

Original Article

2-BFI inhibits hippocampal neuron apoptosis and NMDA receptor expression in chronic epileptic rats

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Abstract: This study is to investigate the effects of 2-BFI on rat models of chronic epilepsy and the possible mechanisms. Rat model of chronic epilepsy was established by the intraperitoneal injection of pentylenetetrazol. The 2-BFI was used for treatment. Seizure scores were obtained according to the Racine scale. Pathological changes in the hippocampus were detected by HE staining. The expressions of caspase-3, and NMDA2A and NMDA2B receptors were detected by immunohistochemistry. Epileptic seizure scores were significantly increased in the model group, and 1.5 mg/kg 2-BFI treatment significantly reduced seizure score. The treatments of 2-BFI significantly ameliorated the pathological manifestations, such as karyopyknosis. Furthermore, the expression levels of caspase-3, NMDA2A and NMDA2B receptors in the hippocampus were significantly reduced by 2-BFI. In summary, 2-BFI could ameliorate the chronic epileptic seizure, probably by inhibiting the expression of caspase-3, NMDA2A and NMDA2B receptors in the hippocampus. Therefore, these findings might provide evidence for the application of 2-BFI in the prevention and treatment of chronic epilepsy in future.

Keywords: Chronic epilepsy, 2-(2-benzofuranyl)-2-imidazoline (2-BFI), caspase-3, NMDA2A receptor, NMDA2B receptor

Introduction

Epilepsy is one of the most common central nervous system (CNS) diseases, secondary only to stroke [1]. It has been shown that the imbalance in inhibitory and/or excitatory neurotransmitters (such as γ -aminobutyric acid and glutamate) in the CNS play an important role in the pathogenesis of epilepsy [2]. Over the past few decades, N-methyl-D-aspartate (NMDA) receptor has attracted increasing attention in basic and clinical researches of epilepsy [3]. The total contents of NMDA receptors are elevated in the brains of epileptic patients and transgenic mouse models of epilepsy, with increased affinity to glycine [4]. When treated with glutamate synthase inhibitor or NMDA receptor antagonist, seizure can be reduced [5], indicating that NMDA receptor might be associated with the disease pathogenesis. On the other hand, abnormal activation of NMDA receptor would induce excitotoxicity, lead to excessive calcium influx and subsequent calcium overload, and probably result in cell death, like apoptosis. The family of cysteine

proteases are related with apoptosis, in which caspase-3 is a key enzyme [6].

The compound 2-(2-benzofuranyl)-2-imidazoline (2-BFI) is a newly discovered ligand for imidazoline I2 receptor (I2R), with high affinity and selectivity [7]. Previous studies have found that several I2R ligands, such as agmatine, idazoxan [8, 9], and LSL60101 [10], can exert neuroprotective effects under various physiopathological conditions. Agmatine has been shown to have a significant antiepileptic effect, which might be related to its blocking effect on the NMDA receptor [11, 12]. However, there were few studies concerning the role of 2-BFI in the treatment of epilepsy.

In this study, the anticonvulsive effects of 2-BFI and the related mechanisms were investigated. Rat model of chronic epilepsy was established by pentylenetetrazol injection, and the pathological changes in hippocampal neurons, apoptosis-related proteins, and NMDA-2A and NMDA2B receptors in these models were analyzed.

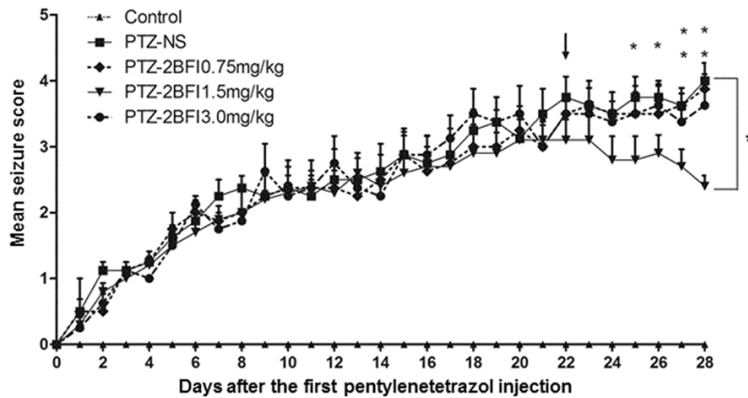


Figure 1. Seizure scores of rat models of chronic epilepsy with or without 2-BFI treatments. The rat model was established by the injection of pentylenetetrazol for 21 d, followed by the administration of 2-BFI for another 7 d (arrow indicated the start point of drug administration). The seizure scores were daily recorded for each animal in the control, model, and 0.75, 1.5, and 3 mg/kg 2-BFI treatment groups, over the whole period. Compared with the model group, * $P < 0.05$, ** $P < 0.01$.

Materials and methods

Animal modeling and grouping

Male Sprague-Dawley (SD) rats, weighing 160–180 g, were provided by the Lakes Company (Shanghai, China). Chronic epilepsy was induced by intraperitoneal injection of 35 mg/kg pentylenetetrazol (Sigma, St Louis, MO, USA) for 21 days. The rats were randomly divided into the following groups: (1) the control group ($n=8$), in which normal rats were injected with saline instead of pentylenetetrazol for 21 days, followed by the injection of saline for another 7 days; (2) the model group ($n=8$), in which pentylenetetrazol-induced epilepsy rats were treated with saline for 7 days; and (3) the treatment groups, in which epileptic rat models were treated with 2-BFI (Tocris, Ellisville, MO, USA) at the concentrations of 0.75 mg/kg ($n=8$), 1.5 mg/kg ($n=10$), and 3.0 mg/kg ($n=8$), respectively, for 7 days. All animal experimental procedures were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Seizure scoring

Seizure scores were obtained during modeling process and drug administration, for totally 28 days, according to the Racine scale [13]: 0, no seizure; 1, facial muscle twitching and rhythmic chewing movement; 2, rhythmic nod-

ding movement or wet-dog shaking (WDS); 3, unilateral clonic forelimb; 4, bilateral clonic forelimb when standing; 5, generalized tonic-clonic seizure, and falling.

Hematoxylin-eosin (HE) staining

After modeling and treatment, these rats were anesthetized with pentobarbital (50 mg/kg) and perfused with 4% paraformaldehyde in PBS, and then sacrificed by decapitation. Brains were removed and fixed in 4% paraformaldehyde at 4°C for 24 h. Brain tissue was then dehydrated, transparented, and embedded in paraffin, and then cut into 5- μ m sections on a paraffin slicing machine (Microm, Walldorf, Germany). The sections were incubated at 60°C for 60 min. After cooling, the sections were subsequently subjected to the treatments of xylene I and II (each for 15 min), 100% alcohol I and II (each for 10 min), 95% alcohol I and II (each for 5 min), and 90% ethanol and 80% ethanol (each for 2 min). Then the sections were stained with hematoxylin and eosin, and observed with a microscope (Leica, Bensheim, Germany).

Immunohistochemistry

Endogenous peroxidases were inhibited by incubation in 3% hydrogen peroxide at 37°C for 10 min. After washing with PBS, sections were blocked with 10% goat serum at room temperature for 10 min, and then incubated with anti-caspase-3 antibody (1:300 dilution; Abcam, Cambridge, MA, USA), anti-NMDA2A receptor antibody (1:1000 dilution; Abcam), and anti-NMDA2B receptor antibody (1:200 dilution; Abcam), respectively, at 4°C overnight. The sections were incubated with HRP-conjugated goat anti-rabbit IgG at 37°C for 30 min, and then dehydrated, sealed, and coverslipped. A Leica microscope equipped with a digital camera was used for the examination. For each section, five high-power fields ($\times 400$) were randomly selected from hippocampal CA1, CA3, and dentate gyrus (DG) regions. Optical density was calculated using the IPP6.0 software, and the mean values were used for statistical analysis.

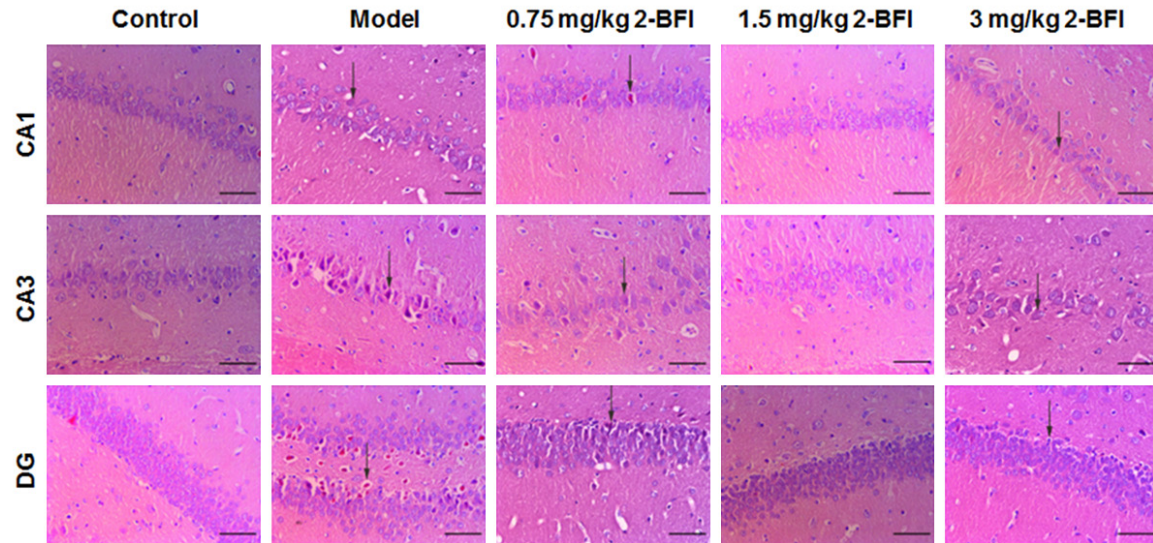


Figure 2. Histological staining of the hippocampus in rat models of chronic epilepsy. After modeling and drug administration, HE staining was performed to detect the histological changes in the hippocampal CA1, CA3, and DG regions in the rat models of chronic epilepsy. The arrows indicate the necrotic neuronal loss and nuclear condensation. Scale bar: 400 μ m.

Statistical analysis

Data are expressed as mean \pm SD. SPSS 17.0 software was used for statistical analysis. One-way ANOVA was performed for the comparison, followed by the Dunnett's test. $P < 0.05$ was considered statistically significant.

Results

2-BFI declines seizure scores of rat models of chronic epilepsy

To investigate the pathogenesis of epilepsy and the effects of 2-BFI on the disease, the seizure scores were daily recorded for each animal, over the modeling and drug administration period. As shown in **Figure 1**, no seizure was observed in the control group over the whole period. In the modeling process, at 3 d after the first injection of pentylenetetrazol, neurological dysfunction occurred in these rat models, as indicated by the aggressive behavior, as well as the scanty and sparse hair; at 7 d, tonic-clonic seizure was observed in rats, i.e., clonic facial and neck muscles; at 13 d, the symptoms become even more serious, characterized by twitching limbs, falling to the ground, generalized tonic-clonic seizure, screaming, and foaming at the mouth; and then at 20 d, continuous level 4-5 attacks were commonly seen in most of these rats.

However, when treated with 2-BFI, the seizure scores of these chronic epileptic rats were declined. Especially, compared with the model group, the seizure scores were significantly decreased in the 1.5 mg/kg 2-BFI treatment group, starting from the fourth day of drug administration ($P < 0.05$ for days 4 and 5; $P < 0.01$ for days 6 and 7) (**Figure 1**). These results suggest that 1.5 mg/kg 2-BFI treatment could decrease seizure scores of chronic epilepsy in rat models induced by pentylenetetrazol.

2-BFI alleviates histopathological changes in rat models of chronic epilepsy

To investigate the effects of 2-BFI on the histological changes in rat models of chronic epilepsy, the brains of these rats were subjected to the HE staining. Our results showed that, in the control group, the neurons in the hippocampal CA1, CA3, and DG regions were arranged neatly, in regular shapes, with round and uniform nuclei. On the other hand, in the model group, the gap between the hippocampal neurons was dramatically increased, and the cells were arranged irregularly, with partial karyopyknosis or even nucleolus disappearing, especially in the CA1 and CA3 regions. However, all the histological changes were drastically less pronounced in the 2-BFI treatment groups. Particularly, in the 1.5 mg/kg 2-BFI group, the histological changes were much less

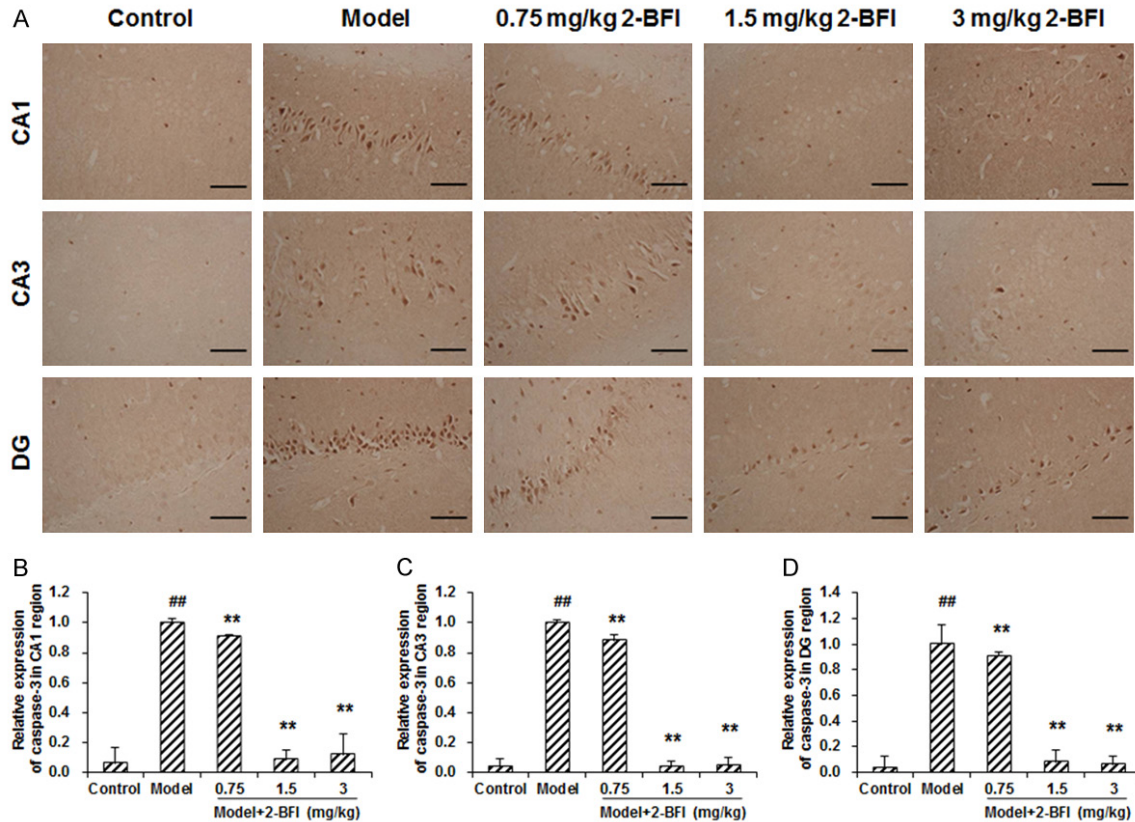


Figure 3. Effects of 2-BFI on the caspase-3 expression in the hippocampus in rat models of chronic epilepsy. (A) After modeling and drug administration, immunohistochemistry was performed to detect the expression of caspase-3 in the hippocampal CA1, CA3, and DG regions in the rat models of chronic epilepsy. Scale bar: 400 μ m. (B-D) Statistical analysis of the caspase-3 expression levels in the hippocampal CA1 (B), CA3 (C), and DG (D) regions. Compared with the control group, ^{##} $P < 0.01$; compared with the model group, ^{**} $P < 0.01$.

intensive in the hippocampal CA1, CA3, and DG regions (**Figure 2**). These results suggest that 2-BFI treatment could alleviate the histopathological changes in rat models of chronic epilepsy induced by pentylenetetrazol.

2-BFI inhibits neuronal apoptosis in hippocampus in rat models of chronic epilepsy

To investigate the effects of 2-BFI on the neuronal apoptosis in the hippocampus in the chronic epileptic rats, the expression of apoptosis-related protein, caspase-3, was detected with immunohistochemistry. Our results showed that neuronal cells positive for caspase-3 could be observed in the hippocampal CA1, CA3, and DG regions in all the control, model, and treatment groups (**Figure 3A**). Compared with the control group, the expression levels of caspase-3 (as indicated by the average IOD value) were significantly elevated in the hippocampal CA1, CA3, and DG regions (all $P < 0.01$), in the model group (**Figure 3B-D**).

However, the treatments of 2-BFI significantly inhibited the expression levels of caspase-3 in all the hippocampal CA1, CA3, and DG regions (all $P < 0.01$), with the most obvious effect for the 1.5 mg/kg 2-BFI treatment group (**Figure 3B-D**). These results suggest that 2-BFI treatment could inhibit the neuronal apoptosis in hippocampal CA1, CA3, and DG regions in rat models of chronic epilepsy induced by pentylenetetrazol.

2-BFI decreases NMDA receptor expression in rat models of chronic epilepsy

To further investigate the mechanism through which 2-BFI exerted the neuroprotective effects in chronic epileptic rats, the expressions of NMDA2A and NMDA2B receptors were detected with immunohistochemistry. For the detection of NMDA2A receptor, our results showed that positive staining of NMDA2A receptor could be observed in the hippocampal CA1, CA3, and DG regions in all the control, model,

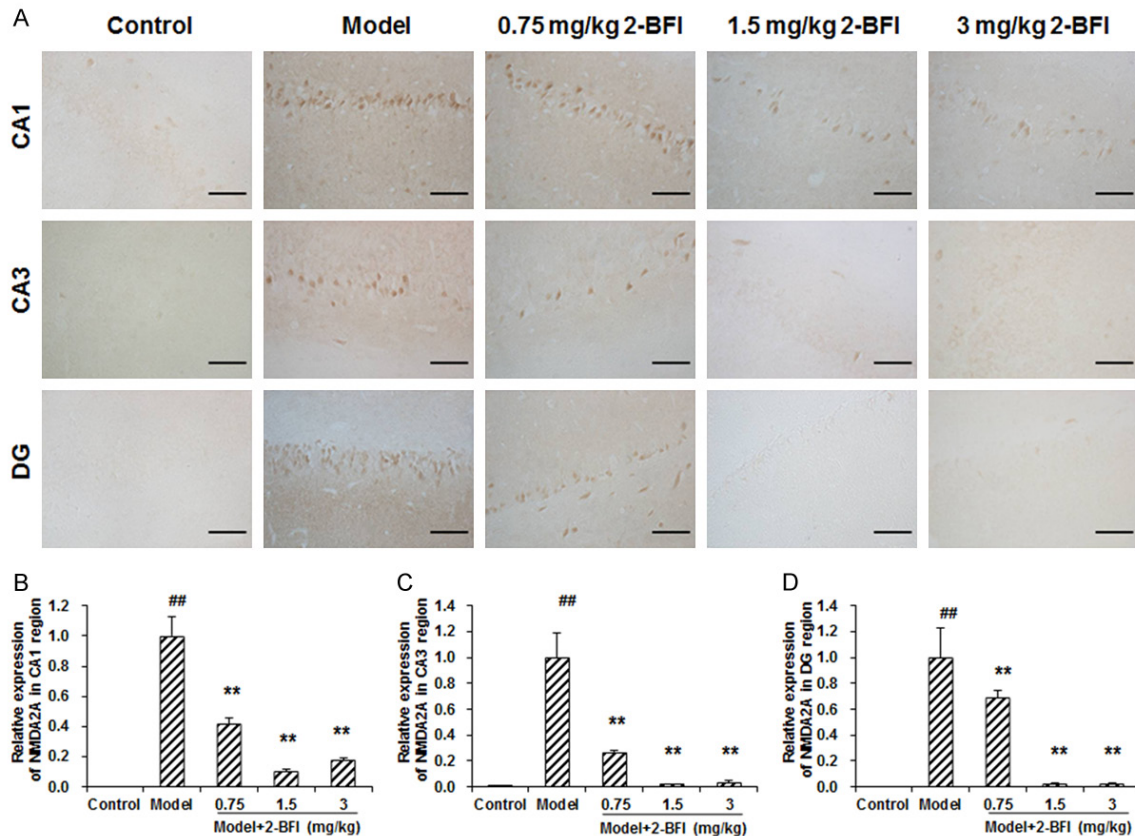


Figure 4. Effects of 2-BFI on the expression of NMDA2A receptor in the hippocampus in rat models of chronic epilepsy. (A) After modeling and drug administration, immunohistochemistry was performed to detect the expression of NMDA2A receptor in the hippocampal CA1, CA3, and DG regions in the rat models of chronic epilepsy. Scale bar: 400 μ m. (B-D) Statistical analysis of the NMDA2A receptor expressions in the hippocampal CA1 (B), CA3 (C), and DG (D) regions. Compared with the control group, ^{##} $P < 0.01$; compared with the model group, ^{**} $P < 0.01$.

and treatment groups (Figure 4A). Compared with the control group, the expression levels of NMDA2A receptor were significantly elevated in the hippocampal CA1, CA3, and DG regions (all $P < 0.01$), in the model group (Figure 4B-D). However, 2-BFI treatments significantly declined the expression levels of NMDA2A receptor in all the hippocampal CA1, CA3, and DG regions (all $P < 0.01$), with the most dramatic decline for the 1.5 mg/kg 2-BFI treatment group (Figure 4B-D). Similar results were obtained for the detection of the NMDA2B receptor (Figure 5). These results suggest that 2-BFI treatment could decrease the expression levels of NMDA2A and NMDA2B receptors in rat models of chronic epilepsy induced by pentylenetetrazol.

Discussion

In this study, we found that 2-BFI, a highly selective I2R ligand [14], could alleviate pentylenetetrazol-

tetrazol-induced chronic epilepsy. Our results showed that, 1.5 mg/kg 2-BFI treatments could significantly reduce the epileptic seizure score and ameliorate the neuronal damages in the hippocampus. Moreover, the expression level of caspase-3 in the model group was significantly higher than in the control group, which could be decreased by 2-BFI. Similar results were obtained with the expressions of NMDA2A and NMDA2B receptors. These results suggest that the mechanism through which 2-BFI exerts the anticonvulsant effects may be related to inhibiting apoptosis and blocking NMDA receptors.

I2R is an imidazoline receptor characterized by the high affinity [15]. Previous studies have shown that I2R ligands, such as idazoxan, 2-BFI, and agmatine, have neuroprotective effects under various physiopathological conditions [16, 17]. Agmatine is an endogenous ligand of I2R [18], which has been shown to

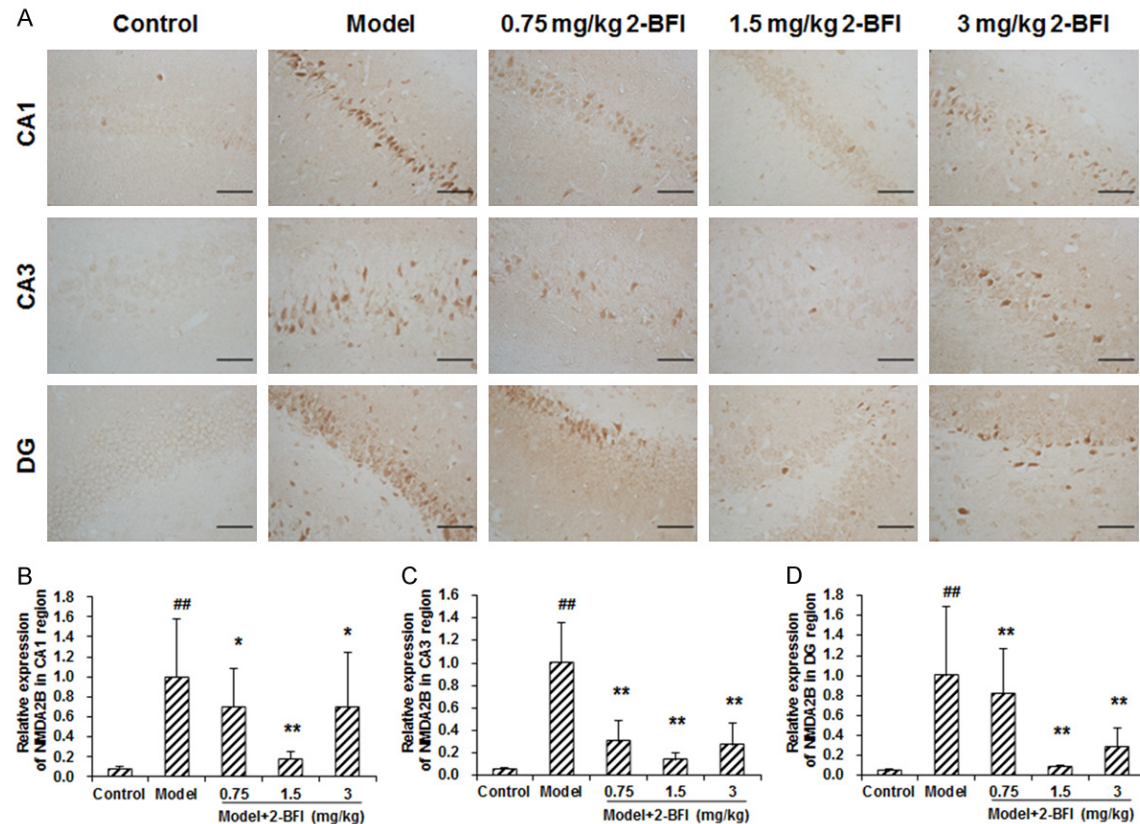


Figure 5. Effects of 2-BFI on the expression of NMDA2B receptor in the hippocampus in rat models of chronic epilepsy. (A) After modeling and drug administration, immunohistochemistry was performed to detect the expression of NMDA2B receptor in the hippocampal CA1, CA3, and DG regions in the rat models of chronic epilepsy. Scale bar: 400 μ m. (B-D) Statistical analysis of the NMDA2B receptor expressions in the hippocampal CA1 (B), CA3 (C), and DG (D) regions. Compared with the control group, ## $P < 0.01$; compared with the model group, ** $P < 0.01$.

have a significant antiepileptic effect [17-19]. Our previous studies show that, compared with agmatine, 2-BFI is another I2R ligand with potent and long-lasting neuroprotective effects against glutamate-induced excitotoxicity [20]. 2-BFI can significantly reduce the infarct volume in rat models of cerebral ischemia and improve the neurological dysfunction [21]. Moreover, 2-BFI can alleviate the spinal cord injury in EAE mice [22]. The neuroprotective effects of 2-BFI have been considered to be related to the antagonism of NMDA receptors, which reversibly inhibits the NMDA receptor-mediated calcium influx, increasing transmembrane potential, in primary cortical neurons [20]. Therefore, in this study, the mechanism for the neuroprotective effects of 2-BFI against chronic epilepsy might be associated with the inhibition of neuronal apoptosis and the blocking of NMDA receptor-mediated intracellular calcium influx.

During the model establishment, Racine level 1 seizure was observed at as early as 3 d after the first injection of pentylenetetrazol; at 20 d, continuous level 4-5 attacks were commonly seen in most of the rat models. Then some of the rat models were treated with 2-BFI for 7 d. Our results showed that the average seizure score was significantly lower in the 1.5 mg/kg 2-BFI treatment group than in the model group, starting from 4 d after the first drug administration, indicating that 2-BFI could alleviate the chronic epileptic seizure. At 6 d, the attack intensity was further decreased. These results suggest that 2-BFI needs no less than 4 d to exert its biological function in these rat models. Of course, further in-depth studies are still needed to determine the optimal drug delivery time.

Pathogenesis and development of chronic epilepsy is a vicious cycle, which is characterized

by neuronal death. Chronic epileptic seizure can induce cell death in neurons, and neuronal death in turn may aggravate the injury [23]. Caspase family play an important role in apoptotic process, and caspase-3 is one of the core members in the protein family. Studies have found that the expression level of caspase-3 is increased in epileptic seizure [24]. In a rat model of ischemic stroke, it has been shown that 3 mg/kg 2-BFI intervention can significantly reduce the apoptosis of brain cells [25, 26]. In this study, our results showed that, compared with the control group, both the hippocampal neuronal cells positive for caspase-3 and the expression level of caspase-3 were significantly increased in the model group. However, 2-BFI treatment could dramatically reduce the caspase-3 expression level and the apoptotic neurons in the hippocampal region, with the most obvious effects observed in the 1.5 mg/kg 2-BFI group, which was consistent with previous studies [27, 28].

Increased excitability and reduced brain inhibition have been associated with the occurrence of epilepsy. In the CNS, γ -aminobutyric acid and glutamate are the major inhibitory and excitatory neurotransmitters, respectively, which might be closely related to the disease pathogenesis. Studies have shown that accumulated glutamate in the brain can affect NMDA receptors and the ion channels to induce synaptic hyperexcitability and finally seizure [29-31]. NMDA receptors are ligand-gated receptors and glutamate-sensitive ion channels, which are mainly composed of the basic subunit NR1 and the regulatory subunit NR2 [32]. The NR2 subunit plays important roles in the receptor assembly, expression, transportation, and synaptic location. NMDA receptor subtypes are determined by the NR2 subunits, including NMDA2A, NMDA2B, NMDA2C, and NMDA2D receptors [21]. NMDA2A and NMDA2B receptors are the most common subtypes in the hippocampus and cerebral cortex, which were therefore mainly focused in the present study [22]. Jiang *et al.* [20] found that idazoxan and 2-BFI reversibly inhibited the NMDA receptor-mediated calcium influx in cultured cortical neurons, and they claimed that this effect was achieved by blocking the NMDA receptor, rather than directly inhibiting the activity of calpain. Idazoxan and 2-BFI can effectively reduce the calcium influx and calcium

overload mediated by the NMDA receptor in rat cortical neurons, and protect the neurons against the toxic effects of excitatory amino acids. In this study, we found that the expressions of NMDA2A and NMDA2B receptors were increased in the hippocampal CA1, CA3, and DG regions in rat models of chronic epilepsy, and the 2-BFI treatment could significantly decrease the receptor expressions, suggesting that the anticonvulsant effect of 2-BFI may be related with the regulation of these receptors.

In summary, our results showed that 2-BFI treatment (at the dosage of 1.5 mg/kg) could ameliorate the chronic epileptic seizure in rat models. 2-BFI could alleviate the histopathological changes, inhibit the expression of caspase-3, and suppress the expressions of NMDA2A and NMDA2B receptors in the hippocampus in these rat models of chronic epilepsy. These findings might provide evidence for the application of 2-BFI in the prevention and treatment of chronic epilepsy in future.

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Disclosure of conflict of interest

None.

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