-95 within the entire hippocampus in mice following moderate TBI. Postsynaptic density protein-95 expression was normalized to β-actin to control for equal protein loading. Brain tissue sections (right) stained with cresyl violet are shown to further document hippocampal injury by Day 7 postinjury. **B:** Bar graph demonstrating quantification of the Western blotting data featured in panel A by using densitometry. Data are expressed as the ratio of PSD-95/β-actin and are presented as the means ± SEM. *p < 0.05, compared with sham-operated control mice. d = day; h = hours.
group, changes in NeuN or PSD-95 immunostaining were not observed for the first 48 hours postinjury, a significant reduction in immunoreactivity for both markers was seen by 3 days postinjury, a time point that preceded the reduction noted by Western blotting. Consistent with the immunoblotting data, PSD-95 immunostaining was absent by Day 7 postinjury. Interestingly, NeuN immunoreactivity was also lost at this time point, suggesting either a loss of antigenicity following delayed injury or significant cell death. In support of this notion, we noted that increased activity of the proapoptotic mediator caspase-3 paralleled the loss of PSD-95 at Day 7 post-TBI (Fig. 3) but not at earlier time points (data not shown). These data suggest that a loss of PSD-95 may precede the activation of apoptotic signaling cascades.

Delayed Cognitive Deficits Following Moderate TBI in Mice

There was a delayed reduction of PSD-95 within the hippocampus following TBI; however, it remained unclear whether this change was associated with neurological demise. To establish whether PSD-95 levels correlated with cognitive deficits, the open-field activity test and the 2-object novel recognition test were performed. Both tests are widely applied and permit the evaluation of learning and memory and hippocampal function. Following TBI and sham operation, open-field activity was increased between Days 2 and 6 postinjury, although these differences failed to reach statistical significance. On Day 7 postinjury open-field activity was significantly increased (consistent with hippocampal impairment), whereas PSD-95 expression declined (Figs. 1A and 4 upper). The hippocampus is important for spatial memory and is also implicated in recognition memory. Thus, the time spent exploring a novel object is considered a sensitive test of memory functioning and may relate to hippocampal integrity. Consistent with this assertion, the time spent exploring a novel object was significantly reduced by Day 7 post-TBI, further suggesting functional deficits within the hippocampus (Fig. 4 lower). Together, these findings indicate both that the loss of PSD-95 directly correlates with cognitive demise and that there is a relationship between hippocampal structure and functional outcome following TBI.
Demonstrating the results of open-field activity testing in mice at various time points after TBI or sham operation. Activity, as measured by the average number of crosses per trial, was recorded. For sham-operated control mice, the average measure of recognition memory, was recorded and compared with that for sham-operated control mice. For all experiments, there were 6–8 mice/group. *p < 0.05, compared with sham-operated mice.

For sham-operated mice at Day 7 post-TBI or post–sham operation, when PSD-95 expression was the lowest. The average time spent exploring a novel object, a measure of recognition memory, was recorded and compared with that for sham-operated control mice. For all experiments, there were 6–8 mice/group. *p < 0.05, compared with sham-operated mice.

**Discussion**

The manifestation of cognitive deficits following head trauma confounds rehabilitation and contribute to a poor quality of life. Remediation of learning and memory deficits remains a significant goal of TBI research; unfortunately, many promising therapies for TBI are limited by a narrow therapeutic window. Data in the present report document a delayed reduction in the hippocampal expression of PSD-95, an abundant protein within excitatory synapses, following TBI. Notably, the loss of PSD-95 directly correlated with a reduction in cognitive function, suggesting a possible cellular mechanism that could, at least in part, explain the neurological deficits observed weeks and even months after the initial trauma. The timing of these delayed secondary changes may permit the institution of therapy in most, if not all, patients.

Neuronal loss, even in the absence of elevated intracranial pressure, is observed within the hippocampus in over 80% of fatal TBIs in humans, and apoptotic neurons are observed in the human hippocampus up to 1 year after the initial trauma. Prior studies have shown that hippocampal LTP, which contributes to learning and memory formation, was disrupted 7 days following lateral fluid percussion injury in rats. Although the precise cellular mechanisms underlying these actions were not established, a reduction in NMDA potentials, attenuated glutamate-induced excitatory currents, and decreased expression of α-calcium/calmodulin-dependent protein kinase II (αCAMKII) were observed. In the present report, PSD-95 expression was maximally reduced throughout the hippocampus at a time point that paralleled impaired LTP in rats. Notably, the overexpression of PSD-95 mimicked several key aspects of LTP and experience-driven synaptic potentiation, including the enhancement of AMPA receptor–mediated transmission and translocation of GluR1 into synapses, whereas a dominant-negative PSD-95 construct blocked LTP in brain slice cultures and attenuated experience-driven plasticity via a reduction in AMPA-mediated currents. Although the mechanisms underlying LTP disruption following TBI remain poorly understood, our findings of a time-dependent reduction in hippocampal PSD-95 expression may permit the future characterization of these processes.

In this study, hippocampal injury was delayed by several days from the primary traumatic event, despite the presence of acute cellular necrosis and edema formation in the pericontusional cortex, processes that began immediately after injury. Although the mechanisms underlying delayed synaptic loss and cell death within the hippocampus remain unresolved, recent work suggests a prominent role for oxidative stress in promoting neuronal injury following experimental TBI in rats. Indeed, PSD-95 couples NMDA receptor activation with nitric oxide–mediated neurotoxicity, supporting a causative role for PSD-95 in neuronal excitotoxicity; however, this explanation may not fully explain why a focal cortical injury induces diffuse, widespread hippocampal damage several days after the initial injury. Notably, bilateral entorhinal cortical deafferentation induced dendritic atrophy in the hippocampus and potentiated cognitive morbidity following fluid percussion injury in rats in an NMDA–dependent manner, suggesting that damage to the cortical projections that innervate the hippocampus may promote secondary injury within the hippocampus, resulting in cognitive decline.

**Conclusions**

The correlation between the delayed loss of hippocampal synapses and behavioral deficits following TBI in mice may reveal a cellular mechanism to explain the delayed learning and memory deficits in humans. Taken together, findings in this study provide a conceptual framework for testing novel therapeutic strategies to preserve synaptic integrity or increase synaptogenesis within the hippocampus.

**Disclosure**

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