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Effects of diazepam on glutamatergic synaptic transmission in the hippocampal CA1 area of rats with traumatic brain injury

Lei Cao[1, 2], Xiaohua Bie[1], Su Huo[2], Jubao Du[2], Lin Liu[2], Weiqun Song[2]

1 Department of Functional Neurosurgery, Xi'an Red Cross Hospital, Xi'an, Shaanxi Province, China
2 Department of Rehabilitation Medicine, Xuanwu Hospital, Capital Medical University, Beijing, China

Lei Cao and Xiaohua Bie contributed equally to this work.

Corresponding author:
Weiqun Song, Ph.D., Department of Rehabilitation Medicine, Xuanwu Hospital, Capital Medical University, Beijing 100053, China, songwq66@vip.163.com.

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Abstract

The activity of the Schaffer collaterals of hippocampal CA3 neurons and hippocampal CA1 neurons has been shown to increase after fluid percussion injury. Diazepam can inhibit the hyperexcitability of rat hippocampal neurons after injury, but the mechanism by which it affects excitatory synaptic transmission remains poorly understood. Our results showed that diazepam treatment significantly increased the slope of input-output curves in rat neurons after fluid percussion injury. Diazepam significantly decreased the numbers of spikes evoked by super stimuli in the presence of 15 μmol/L bicuculline, indicating the existence of inhibitory pathways in the injured rat hippocampus. Diazepam effectively increased the paired-pulse facilitation ratio in the hippocampal CA1 region following fluid percussion injury, reduced miniature excitatory postsynaptic potentials, decreased action-potential-dependent glutamine release, and reversed spontaneous glutamine release. These data suggest that diazepam could decrease the fluid percussion injury-induced enhancement of excitatory synaptic transmission in the rat hippocampal CA1 area.

Key Words: *nerve regeneration; traumatic brain injury; fluid percussion injury; excitatory synaptic transmission; hippocampal CA1 pyramidal neurons; paired-pulse facilitation; miniature excitatory postsynaptic potential; gamma-aminobutyric acid; post-traumatic hyperactivity; intracellular recording; NSFC grant; neural regeneration*

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Introduction

Traumatic brain injury often results in persistent cognitive impairment, which severely reduces the quality of a patient's life (Azouvi et al., 2009; Risdall and Menon, 2011; Moreau et al., 2013). In addition to the risk of acute mortality, severe traumatic brain injury is a risk factor for the development of post-traumatic epilepsy (Liesemer et al., 2011; Lusardi et al., 2012; Yeh et al., 2013). Post-traumatic epilepsy is one of the major contributors to compromised functional outcome and quality-of-life in patients with traumatic brain injury (Andelic et al., 2009; Chen et al., 2013). Animal models provide an efficient way to study the pathophysiology of traumatic brain injury (O'Connor et al., 2011; Long et al., 2013; Xiong et al., 2013). Fluid percussion injury of the rat brain is one of the most extensively used and best characterized animal models of human traumatic brain injury (D'Ambrosio et al., 2004; Thompson et al., 2005; Kharatishvili et al., 2006). Fluid percussion injury produces hyperexcitability of hippocampal CA1 neurons by increasing the activity of the Schaffer collaterals of hippocampal CA3 neurons (Akasu et al., 2002; Dinocourt et al., 2011; Zhang et al., 2011).

Glutamate receptor antagonists have been shown to be neuroprotective (Bernert and Turski, 1996; Allen et al., 1999; Gasparini et al., 1999; Movsesyan and Faden, 2006). On the other hand, facilitating inhibitory synaptic transmission is another way to prevent excitotoxicity following traumatic brain injury (Luo et al., 2011). Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain, being found in 30–40% of all synapses (Noh et al., 2010; Ben-Ari et al., 2012). Diazepam, a benzodiazepine derivative drug, has an anxiolytic action, with sedative and hypnotic effects (Manna and Umathe, 2011; Liu et al., 2013). Diazepam enhances the efficacy of gamma-aminobutyric acid type A ($GABA_A$) receptor-Cl^- channels, which is beneficial for mortality and cognitive impairment following traumatic brain injury (O'Dell et al., 2000; Richter et al., 2012). Moreover, recent research has shown that diazepam attenuates the post-traumatic hyperactivity of rat hippocampal

CA1 neurons (Ooba et al., 2008; Ma et al., 2014). In the present study, we sought to explore the effects of diazepam on excitatory synaptic transmission following brain trauma by intervention with the $GABA_A$ receptor antagonist dicentrine (Palombi and Caspary, 1992).

Materials and Methods

Animals

Twenty-four clean, healthy, 6-week-old male Wistar rats weighing 280–320 g were used in the experiments. All rats were obtained from Experimental Animal Laboratories of the Academy of Military Medical Sciences (Beijing, China) (license No. SCXK (Army) 2007-0004). Rats were maintained under a 12-hour light/dark cycle in the animal facility, and were allowed free access to food and water. All procedures in this study were approved by the Animal Care and Use Committee of Capital Medical University, China. All animals were equally and randomly divided into a control group (without any treatment), a diazepam group, a fluid percussion injury group, and a fluid percussion injury + diazepam group.

Establishment of fluid percussion injury model

A fluid percussion injury model was established as previously reported with some modifications (Smith et al., 2005). Briefly, animals were intraperitoneally anesthetized with pentobarbital sodium 50–60 mg/kg, placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). A scalp incision was made and the scalp and temporal muscles were exposed. A craniotomy (3 mm × 3 mm) was performed 3 mm caudal from the coronal suture and 3 mm lateral to the sagittal suture on the left parietal bone without impairing the dura. A connecting cap was placed over the craniotomy and connected with the fluid percussion injury device. Animals were subjected to a fluid percussion of 3.8–4.8 atm (385.0–486.4 kPa) onto the cerebral cortex of the left hemisphere.

Diazepam treatment post-injury

At 30 and 90 minutes following fluid percussion injury, animals in the fluid percussion injury + diazepam groups were intraperitoneally administered diazepam ($C_{16}D_5H_8ClN_2O$, molecular weight 284.76; 10 mg/kg, in 0.9% saline) (Product ID: D-910; Sigma, St. Louis, MO, USA). The rats in the control group received an equal volume of 0.9% saline at the corresponding time points.

Hippocampal brain slice preparations and intracellular recording

After a survival period of 7–8 days, the rats were decapitated and brains were harvested for slice preparation. Brain tissue including the hippocampal CA1 area was cut into 400-μm horizontal slices, which were immersed in a cooled artificial cerebrospinal fluid that was pre-bubbled with 95% O_2–5% CO_2 in a manner similar to that described previously[21]. Each slice was cut into two slices, one containing the left (ipsilateral to the impact) hippocampus and the other one containing the right (contralateral) hippocampus. Intracellular recording of hippocampal CA1 pyramidal neurons was conducted after 1 hour. Excitatory postsynaptic potentials were evoked through a concentric bipolar electrode (Nihon Kohden SEN-7103, Tokyo, Japan) placed on the Schaffer collaterals in the hippocampal CA2 region. Data were recorded when the resting membrane potential of the neuron had remained stable for more than 20 minutes. Input–output relationship curves were generated based on the stimulus intensity (input) and initial slopes of the excitatory postsynaptic potentials. With the greater stimulation intensities (10, 20 and 30 V, 200 μs duration), recordings of the numbers of spikes were conducted in the presence of 15 μmol/L bicuculline (Product ID: 285269; Sigma). Bicuculline ($C_{20}H_{17}NO_6$, molecular weight 284.76) is a $GABA_A$ receptor antagonist (Palombi and Caspary, 1992). The consecutive excitatory postsynaptic potentials were evoked by paired-pulse stimuli with intervals from 50 to 160 ms repeated every 10 seconds in the presence of bicuculline (15 μmol/L), and the values for amplitude ratios of excitatory postsynaptic potentials (P2/P1) were recorded. Miniature excitatory postsynaptic potentials were recorded in the presence of 15 μmol/L bicuculline and 1 μmol/L tetrodotoxin (Product ID: T8024; $C_{11}H_{17}N_3O_8$, molecular weight 319.27; Sigma), and the numbers of miniature excitatory postsynaptic potentials during 300 seconds were analyzed by Mini-analysis software (Version 6; Synaptosoft, Decatur, GA, USA).

Statistical analysis

Quantitative data were expressed as the mean ± SD and analyzed by one-way analysis of variance using SPSS 16.0 (SPSS, Chicago, IL, USA). Student-Newman-Keuls tests were used for specific comparisons. Statistical significance was set at a level of $P < 0.05$.

Results

Diazepam decreased fluid percussion injury-induced hyperactivity in rat hippocampal CA1 pyramidal neurons

The slope of the input–output curve was greater in the ipsilateral hippocampus with the fluid percussion injury group than in the control group ($P < 0.05$). Compared with the fluid percussion injury group, the slope of the input–output was significantly lower in the fluid percussion injury + diazepam group ($P < 0.05$). However, no significant difference in slope was detected among the control, fluid percussion injury and fluid percussion injury + diazepam groups in the contralateral hippocampus ($P > 0.05$; **Figure 1A**). These observations suggested that the efficacy of excitatory synaptic transmission in the hippocampal CA1 area was enhanced at 1 week after fluid percussion injury; however, diazepam hampered this enhancement.

To examine whether the fluid percussion injury-induced facilitation of synaptic transmission is due to functional damage of GABAergic inhibitory neurons in ipsilateral hippocampal slices, bicuculline (15 μmol/L), a selective blocker of $GABA_A$ receptors, was applied. In normal artificial cerebrospinal fluid, the numbers of spikes induced by different stimulus intensities (10, 20 and 30 V, 200 μs

duration) were not signifi cantly different between the fluid percussion injury group and the fluid percussion injury + diazepam group ($P > 0.05$). However, in the presence of 15 μmol/L bicuculline, diazepam significantly reduced the number of spikes ($P < 0.05$; **Figure 1B**). These results suggested that the inhibitory pathways are fully functional in the hippocampi of fluid percussion injury rats.

Taken together, these results indicated that diazepam significantly decreases the fluid percussion injury-induced hyperactivity of rat hippocampal CA1 pyramidal neurons in the ipsilateral hippocampus following fluid percussion injury.

Diazepam increased fluid percussion injury-induced paired-pulse facilitation in rat hippocampal CA1 pyramidal neurons

The results described above revealed the preservation of inhibitory pathways; therefore, we concluded that fluid percussion injury directly enhanced the function of the glutamatergic excitatory pathway in the hippocampal CA1 area. To test this, Schaffer collaterals were stimulated by a pair of electrical pulses at 50–160-ms intervals, repeated every 10 seconds. As shown in **Figure 2**, the paired-pulse facilitation ratio (P1/P2 ratio) decreased with prolonged stimulus interval. P2/P1 ratios were significantly increased in the fluid percussion injury + diazepam group compared with the fluid percussion injury group ($P < 0.05$), to a level that showed no significant difference from the control group ($P > 0.05$). These results suggested that diazepam effectively diminishes the probability of action-potential-dependent glutamine release from the terminals of Schaffer collateral terminals in the ipsilateral hippocampus CA1 area following fluid percussion injury.

Diazepam diminished the number of miniature excitatory postsynaptic potentials in fluid percussion injury-induced rat hippocampal CA1 pyramidal neurons

To investigate a possible contribution of postsynaptic mechanisms in this enhancement, we analyzed the number of miniature excitatory postsynaptic potentials. Under the experimental conditions, the spontaneous miniature excitatory postsynaptic potentials were recorded as subliminal depolarization waveforms. During the recording time of 300 seconds, the number of miniature excitatory postsynaptic potentials recorded in the ipsilateral hippocampus following fluid percussion injury was significantly decreased by the application of diazepam ($P < 0.05$; **Figure 3**). These results showed that, in the ipsilateral rat hippocampus CA1 area following fluid percussion injury, diazepam effectively reversed the increase in spontaneous glutamate release from the Schaffer collateral terminals.

Discussion

Studying the mechanisms underlying post-traumatic epilepsy is complicated by its long latency, often occurring months or years after a traumatic event (Lusardi et al., 2012). Early pharmacological intervention following traumatic brain injury is especially important for preventing post-traumatic epilepsy. The present study was designed to evaluate the ability of diazepam to prevent the development of hyperexcitability in rats with traumatic brain injury. We found that diazepam could decrease fluid percussion injury-induced hyperactivity and increase fluid percussion injury-induced paired-pulse facilitation in rat hippocampal CA1 pyramidal neurons. Diazepam also diminished the number of miniature excitatory postsynaptic potentials.

Lateral fluid percussion injury is one of the most commonly used experimental models of human traumatic brain injury in rodents (Morales et al., 2005). Early electrophysiological studies using acute hippocampal slices demonstrated increased excitability at 1 week (Santhakumar et al., 2000) and as late as 15 weeks (Golarai et al., 2001) after brain injury. Based on a previous study (Cao et al., 2006), we used 1 week as the time point at which to study the change in excitabilities after fluid percussion injury. The present results show that diazepam markedly decreases fluid percussion injury-induced hyperactivity of rat hippocampal CA1 pyramidal neurons, which may be realized through effectively diminishing the probability of action potential-induced glutamate release from Schaffer collateral branches, and inhibiting spontaneous glutamate release in Schaffer collateral branches.

Diazepam is effective in treating chronic seizures (Han et al., 2011; Rossetti et al., 2012). Glutamatergic synaptic transmission in the hippocampal CA1 area is facilitated through presynaptic mechanisms after traumatic brain injury (Cao et al., 2006). Electrophysiological experiments have clarified that diazepam binds to an allosteric site for GABAA receptor chloride channels to enhance the efficacy of inhibitory synapses (Ooba et al., 2008). The present findings indicate that diazepam effectively attenuates fluid percussion injury-induced glutamatergic synaptic transmission in the hippocampal CA1 area, and also affects hyperexcitability after traumatic brain injury. This observation suggested that weakened glutamatergic synaptic transmission is the cause, rather than the result, of hyperexcitability after traumatic brain injury. Diazepam is a well-characterized and widely consumed drug; however, there has been little clinical research on using diazepam to treat post-traumatic epilepsy. Our findings indicate that diazepam has the potential to prevent post-traumatic epilepsy following traumatic brain injury and may reduce brain excitability long term, thus presenting a theoretical basis for the treatment and prevention of post-traumatic epilepsy after traumatic brain injury.

In summary, diazepam significantly decreased fluid percussion injury-induced hyperexcitability in the rat hippocampal CA1 area. The mechanism underlying this effect includes decreased probability of action potential-dependent and spontaneous release of glutamate from Schaffer collateral terminals.

Author contributions: *The study was carried out in collaboration among all authors. Cao L, Bie XH and Song WQ were responsible for the concept and design of study. Cao L and Bie XH were responsible for the acquisition of data. Huo S and Du JB performed the statistical analysis. Liu L analyzed the data*

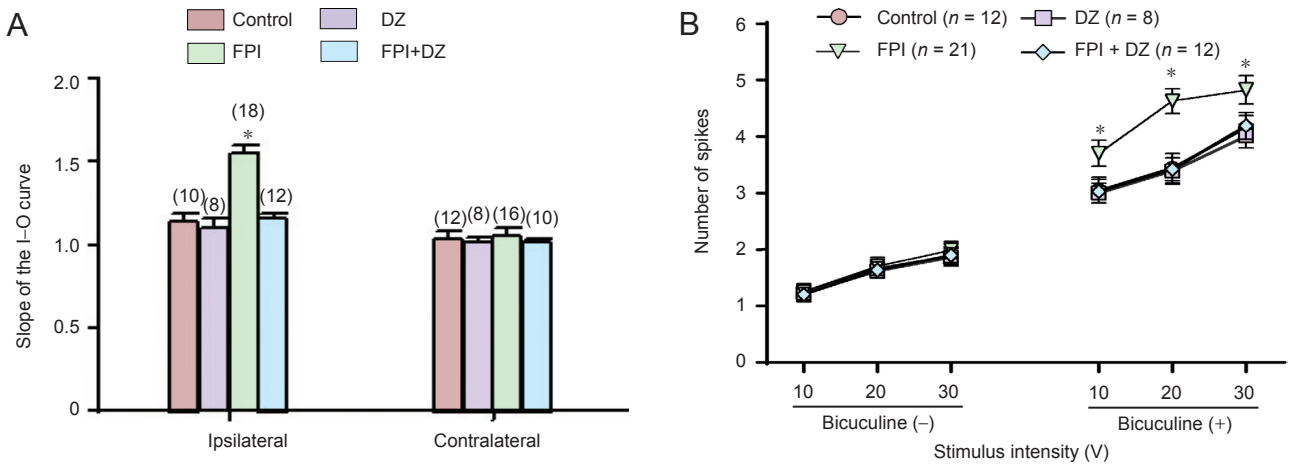

Figure 1 Diazepam (DZ) decreases fluid percussion injury (FPI)-induced hyperactivity of rat hippocampal CA1 pyramidal neurons.
(A) Effect of DZ on the input–output (I–O) relationship of monosynaptic excitatory postsynaptic potentials obtained from FPI rat hippocampal CA1 pyramidal neurons induced by stimulating the Schaffer collaterals. The figure shows pooled values for slopes of the I–O curves of the four groups. The number in the brackets represents the number of rats. (B) Effect of DZ on the number of spikes induced by super-threshold orthodromic stimulation (stimulation intensity (10, 20 and 30 V, 200 μs duration)) of the Schaffer collaterals in the FPI rat hippocampal CA1 region. The figure shows pooled data for the relationship between the number of spikes and stimulus intensity in the FPI and FPI + DZ group in (bicuculline (+)) and without (bicuculline (–)) the presence of 15 μmol/L bicuculline. *n*: Number of rats. Quantitative data were expressed as the mean ± SD and analyzed by one-way analysis of variance. Student-Newman-Keuls tests were used for specific comparisons. $*P < 0.05$, *vs.* other groups.

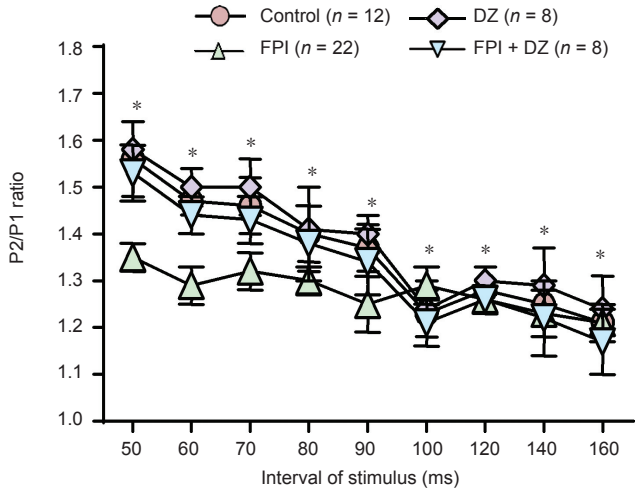

Figure 2 Effects of diazepam (DZ) on the excitatory postsynaptic potential paired-pulse facilitation of fluid percussion injury (FPI) rat hippocampal CA1 neurons.
The two consecutive excitatory postsynaptic potentials were evoked by paired-pulse stimuli with intervals from 50 to 160 ms repeated every 10 seconds in the presence of bicuculline (15 μmol/L). Sample records (average of six consecutive pairs) of excitatory postsynaptic potential pairs (stimulus interval is 60 ms) were obtained in the control, DZ, FPI and FPI + DZ groups. P1 and P2 indicate the first and second excitatory postsynaptic potentials, respectively. The figure shows pooled data for the effect of DZ on the P2/P1 ratio with stimulation intervals from 50 ms to 160 ms on the FPI ipsilateral side. *n*: Number of rats. Quantitative data were expressed as the mean ± SD and analyzed by one-way analysis of variance. Student-Newman-Keuls tests were used for specific comparisons. $*P < 0.05$, *vs.* other groups.

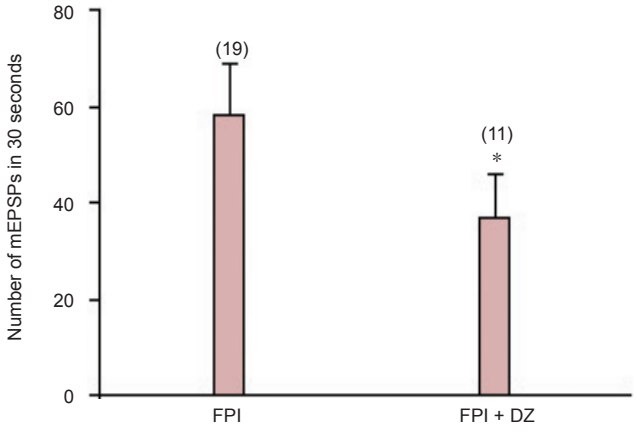

Figure 3 Effect of diazepam (DZ) on the miniature excitatory postsynaptic potentials (mEPSPs) in rat hippocampal CA1 pyramidal neurons following fluid percussion injury (FPI).
The number in the brackets represents the number of rats. There were 19 and 12 pyramidal neurons in FPI and FPI + DZ groups, respectively. Quantitative data were expressed as the mean ± SD and analyzed by one-way analysis of variance. Student-Newman-Keuls tests were used for specific comparisons. $*P < 0.05$, *vs.* FPI group.

and interpreted the results. Cao L and Song WQ discussed analyses, interpretation, and presentation, and wrote the manuscript. Song WQ was responsible for the manuscript authorization. All authors approved the final version of the paper.

Conflicts of interest: *None declared.*

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