

## Original Article

# Expression of BDNF and Nogo-A after singular and repetitive mild traumatic brain injury in rats

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**Abstract:** Mild traumatic brain injury (mTBI) potentially causes long-lasting cognitive deficits, emotional difficulties and behavioral disturbances. Owing to a lack of observable damages following mTBI, the early diagnosis and long-term impact of the insult are limited. In the present study, we assessed the effects of singular and repetitive experimental mTBI (smTBI and rmTBI) in rats on the cognitive, motor function and anxiety-like behavior as well as the time-point changes of BDNF and Nogo-A within 24 days following injuries. Experimental brain injury was induced using a concussive head trauma with a weight-drop device. The single and repetitive mTBI injured rats and sham-operated rats were tested for cognitive, motor function and anxiety-like behavior at 1, 7 and 14 days post-trauma. The results showed that smTBI rats showed impaired cognitive, motor function and anxiety-like behavior within one week after injuries. rmTBI resulted in motor functional impairment of rats within one week while the cognitive deficits and anxiety behavior lasted until two weeks after the injury. Furthermore, brain BDNF peaked at day 2 in smTBI hippocampus CA1 sections and at day 4 for rmTBI rats. Meanwhile, the hippocampus Nogo-A significantly decreased at day 4 in smTBI rats and at day 8 for rmTBI rats. Likewise, BDNF and Nogo-A fluctuated at each time point tested in secondary motor cortex M2-1 of the rat post-smTBI and rmTBI. In conclusion, the time-course change of BDNF and Nogo-A might provide clues for the early diagnosis and treatment of single and repetitive mild TBI.

**Keywords:** BDNF, Nogo-A, singular mTBI, repetitive mTBI, cognitive impairment

## Introduction

Traumatic brain injury (TBI), defined as an insult to the brain from an external mechanical force that causes neurological and consciousness impairments, is one of the leading causes of injury that affects approximately 10 million people world-wide annually, especially in children and young adults [1, 2]. TBI increases the risk of death and disability and is a significant public health problem. In human, severe TBI causes chronic physical, neurological and cognitive impairments and most individuals suffered mild TBI show no apparent physical changes. In recent years, the impacts of repetitive mild TBI (rmTBI) have become of particular concern for individuals at high risk of repeated concussion, such as certain sports athletes and military populations [3]. Compelling evidence from both clinical and experimental settings revealed that repeat injuries within spe-

cific time frames after the initial insult can cause exacerbated brain pathology and related behavioral morbidity both in short and long term [4, 5]. It is showed that rmTBI has been associated with chronic traumatic encephalopathy or chronic neurodegenerative syndromes with pathological manifestations of brain volume loss, presence of tau-immunoreactive neurofibrillary tangles and amyloid beta deposition [3, 4, 6]. Diagnosis of mTBI is challenging now due to the lack of any immediate symptoms and identified pathological changes [7]. In recent years, more attention is paid in describing potential biomarkers in the early diagnosis of mTBI [8].

Brain-derived neurotrophic factor (BDNF) is a highly expressed neurotrophic factor in the brain that affects neuronal survival and regeneration [9, 10]. Accumulated *in vivo* studies have shown that BDNF attenuates neuronal cell

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death caused by excitotoxicity, ischemia, or axotomy and regulates synaptic plasticity [9]. However, controversy still exists regarding the effect of BDNF on hippocampal neurons and cognitive functions after TBI [11]. Moreover, abnormal regulation of myelin associated inhibitors (MAIs) repress neuron regeneration, sprouting, and plasticity in both the intact and lesioned central nervous system [12]. As a critical member of MAIs, Nogo-A is shown to initiate growth cone collapse and inhibit neurite outgrowth *in vitro* [13]. BDNF is gaining recognition as a main modulator of MAG and Nogo-A inhibition [14]. Our study will focus the longitudinal BDNF and Nogo-A patterns and their relations to motor and cognitive functions in Sprague-Dawley rats after singular mTBI and rmTBI insults.

### Materials and methods

#### *Animal model*

Healthy adult Sprague-Dawley rats (weight  $280 \pm 30$  g) were obtained from Experimental Animal Center of Kunming Medical University and housed in standard polyethylene cages in an environmentally controlled room (22-24°C, 12 h light/dark cycle) with access to standard food and water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Kunming Medical University and were conducted in accordance with standards published by the National Research Council.

All rats were randomized to undergo singular mTBI (n=60) and repetitive mTBI (n=60). Before the injuries, all the rats were recorded of respiratory rate, corneal reflection, pain response, external auditory canal reaction and righting reflex. Afterwards, the rat was fixed on the platform and positioned with head directly under the metal bolt. The bolt was elevated 95°~105° from its initial status and then was set for free-falling. After the injury, above parameters were determined and recorded. With regarding to the establishment of rmTBI model, the above operation was repeated in three consecutive days and once every day. Rats with subdural hemorrhage or characteristics of severe trauma were excluded from the study. For sham injuries, the same procedure was performed except that the impact device was discharged in the air. All rats were recovered in room air.

#### *Assessment of motor function*

Motor ability and function of the injured and control animals were assessed on an accelerating Rota-Rod as described by Luo et al [3]. In brief, the Rota-Rod consists of a rotating drum (4 cm in diameter) and the test rat is placed on the drum. The Rota-Rod was set to accelerate from 5 to 30 rpm during a test period of 5 min. The rat was tested three times with an inter-trial interval of 20 min, and the time between placement on the Rota-Rod and fall off from the Rota-Rod onto the transducer platform was automatically recorded. The average latency of the three trials was calculated for the analysis.

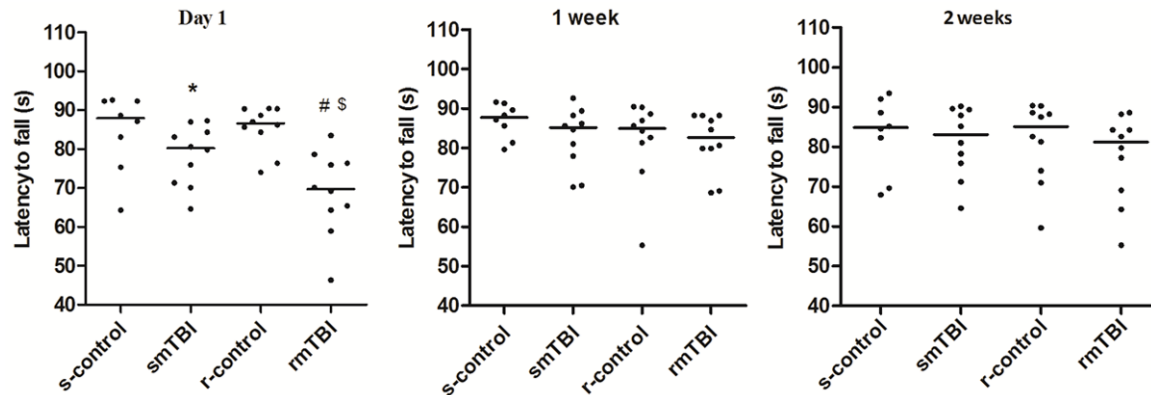
#### *Assessment of spatial learning and memory*

Spatial learning and memory were evaluated using the hidden trials of Morris water maze (MWM) that has been described by [15]. In brief, circular tank (2.0 m in diameter; 60 cm in depth) was filled with water to a depth of 29 cm and with water temperature maintained at 25°C. A clear platform (15 cm in diameter) was submerged 2.0 cm below water surface in the southwest quadrant 15 cm from the wall. Rat was placed facing the wall and randomized to 1 of 4 starting locations (north, south, east, and west) during the hidden trail. All rats were given 120 s to find the location of the hidden platform, mount the platform, and remain on it for 30 s. The time until the rat mounted the platform (escape latency) was recorded. Rat that failed to mount the platform within 120 s was placed on the platform by the examiner and stayed for 10 s and was recorded with an escape latency of 120 s. After finishing the test in southwest quadrant, rat was then rewarded by placement under a heat lamp for 4 min and followed by another trail.

#### *Elevated plus maze testing*

The mild TBI caused anxiety-like behavior in rat was determined using elevated plus maze (EPM) testing as described [16]. In brief, the plus maze elevated 1 m from the floor with two intersecting runways (12 cm wide × 100 cm long) that perpendicular from each other. Two of the runways had no walls while the others had walls with height of 40 cm. The tested rat was placed in the center of the maze facing a closed arm and allowed to freely explore for 5 min. Time spent in open arms was recorded.

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**Figure 1.** Effects of singular and repetitive mild TBI on motor function of injured rats. Rota-Rod tests were performed and the time of rats fall to were recorded on day 1, 7, and 14 after initial injuries (n=8 for s-sham at day 1, 7, and 14; n=10 for s-mTBI, r-sham and r-mTBI group at day 1, 7, and 14). \*P<0.05, compared to s-control; #P<0.05, compared to r-control; \$P<0.05, compared to smTBI.

### Immunohistochemical analysis

After behavior tests, smTBI or rmTBI and control rats were sacrificed 1, 2, 4, 8, 16 and 24 days post-trauma (n=8 for each time point). The animals were anaesthetized intraperitoneally with 1% pentobarbital sodium and perfused transcardially with saline solution with heparin. The brains were resected and fixed in 4% paraformaldehyde for 48 h at room temperature, then cut into three consecutive coronal sections. Then the sections were processed and embedded with OTC, a series of consecutive sections were cut through a freezing microtome and then were pasted on slides. The sections were then washed in TBS and endogenous peroxidases were quenched with 3% H<sub>2</sub>O<sub>2</sub> in distilled water for 15 min. After incubation in normal nonimmunone serum blocking solution for 15 min, the sections were reacted with rat monoclonal antibodies against BDNF (1:350 dilution, Chemicon, USA) and Nogo-A (1:250, R&D system, USA) overnight at 4°C that were then detected using secondary biotin-conjugated IgG antibodies (Fuzhou Maxim Biotech, China) for 1 h at room temperature. Immunoreactions were visualized with a diaminobenzidine kit and analyzed with a light microscope (Nikon Eclipse 8600, Melville, NY).

### Statistical analysis

Data are expressed as means  $\pm$  SEM. All statistical analyses were carried out using SPSS 13.0 (SPSS, Chicago, IL). Statistical comparisons were performed by one-way analysis of variance followed by the Student-Newman-

Keuls test; Correlations between BDNF and Nogo-A were analyzed using Pearson test. P<0.05 was considered statistically significant.

## Results

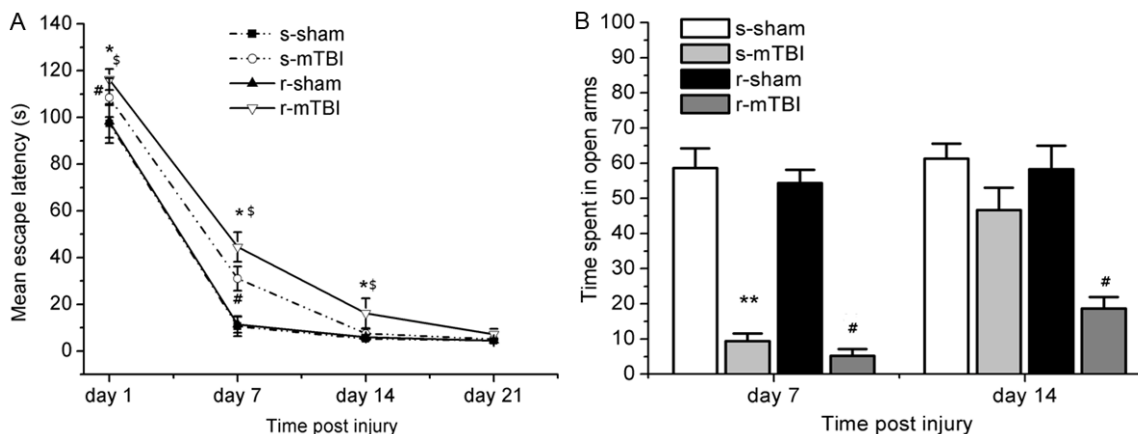
### Impairment of motor function by singular mild TBI and repetitive mild TBI

To evaluate the impact of smTBI and rmTBI on motor function and activity of the injured animals, a Rota-Rod test was applied and the latency to fall of each rat was recorded one day, one week and two weeks after injury, respectively. The data showed that smTBI and rmTBI showed impaired performance in Rota-Rod testing on days 1 post-trauma as reflected by the significantly reduced time to fall compared with the corresponding sham-injured animals of s-control and r-control group. Meanwhile, rmTBI rats exhibited decreased latency to fall than that of smTBI rats (**Figure 1A**). smTBI rats showed recovered motor function one week after the injury, while, rmTBI rats still had persistently decreased latency compared with sham-injured rats until two week later (**Figure 1B** and **1C**).

### Impacts of singular and repetitive mild TBI on cognitive function and anxiety-like behavior

Morris water maze test was applied to evaluate the spatial learning and memory of rats in response to singular mild TBI and repetitive mild TBI. The data showed that following testing in the Morris water maze (MWM) on days 1-7 after initial injury, both smTBI and rmTBI rats

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**Figure 2.** Effects of singular and repetitive mild TBI on cognitive function and anxiety-like behaviors of injured rats. A. Morris water maze (MWM) tests were performed and the mean escape latency was recorded to indicate the cognitive function of the injured rats within 21 days. B. Anxiety-like behavior of injured rats was evaluated using an elevated plus mazing test on day 7 and day 14. \* $P < 0.05$ , compared to s-control; # $P < 0.05$ , compared to r-control; \$ $P < 0.05$ , compared to smTBI.

had significantly longer MWM escape latency than the matched sham-operated animals, and rmTBI rats showed longer mean escape latency than the smTBI animals until 14 days after the injuries. However, rmTBI did not significantly affect the performance of the MWM tests three weeks after brain injury (Figure 2A). Moreover, rmTBI also resulted in increased unconditioned anxiety-like behavior on day 7 and day 14 after injuries as reflected by the significantly reduced time spent in open arms during the elevated plus maze (EPM) tests compared to r-sham group ( $P < 0.05$ ). In addition, smTBI rats had less time spent in open arms than the s-sham rats on day 7 after injury and no significant difference was shown in EPM tests after 14 days after injury (Figure 2B).

### *BDNF expression in response to the mild TBI and repetitive mild TBI*

We determined the longitudinal expression of BDNF in hippocampus CA1 area and secondary motor cortex M2-1 area using IHC within 24 days after the injuries. smTBI rats showed marked increase of BDNF in brain CA1 area after 2 days of injury and no significant change has been shown within 4-24 days compared to the s-sham animals ( $P < 0.05$ ). Moreover, the BDNF expression dramatically reduced immediately after the repeated insults while increased and peaked on day 4. After another decrease on day 8 the BDNF returned to the normal level and profoundly increased afterwards (Figure 3A and 3B). In secondary motor

cortex M2-1 area, BDNF was downregulated during 2 days after the singular mild TBI and returned to initial levels until the ending of the test. However, BDNF is mainly upregulated within the frame of the tests and peaked at day 1 and day 16 post-trauma (Figure 3C).

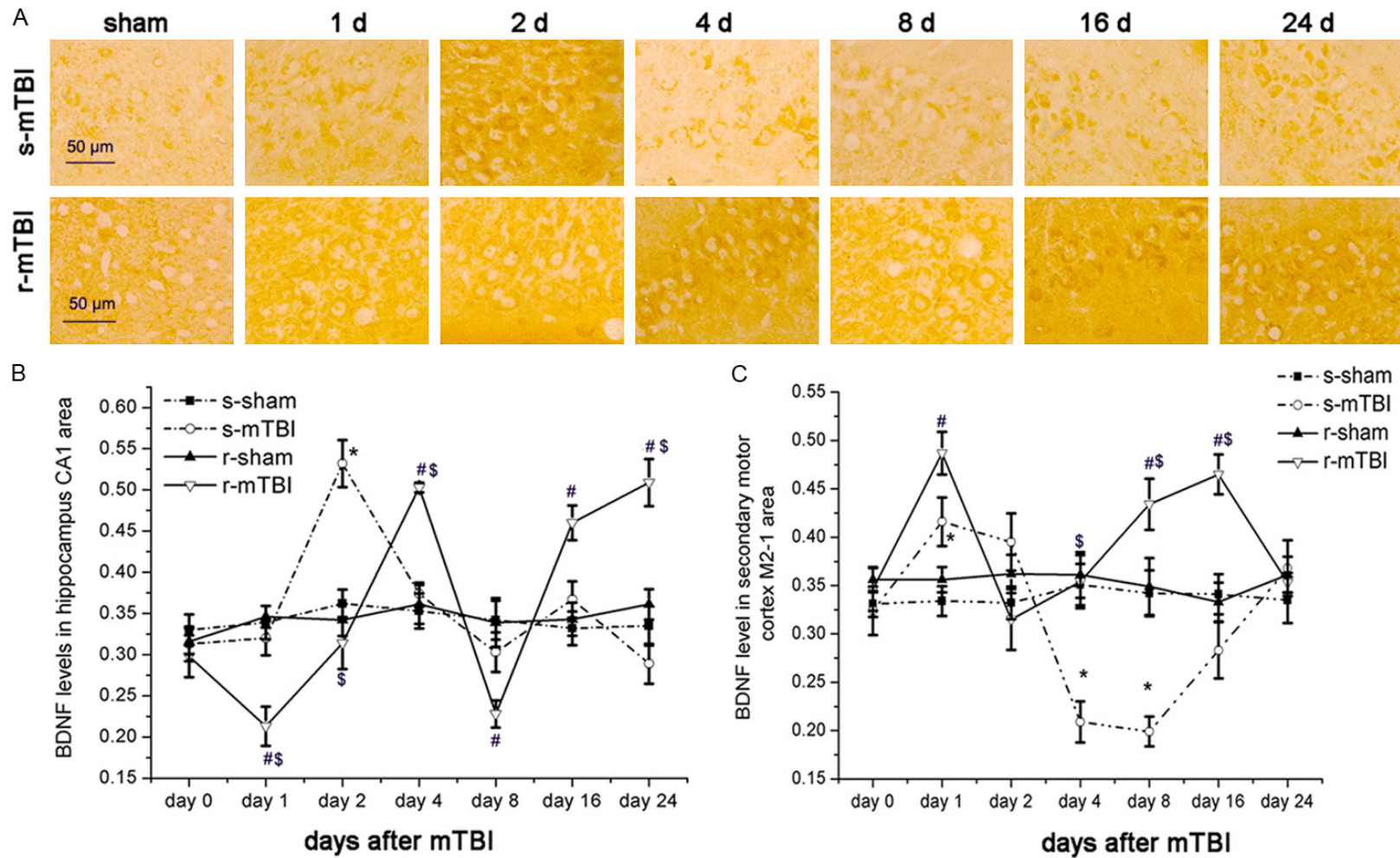
### *Altered expression of Nogo-A after singular mild TBI and repetitive mild TBI*

We further detected the levels of Nogo-A in hippocampus CA1 area and secondary motor cortex M2-1 within 24 days post-trauma. As shown in Figure 3A and 3B, Nogo-A showed the similar fluctuation tendency in hippocampus CA1 area of smTBI rats as that in the rmTBI rats, though rmTBI seemed to have a delayed change of Nogo-A than the smTBI rats (Figure 4A and 4B). However, in secondary motor cortex M2-1 area, Nogo-A persistently had a dramatically increase after repetitive mTBI within 24 days compared with the sham control. Moreover, after singular TBI, Nogo-A expression is significantly higher on day 1-2 than that of the control animals, and it gradually decreased until day 16 after the injury followed by another increase at day 24 (Figure 4C).

### *Association of the expression between BDNF and Nogo-A in TBI-subjected mouse*

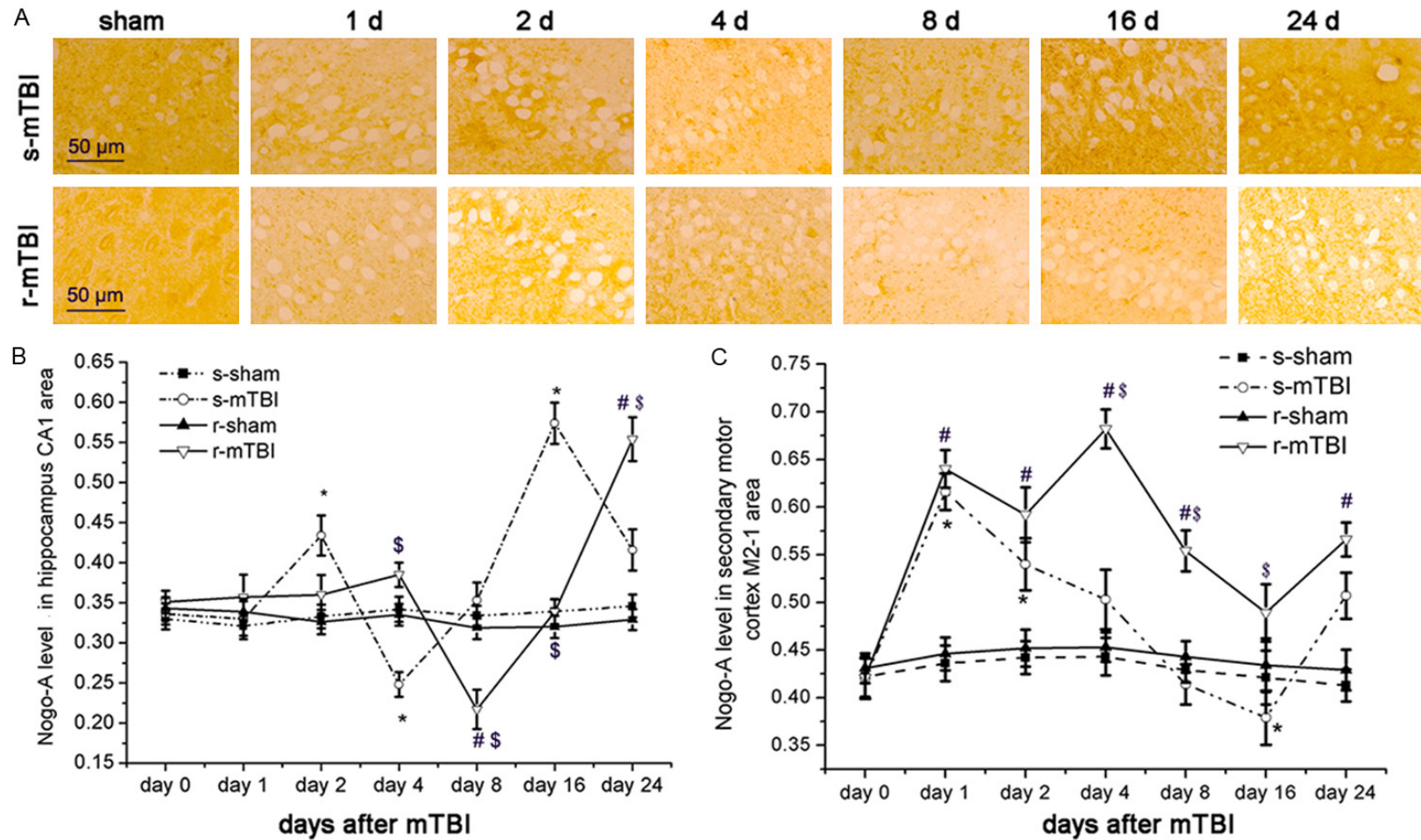
BDNF facilitates the repair of neuron injury while Nogo-A seems to have adverse effect on nerve growth by inhibiting neuron regeneration. Therefore, we sought to investigate the associ-

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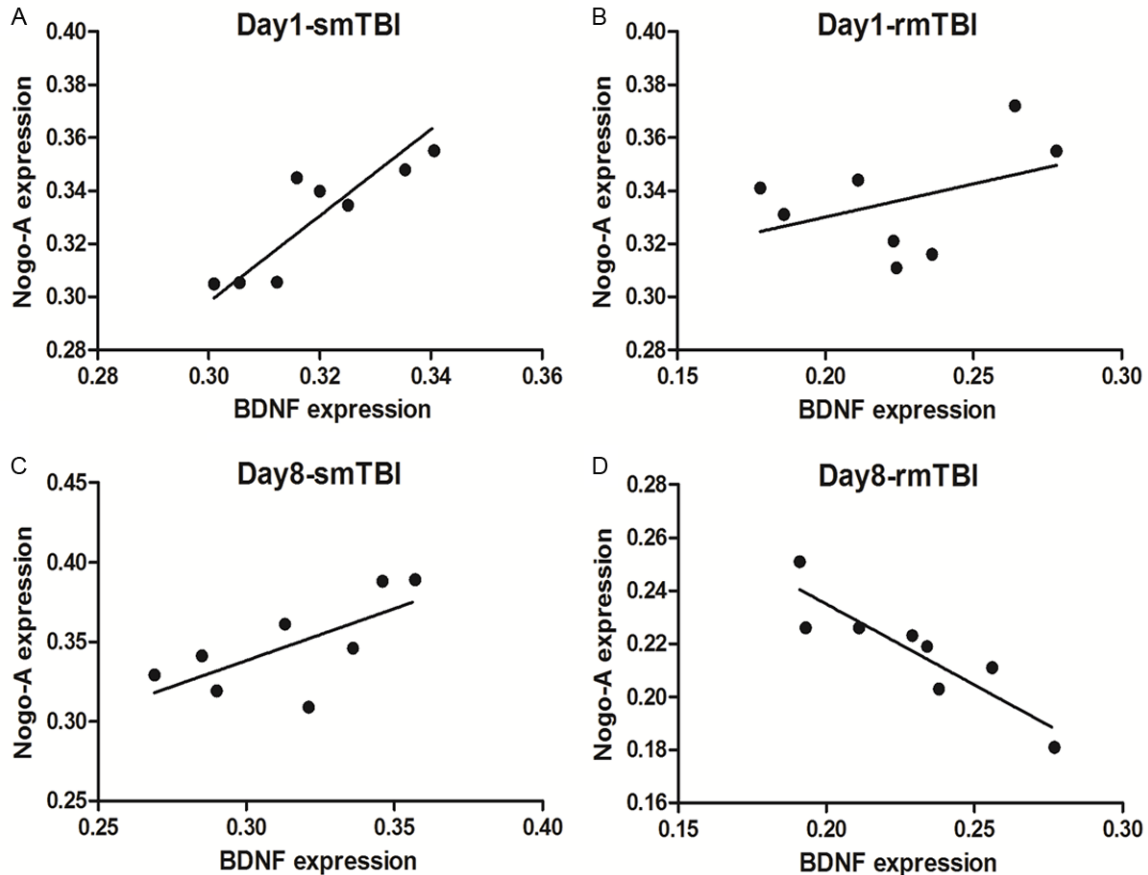
**Figure 3.** Longitudinal change of BDNF in hippocampus CA1 area and secondary motor cortex M2-1 area within 24 days after singular and repetitive injuries. IHC analysis (A) and quantification (B) of BDNF staining in hippocampus CA1 area of injured rats (n=8 for smTBI or rmTBI group at each time point; n=5 for s-sham or r-sham group at each time point), scale bar =50  $\mu$ m (C) Quantification of BDNF positive staining in secondary motor cortex M2-1 area of injured rats within 24 days (n=8 for smTBI or rmTBI group; n=5 for s-sham or r-sham group). \*P<0.05, compared to s-control; #P<0.05, compared to r-control; \$P<0.05, compared to smTBI.

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**Figure 4.** Longitudinal change of Nogo-A in hippocampus CA1 area and secondary motor cortex M2-1 area within 24 days after singular and repetitive injuries. IHC analysis (A) and quantification (B) of Nogo-A staining in hippocampus CA1 area of injured rats (C) Quantification of Nogo-A positive staining in secondary motor cortex M2-1 area of injured rats within 24 days (n=8 for smTBI or rmTBI group; n=5 for s-sham or r-sham group). Scale bar =50  $\mu$ m. \*P<0.05, compared to s-control; #P<0.05, compared to r-control; \$P<0.05, compared to smTBI.

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**Figure 5.** Correlation analysis of the expression between BDNF and Nogo-A in hippocampus CA1 area. BDNF and Nogo-A in brain hippocampus CA1 sections were stained and the optical density (OD) were recorded. OD values of BDNF against Nogo-A of each rat after smTBI (A and C) and rmTBI (B and D) were scatter plotted.

ation between the levels of BDNF and Nogo-A in hippocampus neural area on day 1 and day 8 after smTBI and rmTBI. A significant positive correlation between BDNF and Nogo-A was shown in smTBI rats on day 1 ( $r=0.87$ ,  $P=0.005$ ) (Figure 5A). Negative correlation between BDNF and Nogo-A was shown in rmTBI rats 8 day post-injury ( $r=-0.894$ ,  $P=0.003$ ) (Figure 5D). Meanwhile no significant correlations were indicated between BDNF and Nogo-A in rmTBI rats 1 day post-injury ( $r=0.416$ ,  $P=0.305$ ) (Figure 5B) and smTBI animals 8 days post-injury ( $r=0.069$ ,  $P=0.061$ ) (Figure 5C). Above all, fluctuations of BDNF and Nogo-A seemed not follow a consistent rule and their correlations may vary with spatial-temporal difference of individuals.

### Discussion

Traumatic brain injury is one among the most frequent neurological disorders, with an esti-

mated 80% of overall TBI classified as falling in the mild range that manifested with loss of consciousness lasting less than 30 minutes, an initial Glasgow Coma Score of 13 to 15, and posttraumatic amnesia lasting less than 24 hours [17, 18]. It is claimed that mTBI is a highly individualized injury. Most people recover relatively quickly and fully while others have long-term problems [19]. mTBI increases the risk of the development of subacute and chronic sequelae such as depression, posttraumatic stress disorder, mild cognitive impairment and chronic traumatic encephalopathy (CTE). Meanwhile, a higher incidence of mTBI sequelae is associated with the ages of the victims and the frequency of concussions [20]. Therefore, in our study, the experimental brain injury rodent animals were established and the impacts of singular and repetitive mTBI on the motor and cognitive function were evaluated and the fluctuations of BDNF and Nogo-A during the neuron repair were described.

## Changes of BDNF and Nogo-A after mTBI

In the current study, we assessed the smTBI and rmTBI on the motor and cognitive functions of the experimental brain injured rats and found that motor function in both smTBI and rmTBI rats were impaired immediately after the insults and this adverse effect disappeared after one week. The cumulative effects of repetitive mTBI on cognitive deficits and depression-like behaviors lasted for two weeks post-trauma while these symptoms relieved in smTBI rats after one week of injuries. Our conclusions are consistent with reports in the human population in which those suffering from mild head injury often return to their preconcussive status within a week [21]. Moreover, Mouzon reported that in an experimental mice model (total of 5 hits with an interval of 48 hours), the long-term effects of repetitive TBI on chronic neuropathological and neurobehavioral changes have been observed until 18-months post-traumatic period [22]. We considered the experimental settings and assessing periods may cause the difference in the observations of the effects of rmTBI.

Mild traumatic brain injury may cause neuronal cell death and traumatic axonal injury in both humans and animals [23, 24]. As a critical neurotrophic factor that affects neuronal survival and regeneration, change of BDNF is associated with the recovery of cognitive defects from injury. A compelling clinical study has shown that day-of-injury circulating BDNF is associated with TBI diagnosis and also provides 6-month prognostic information regarding recovery from TBI [10]. In addition, acute BDNF is associated with memory recovery following TBI that may implicate hippocampal damage/degeneration [25]. In our study, BDNF peaked on day 4 for rmTBI rats and on day 2 for smTBI rats in hippocampal neurons. This signature may serve as a biomarker for the early diagnosis of mTBI. More details about the longitudinal expression of BDNF during single and repetitive TBI are needed.

Furthermore, Nogo-A is demonstrated to inhibit neurite outgrowth *in vitro*. Inhibition of Nogo-A using neutralizing antibody has been shown to enhance axonal outgrowth and sprouting to enhance cognitive recovery following experimental TBI in rats [26]. However, Marklund *et al* showed that Nogo-A/B deficient mice exhibited less recovery from neurological motor and cog-

nitive deficits compared to brain-injured WT mice [27]. Moreover, in our study, the time-course change of BDNF and Nogo-A failed to follow a consistent rule. Until now, the definite association between BDNF and Nogo-A has not been documented and further details are required.

Above all, in the present study, we created single and repetitive mild TBI rat model using a weight-drop device and evaluated the change of animal motor and cognitive performance, the time-course change of BDNF and Nogo-A within 24 days after the injuries. The results demonstrated that repetitive mTBI injured rats exhibited long-lasting motor and cognitive deficiency and anxiety-like behavior than the rats underwent singular mTBI insult. BDNF and Nogo-A showed fluctuations in a spatial-temporal dependent manner in the hippocampus CA1 area and secondary motor cortex M2-1 within 24 days post-trauma. The time-course change of BDNF and Nogo-A might provide clues for the early diagnosis and treatment of single and repetitive mild TBI.

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### Disclosure of conflict of interest

None.

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