Treatment with harmine ameliorates functional impairment and neuronal death following traumatic brain injury

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Abstract. Traumatic brain injury (TBI) is a leading cause of mortality in young individuals, and results in motor and cognitive deficiency. Excitotoxicity is an important process during neuronal cell death, which is caused by excessive release of glutamate following TBI. Astrocytic glutamate transporters have a predominant role in maintaining extracellular glutamate concentrations below excitotoxic levels, and glutamate transporter 1 (GLT-1) may account for >90% of glutamate uptake in the brain. The β-carboline alkaloid harmine has been demonstrated to exert neuroprotective actions in vivo, and the beneficial effects were specifically due to elevation of GLT-1. However, whether harmine provides neuroprotection following TBI remains to be elucidated. The present study performed intraperitoneal harmine injections in rats (30 mg/kg per day for up to 5 days), in order to investigate whether harmine treatment attenuates brain edema and improves functional recovery in a rat model of TBI. The neuronal survival ratio and the protein expression of apoptosis-associated caspase 3 were also assessed in the hippocampus of the rat brain. Furthermore, the expression levels of GLT-1 and inflammatory cytokines were detected, in order to determine the underlying mechanisms. The results of the present study demonstrated that administration of harmine significantly attenuated cerebral edema, and improved learning and memory ability. In addition, harmine significantly increased the protein expression of GLT-1, and markedly attenuated the expression levels of interleukin-1β and tumor necrosis factor-α, thereby attenuating apoptotic neuronal death in the hippocampus. These results provided in vivo evidence that harmine may exert neuroprotective effects by synergistically reducing excitotoxicity and inflammation following TBI.

Introduction

Traumatic brain injury (TBI) is one of the leading causes of mortality and morbidity in adults and children worldwide, which results in immediate and delayed motor and cognitive deficiency (1). TBI-induced tissue loss and cell death occur following primary injury (direct physical disruption of the tissue) and secondary injury (delayed molecular pathophysiological changes) (2). Numerous pharmacological interventions are currently being investigated to attempt to reduce the severity of secondary injury following primary injury; however, current therapies are limited in their utility and efficacy (1).

Excitotoxicity is widely recognized as an important process in nervous cell death, produced by excessive release of glutamate following acute brain injury. In order to protect neurons from glutamate-mediated excitotoxicity, astrocytes transmit excessive glutamate from the extracellular space (3,4). Previous data confirmed that excitatory amino acid transporters are important in maintaining extracellular glutamate concentrations below excitotoxic levels (3). In total, five subtypes of sodium-dependent glutamate transporters have been identified: Glutamate aspartate transporter (GLAST; EAAT1), glutamate transporter-1 (GLT-1; EAAT2), EAAT3, EAAT4 and EAAT5 (5). Of these transporters GLT-1 has a principal role, and accounts for >90% of glutamate uptake in the brain (6). Previous studies have demonstrated that GLT-1 dysfunction is associated with the pathogenesis of multiple neurological disorders, including Alzheimer's disease, amyotrophic lateral sclerosis, stroke, several forms of epilepsy and TBI (7-11). Therefore, drugs and agents that increase glutamate transport activity may be significant in the treatment of neurological diseases.

Harmine is a β-carboline alkaloid, which was isolated >100 years ago (12). Harmine exerts a series of pharmacological actions, including antioxidative, antigenotoxic, antidepressant-like and antiplasmodial actions (13-16). Previously, harmine was observed to exert neuroprotective
actions in an in vivo investigation, and the beneficial effects were specifically due to the elevation of GLT-1 (17). However, no experiments have been performed to determine whether harmine provides neuroprotection following TBI.

In the present study, intraperitoneal (i.p.) injection of harmine (30 mg/kg per day, for up to 5 days) into rats was performed in order to investigate whether harmine attenuates brain edema and improves functional recovery in a rat model of TBI. Furthermore, to determine whether the mechanism underlying the neuroprotective effects of harmine are associated with glutamate transport and inflammatory factors, the expression levels of GLT-1 and inflammatory cytokines were examined in the hippocampus of the rat brain. These investigations aimed to investigate a potential novel therapeutic modality for the treatment of TBI.

Materials and methods

Animals and a controlled TBI model. All experimental procedures were approved by The Third Military Medical University Committee (Chongqing, China) for the use and care of animals in research, and were performed in accordance with the The Third Military Medical University’s guidelines for the care and use of laboratory animals. A total of 150 male Sprague-Dawley rats (age, 10-12 weeks; weighing, 280-320 g; obtained from The Third Military Medical University Animal Center) were used in the present study. All the rats had free access to food and water, and were housed under a standard 12-h light/dark cycle. The housing temperature was 25-28˚C and the rats were randomly placed into a cage to maintain body temperature.

A previously described TBI procedure (18) was performed in the present study. Briefly, the rats were anesthetized with sodium pentobarbital i.p. 50 mg/kg; Bio-Rad Laboratories (Shanghai) Co., Ltd., Shanghai, China] and a 5-mm craniotomy was performed over the left parietal cortex, which was centered on the coronal suture [Bio-Rad Laboratories (Shanghai) Co., Ltd.] and 3 mm lateral to the sagittal suture. The TBI was performed using a pneumatic piston [Bio-Rad Laboratories (Shanghai) Co., Ltd.] with a rounded metal tip (2.5 mm diameter), which was angled 22.5˚ to vertical to ensure that the tip was perpendicular with the brain surface at the center of the craniotomy. A velocity of 4 m/s and a deformation depth of 2 mm below the dura were used to generate the TBI in the procedure. The bone flap was immediately replaced and sealed, and the scalp was closed using sutures. Body temperature was monitored throughout the surgery, to ensure it was maintained at 37.0±0.5˚C. Whilst the rats were recovering from anesthesia, they were placed in a heated cage to maintain body temperature.

Group and drug administration. The rats were randomly divided into three groups: Sham-operated group (sham; n=15); the TBI group (TBI; n=35) and the TBI + harmine (Beijing Aoboxing Biotechnology Co., Ltd., Beijing, China)-treated group (Harmine; n=35). Harmine was administered immediately following TBI (i.p. 30 mg/kg per day) for up to 5 days. The sham and TBI groups received equal volumes of 0.9% saline solution (i.p.).

Measurement of brain edema. Brain edema was evaluated by analyzing the brain water content, as described previously (19). Following anesthetization, as described above, and sacrifice by exsanguination, the rat brains were separated and weighed immediately using a chemical balance, in order to obtain the wet weight (WW). Following drying in a desiccating oven for 24 h at 100˚C, the dry tissues were weighed to obtain the dry weight (DW). The water percentage in the brain was calculated according to the following formula Brain water % = (WW - DW) / WW x 100.

Behavioral recovery. The neurobehavioral status of the rats was evaluated using a set of 10 tasks, collectively termed the neurologic severity score (NSS) (20), which assesses reflexes, alertness, coordination and motor abilities. A score of one is awarded for failure to perform a particular task; therefore, a score of 10 reflects maximal impairment, whereas a normal rat scores 0. The rats were grouped as follows for examination of behavioral recovery: Sham, n=3; TBI, n=7; and Harmine, n=7. Following TBI, the NSS was evaluated at 1, 3 and 5 days. Each rat was assessed by an observer who was blinded to the animal treatment. The difference between the initial NSS and at any later time-point was calculated for each rat, and this value (ANSS) reflects the spontaneous or treatment-induced recovery of motor function. Furthermore, the spatial learning ability of the rats was assessed using a Morris water maze, as previously described (21). Briefly, the Morris water maze consisted of a black circular pool (diameter, 180 cm; height, 45 cm) filled with water (depth, 30 cm; temperature, 26˚C) and divided into four equal quadrants: North (N), west (W), south (S) and east (E). An escape platform (diameter, 12 cm; height, 28 cm, made opaque with paint and submerged 2 cm) was placed in the center of one of the quadrants, equidistant from the sidewalk and the center of the pool. Rats were trained to locate the platform prior to TBI or undergoing the sham surgery. For each trial, a rat was randomly placed into a quadrant start point (N, S, E or W) facing the wall of the pool and was allowed ≤60 sec to escape to the platform. Rats that failed to escape within 90 sec were placed on the platform for ≤20 sec and returned to the cage for a new trial (intertrial interval, 20 sec). The maze performance was recorded using a video camera suspended above the maze and interfaced with a video tracking system (HVS Imaging; Hampton, UK). The mean escape latency of a total of five trials was calculated. This assessment was performed 1, 3 and 5 days following TBI or sham surgery.

Hematoxylin and eosin (H&E) staining. At 24 h post-surgery, the rats (sham, n=3; TBI, n=7; and Harmine, n=7) were anesthetized, as described above, and perfused intracardially with isotonic sodium chloride solution, followed by 4% (w/v) paraformaldehyde [Bio-Rad Laboratories (Shanghai) Co., Ltd.] in 0.1 M sodium phosphate buffer [pH 7.4; Bio-Rad Laboratories (Shanghai) Co., Ltd.]. The rats were sacrificed via exsanguination. The brains were subsequently removed and fixed for 48 h in 4% (w/v) paraformaldehyde. Following fixation, the brains were embedded in paraffin [Bio-Rad Laboratories (Shanghai) Co., Ltd.], sliced into 4 µm coronal sections at the bregma, and stained with H&E [Bio-Rad Laboratories (Shanghai) Co., Ltd.]. The surviving and dying neurons in the CA1 area per 1 mm were subsequently quantified under an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan).
**Immunofluorescence.** At 24 h post-TBI, coronal sections were incubated with 10% normal donkey serum [Bio-Rad Laboratories (Shanghai) Co., Ltd.] for 30 min at room temperature in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 [Bio-Rad Laboratories (Shanghai) Co., Ltd.]. The sections were then incubated at 4°C overnight with the following primary antibodies: Goat anti-rat neuron-specific nuclear protein [NeuN; 1:200 (cat. no. sc-31154); Santa Cruz Biotechnology, Inc., Dallas, TX, USA] and rabbit anti-rat caspase 3 [1:50; (cat. no. sc-7148); Santa Cruz Biotechnology, Inc.]. The sections were then washed with PBS four times at room temperature, followed by an incubation with appropriate fluorescein-labeled secondary antibodies [1:200 (cat. nos. sc-2342 and sc-2341) Santa Cruz Biotechnology, Inc.] for 1 h at room temperature. The sections were incubated with DAPI (1 ng/Nl; Beijing Aobxing Biotechnology Co., Ltd.) to counterstain the nucleus. Subsequently, the sections were washed with PBS and mounted using water-based mounting medium containing anti-fading agents (Biomeda; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Confocal images were captured using a laser scanning confocal microscope (Olympus FV1000; Olympus Corporation, Tokyo, Japan) and digital imaging software (FV10-ASW 1.5 Viewer; Olympus Corporation).

**Western blot analysis.** The rats (sham, n=3; TBI, n=7; and Harmine, n=7) were anesthetized and underwent intracardiac perfusion with 0.1 mol/l PBS (pH 7.4). The cortex region of the brain was rapidly isolated and the brain tissues were homogenized using a homogenizer, total proteins were extracted using protein extraction reagent [both Bio-Rad Laboratories (Shanghai) Co., Ltd.] and protein concentration was determined using a bicinechonic acid reagent (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) method. The protein samples (5 mg) were separated by 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [Bio-Rad Laboratories (Shanghai) Co., Ltd.] and were subsequently transferred onto polyvinylidene fluoride membranes (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). The blots were then blocked with 5% fat-free dry milk for 1 h at room temperature. Following blocking, the membranes were incubated with the following primary antibodies overnight at 4°C: Rabbit anti-GLT-1 polyclonal antibody (cat. no. sc-15317), rabbit anti-interleukin (IL)-1β polyclonal antibody (cat. no. sc-7884), rabbit anti-tumor necrosis factor (TNF)-α polyclonal antibody (cat. no. sc-7895), rabbit anti-caspase 3 polyclonal antibody (cat. no. sc-7148) and mouse anti-β-actin monoclonal antibody (cat. no. sc-7210) all 1:500, Santa Cruz Biotechnology, Inc.). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; cat. no. sc-2027) and anti-mouse IgG (cat. no. sc-2025) for 2 h at room temperature (1:5,000, Santa Cruz Biotechnology, Inc.). Following incubation with the secondary antibody, the immunoblots were then visualized following development with an enhanced chemiluminescence detection system [Bio-Rad Laboratories (Shanghai) Co., Ltd., and densitometric signals were quantified using an enhanced chemiluminescence detection system [Bio-Rad Laboratories (Shanghai) Co., Ltd.]. The western blotting results were analyzed using ImageJ 1.4i software (National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** Data are expressed as the mean ± standard error of the mean. SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the data. Statistical analysis
Treatment with harmine attenuates cerebral edema. The wet-dry weight method was used to evaluate brain edema. As shown in Fig. 1, brain water content was significantly increased in the TBI group, compared with the sham group at 1, 3 and 5 days following trauma. Treatment with harmine significantly reduced the tissue water content at 1, 3 and 5 days, compared with the TBI group.

Results

**Treatment with harmine attenuates TBI-induced learning and spatial memory dysfunction.** Since treatment with harmine successfully attenuated brain edema, the present study examined whether harmine also improved spatial learning function, which was assessed using a Morris water maze at 1, 3 and 5 days post-TBI or sham surgery. As shown in Fig. 2, TBI resulted in a significant spatial learning deficit at 1, 3 and 5 days, compared with the sham group, and harmine treatment...
significantly reduced the escape latency at 3 and 5 days, compared with the TBI group.

Treatment with harmine attenuates TBI-induced motor deficits. Temporal variations in the functional recovery of the TBI rats are demonstrated in Fig. 3 and expressed as ΔNSS. Post-TBI administration of harmine significantly improved the motor function recovery of the rats at 1, 3 and 5 days following TBI, compared with the TBI group without harmine treatment.

Treatment with harmine suppresses neuronal death in the hippocampal region following TBI. Neuronal survival was assessed at 24 h using H&E staining. Morphologically, the nuclei of the normal neurons were round and poorly stained, whereas the nuclei of the dying neurons were pyknotic and

Figure 7. Co-localization of NeuN and caspase 3 24 h post-TBI was determined using immunofluorescent staining (magnification, x400). Cell nuclei were counterstained with DAPI. (A) Caspase 3; (B) NeuN; (C) DAPI; (D) A and B merge; (E) A, B and C merge. NeuN, neuron-specific nuclear protein; TBI, traumatic brain injury.

Figure 6. Protein expression levels of IL-1β and TNF-α. (A) Western blot analysis was used to detect the protein expression levels of IL-1β and TNF-α in the hippocampus at 1, 3 and 5 days (d). (B) Quantitative results of IL-1β and TNF-α are presented as the ratio of densitometries of IL-1β and TNF-α to β-actin. Data are presented as the mean ± standard error of the mean (n=5/group). A significant increase in the expression levels of IL-1β and TNF-α were detected in the TBI group at 1, 3 and 5 days, whereas treatment with harmine resulted in a significant downregulation of the expression levels of IL-1β and TNF-α at 1, 3 and 5 days. *P<0.05, vs. the sham group; **P<0.05, vs the TBI group. TBI, traumatic brain injury; IL, interleukin; TNF, tumor necrosis factor.
studies have demonstrated that GLT-1 investigations the mechanisms underlying the α/β against however, β-actin following administration of harmine. expression of IL-1 β expression levels of the inflammatory cytokines in the hippocampus following TBI. As shown in Fig. 5, at 1, 3 and 5 days post-TBI, the expression of caspase 3 was significantly increased in the TBI group, compared with the sham group. However, the administration of harmine significantly reduced the expression of caspase 3, compared with the TBI group.

Treatment with harmine reduces the expression of caspase 3 in the hippocampus following TBI. The expression of caspase 3 in the hippocampus were detected using western blot analysis. As shown in Fig. 8, at 1, 3 and 5 days post-TBI, the expression of caspase 3 was significantly increased in the TBI group, compared with the sham group. Administration of harmine markedly elevated the expression of caspase 3, as well as significantly reduced the protein expression of caspase 3, suggesting that harmine attenuated apoptotic neuronal death in the hippocampus. Previous studies have reported that treatment with harmine significantly attenuate glutamate-induced and oxidative stress-induced neuronal death in neuronal cell cultures (15,17). In addition, a previous in vivo study demonstrated that harmine exerts neuroprotective effect against glutamate-mediated excitotoxicity in a rat model of amyotrophic lateral sclerosis disease (17). These findings, together with the findings of the present study, improve current understanding of harmine-mediated neuroprotection in neurological disorders.

Previous in vivo studies have demonstrated that GLT-1 dysfunction causes acute and chronic brain injury, and that glutamate-mediated excitotoxicity is considered to be an important process in neurological disorders (22,23). The accumulation of excess extracellular glutamate and subsequent overstimulation of glutamatergic receptors increases the production of reactive and excitotoxic oxygen/nitrogen species, which may induce oxidative stress leading to neuronal death (24). Previous studies have reported that elevated activity and expression of GLT-1 induces demonstrated in rat models of various acute and chronic neurologic diseases (7-11), including a rat model of TBI, in which the overexpression of GLT-1 was observed to significantly reduce glutamate overflow, decrease cell death and improve behavioral recovery (20). The results of the present study confirmed that treatment with harmine markedly elevated the expression of GLT-1 in the hippocampus following trauma. These findings may demonstrate one of the predominant mechanisms by which harmine exerts its neuroprotective effects in TBI.

**Figure 8.** Protein expression levels of caspase 3 and β-actin. (A) Western blot analysis was used to detect the protein expression levels of caspase 3 and β-actin in the hippocampus at 1, 3 and 5 days (d) following injury. (B) Densitometry of caspase 3 relative to β-actin. Data are presented as the mean ± standard error of the mean (n=5/group). Expression of caspase 3 was significantly increased in the TBI group at 1, 3 and 5 days, whereas administration of harmine significantly decreased the protein expression of caspase 3. *P<0.01, vs. the sham group; **P<0.05, vs. the TBI group. TBI, traumatic brain injury.

Caspase 3 and NeuN are co-localized in the hippocampus following TBI. The co-localization of NeuN and caspase 3 was detected using immunofluorescent staining 24 h post-TBI. As shown in Fig. 7, the majority of TBI-induced apoptosis occurred in the neurons.

**Discussion**

The present study demonstrated that administration of harmine (30 mg/kg per day) for up to 5 days immediately following TBI had neuroprotective potential in the rats. Treatment with harmine attenuated post-traumatic cerebral edema, and improved learning and memory abilities. Furthermore, the present study investigated the mechanisms underlying the protective effects of harmine on TBI. At the molecular level, the protein expression levels of GLT-1 and inflammatory cytokines in the hippocampus of the rat brain were detected using western blot analysis. Treatment with harmine resulted in a marked elevation in the expression of GLT-1, as well as significant reductions in the expression levels of IL-1β and TNF-α, suggesting that harmine attenuated apoptotic neuronal death in the hippocampus. Previous studies have reported that treatment with harmine significantly attenuated glutamate-induced and oxidative stress-induced neuronal death in neuronal cell cultures (15,17). In addition, a previous in vivo study demonstrated that harmine exerts neuroprotective effects against glutamate-mediated excitotoxicity in a rat model of amyotrophic lateral sclerosis disease (17). These findings, together with the findings of the present study, improve current understanding of harmine-mediated neuroprotection in neurological disorders.
Treatment with harmine not only protects neurons against glutamate insult via elevating the expression level of GLT-1, but it also led to the exertion of anti-inflammatory effects following TBI. Previous studies have demonstrated that inflammatory cytokines can directly and indirectly attenuate the expression of glutamate transporters in astrocytes, which has a significant role in TBI-induced glutamate excitotoxicity, and it has been reported that IL-1β and TNF-α regulate glutamate transmission directly through elevation of the expression of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (25-27). In the present study, the expression levels of inflammatory cytokines in the hippocampus of the rat brain were suppressed following treatment with harmine. Although the mechanism underlying the neuroprotective effects of harmine on TBI remain to be fully elucidated, the data of the present study suggested that attenuation of inflammation may synergistically reduce extracellular glutamate. This suggests that the recruitment of attenuated inflammation occurs in a physiological action, which acts synergically with the GLT-1 response to protect neurons from TBI-induced insult and death, and further investigation of this is required. The present study performed only preliminarily examination of the neuroprotective effects induced by harmine on TBI rats; therefore, further investigations are required to establish the appropriate routes of administration, the exact time-frame, and whether these effects are observed following different injury magnitudes.

The results of the present study demonstrated that administration of harmine significantly attenuated cerebral edema, and improved learning and memory abilities following experimental TBI. Furthermore, treatment with harmine markedly elevated the expression of GLT-1 and significantly attenuated the expression levels of IL-1β and TNF-α, thereby attenuating apoptotic neuronal death in the hippocampus. These findings indicated that administration of harmine (30 mg/kg per day, up to 5 days) following TBI can be neuroprotective, via anti-inflammatory and anti-excitotoxicity effects following experimental TBI in rats. These findings may represent a potential novel therapeutic modality for the treatment of TBI.

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