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Original Article

Modulatory effects of $GABA_BR$ on cognitive function of epileptic rats

Tao Sun[1,2], Yan-Ping Lan[1,2], Chun Zhang[1,2], Zhen Zhang[1,2], Nan Wu[1,2], Feng Wang[1,2]

[1]Department of Neurosurgery, General Hospital of Ningxia Medical University, Yinchuan, Ningxia, China; [2]Ningxia Key Laboratory of Cerebrocranial Disease, Incubation Base of National Key Laboratory, Ningxia Medical University, Yinchuan, Ningxia, China

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Abstract: Objective: To study the modulatory effects and mechanism of $GABA_BR$ on the cognitive function of epileptic rats induced by pilocarpine. Method: The lithium chloride (LiCl)- pilocarpine induced epileptic model was established in SD rats, and those rats were randomly divided into $GABA_BR$ groups (saline group, Baclofen group, CGP group) and Arc groups (Sham operation group, Empty vector group, Virus group). Morris water-maze test and passive avoidance performance test were used to detect the cognition of epileptic rats. The expression of $GABA_BR$ (GB_1, GB_2), and Arc/Arg3.1 in hippocampus of epileptic rats were determined by immunohistochemistry and Western Blotting at protein level. In addition, the expression of $GABA_BR$ (GB_1, GB_2), and Arc/Arg3.1 was detected by RT-PCR at mRNA level. Results: The results of Morris water-maze test and passive avoidance performance test showed that the learning ability and cognitive performance improved in the rats of CGP group but declined in Baclofen group and Virus group, comparing with the control. The data from immunohistochemistry, RT-PCR and Western blotting revealed that the relative expression of Arc/Arg3.1 was significantly increased in CGP group comparing with the other two groups but decreased in Baclofen group and virus group comparing with the other two groups ($P<0.05$). The relative expression ofGB_1 and GB_2 was significantly increased comparing with the other two groups but had no significant difference between virus group and the other two groups. Conclusion: $GABA_BR$ influenced the spatial learning and memory of epileptic rats by regulating Arc/Arg3.1.

Keywords: $GABA_BR$, Arc/Arg3.1, kindled, learning and memory

Introduction

Epilepsy is a very common disease in the central nervous system, which often causes cognitive impairment. The pathophysiological mechanism of cognitive impairment is still unclear [1]. $GABA_B$ receptors ($GABA_BR$) are the major inhibitory transmitter in vertebrate central nervous system, which are known to play a key role in modulating the physiological activities and pathological changes [2, 3]. Recent reports show that $GABA_BR$ exerts an important influence on regulating the activity of hippocampal neurons as well as the maintenance of long-term memory precision [4, 5]. New protein synthesis is required for long-term memory formation. Activity regulated cytoskeletal protein (Arc/Arg3.1), a member of immediate-early gene family, is believed to play a critical role in newly translated protein coupling in learning and cognition related processes [6, 7].

In the present study we focused on the influence on pilocarpine-induced epileptic rats in cognition function and Arc/Arg3.1 expression after treated with $GABA_B$ receptor agonist Baclofen, antagonist CGP35348 or Arc siRNA. The mechanism underlying the regulation of Arc and $GABA_BR$ on the cognitive dysfunction induced by epilepsy was discussed in our study.

Materials and methods

Reagents

Arc siRNA lentiviral vector (titer: 6.5 E+12 V.G/ml) was synthesized and screened by Shanghai Genechem Technology co., LTD. The target sequence was GAAGAACTGGGTGGAGTTCAA. Primary antibodies against Arc/Arg3.1, GB_1R and GB_2R were purchased from Abeam. $GABA_B$ receptor agonist Baclofen and antagonist CGP35348 were obtained from Sigma. First

Strand cDNA Synthesis Kit was purchased from Thermo Fisher. The PCR primers were designed and synthesized by Sangon Biotech (Shanghai). BCA Protein Assay Kit and Total Protein Extraction Kit were purchased from Jiangsu KeyGEN BioTECH Corp., Ltd.

Animals

Male Sprague-Dawley rats (6-8 weeks old, 250-300 g) were provided from the Animal Center of Ningxia Medical University. Each rat was singly housed with an alternating 12: 12 h light/dark cycle. Pilocarpine was used to establish kindling model in rats. These rats were randomly divided into saline group (Ctrl), Baclofen group (Bac), CGP group (CGP), Sham operation group (Sham operation), Empty vector group (Empty vector) and Virus group (Virus) with 20 animals in each group.

Model establishment

Anhydrous lithium chloride (LiCl) was dissolved in saline (127 mg/kg) and injected into the abdominal cavity of the rats. After 18 h, the rats were injected with hyoscyamine sulfate (1 mg/kg, IP.) to minimize the peripheral effects of pilocarpine, and then injected with pilocarpine (30 mg/kg, IP.) 30min later to induce status epilepticus (SE). Rats that did not have behavioral seizures (class 4 or higher on scale of Racine) within 30 min of pilocarpine injection were further injected with a second dose of pilocarpine (10 mg/kg) until SE appeared (total amount of pilocarline should be less than 60 mg/kg). Diazepam (10 mg/kg, IP.) was administered to stop seizure activity 1 h after the onset of status epilepticus or after the rats appeared dehydration symptoms. For $GABA_BR$ group: Baclofen group: After diagnosed as epileptic, the rats were injected with 0.25% (2.5/mL) baclofen (5 mg/kg/d, IP.); CGP group: After diagnosed as epileptic, the rats were injected with GCP35348 (1 mg/kg/d, IP.); Saline group: After diagnosed as epileptic, the rats were injected with saline (2 mL/kg/d, IP.). The administrations continued for 1 week. And behavior tests were conducted 3-4 h after the drug administration every day. Arc group: rats were anesthetized and installed in a stereotaxic frame. Using a precision micro-syringe, rats were bilaterally injected in hippocampus with 2 μL (0.2 μL/min) Arc siRNA viral solution based on the following coordinates: 3 mm posterior to Bregma, ± 2 mm lateral the medial suture, 3 mm ventral to the skull surface. Normal saline and empty vector are used as controls. All viral-injections were conducted according to the rat stereotaxic coordinates. Rats were left to recover for 14 days before behavioral tests.

Morris water-maze

Two weeks after drug treatments the rats were tested in the Morris water-maze. Testing was performed on 8 consecutive days including place navigation test and spatial learning test. After 2-3 h of treatments, the rats were trained in place navigation test lasted for 6 days, and the escape latencies of the rats were recorded. On the seventh and the eighth day, spatial learning test was conducted to record number of crossings in target quadrant and time in target quadrant in 60 s. These parameters were used for reflecting the spatial learning ability of animals.

Passive avoidance performance test (step through test)

Passive avoidance performance test was performed after Morris water-maze test. Each rat was habituated to the dark compartment of the apparatus for 2 min. Subsequently, rats were placed into an illuminated compartment connected to a dark compartment equipped with an electric grid floor. Rats which entered to the dark compartment were punished by an electric shock on the feet. After 5 min training, the rats were returned to their home cage for 24 h. On the following day, the pre-trained rats were placed again into the illuminated compartment. The time that the rat took to enter the dark compartment in 3 min was recorded as retention time and the frequency of the position changes of the rat in the apparatus was recorded as shuttle times. These two parameters were used to evaluate the memory capacity. Data were analyzed by animal behavior video analysis software.

Sample collection

After behavioral tests, the rats were sacrificed following anesthetized with 10% chloral hydrate and the hippocampus tissues of some rats were collected. The other rats were perfused with 4% Paraformaldehyde solution. The

Table 1. Specific primers used in real-time PCR analysis

Gene	Primer	Sequence (5'→3')
GAPDH	FW	GAGTCAACGGATTTGGTCGT
	RV	GACAAGATTCCCGTTCTCAG
Arc	FW	ACAGACACAGCAGATCCAGC
	RV	ATGAATCACTGCTGGGGGC
GB1	FW	AGATTGTGGACCCCTTGCAC
	RV	AGAAAATGCCAAGCCACGTA
GB2	FW	CACCGAGTGTGACAATGCAAA
	RV	CCAGATTCCAGCCTTGGAGG

isolated brains were stored in sucrose solution to dehydrate gradiently and embedded in paraffin.

Immunohistochemistry

The immunohistochemical method of SABC (StreptAvidin Biotin Complex) staining was performed as below. After antigen retrieval using citric acid buffer, the sections were blocked with serum and incubated with antibodies (Arc/Arg3.1, 1:500; GB_1, 1:300; GB_2, 1:500) at 4°C overnight. After washing in PBS, Polink-2 plus polymer HRP detection system for rabbit primary antibody was employed in this experiment. Normal operations of immunohistochemistry were performed afterwards. The hippocampal CA3 areas of immunostained slides were observed and positive cells were quantitatively analyzed using KS400 image analysis system. 6 fields of view were picked for every slide.

RT-PCR

Total RNA of hippocampus tissues was extracted using TRIzol reagent according to the manufacturer's protocol. First-strand cDNA was generated using M-MLV reverse transcriptase. PCR was performed to detect the mRNA expression of each gene. PCR application conditions were described as followed: denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 45 s. RT-PCR products were analyzed and visualized on 4% agarose gel containing ethidium bromide (EB). Images were captured by Tanon 3500 digital gel imaging system. The PCR primers used were listed in **Table 1**.

Western blotting

Hippocampus tissues were lysed with protein extraction kit on ice, and then total protein content was determined by BCA protein determination method. Protein from each sample was separated by SDS/PAGE and transfer on to a PVDF membrane. After blocking with 5% non-fat milk for 1 h, the membranes were immunoblotted with primary antibodies (Arc/Arg3.1, 1:500; GB_1, 1:300; GB_2, 1:500) overnight. After incubated with secondary antibody (1:5000), signal detection was performed by Odyssey infrared laser imaging system, followed by gray intensity analysis.

Statistical analysis

All results are expressed as mean ± SD. Analysis was performed using SPSS 17.0 software. The significance of the difference between groups was measured using one-way ANOVA followed by Dunnett test for comparison between two groups. $P<0.05$ was considered to be statistically significant (*$P<0.05$, **$P<0.01$).

Results

Morris water-maze

Place navigation test: Day 1 and 2 are adaption period for the rats, the escape latencies of three groups showed no statistical difference from each other ($P>0.05$). For $GABA_BR$ group, on the 3, 4, 5, 6 day, the rats of Baclofen group had a longer escape latency compared to other two groups, while the rats of CGP group had a shorter escape latency (**Figure 1A**). These results revealed that Baclofen could inhibit learning and memory capabilities of epileptic rats, while CGP35348 improved learning and memory. For Arc group, the rat of virus group had a longer escape latency compared with the Shame operation group and Empty vector group (**Figure 2A**), suggesting that Arc siRNA inhibited learning and memory capabilities as well.

Spatial learning test: For $GABA_BR$ group, the groups differed significantly ($P<0.05$) in number of crossings in target quadrant and time in target quadrant. Both these measurements decreased significantly in Baclofen group but increased in CGP group (**Figure 1B** and **1C**).

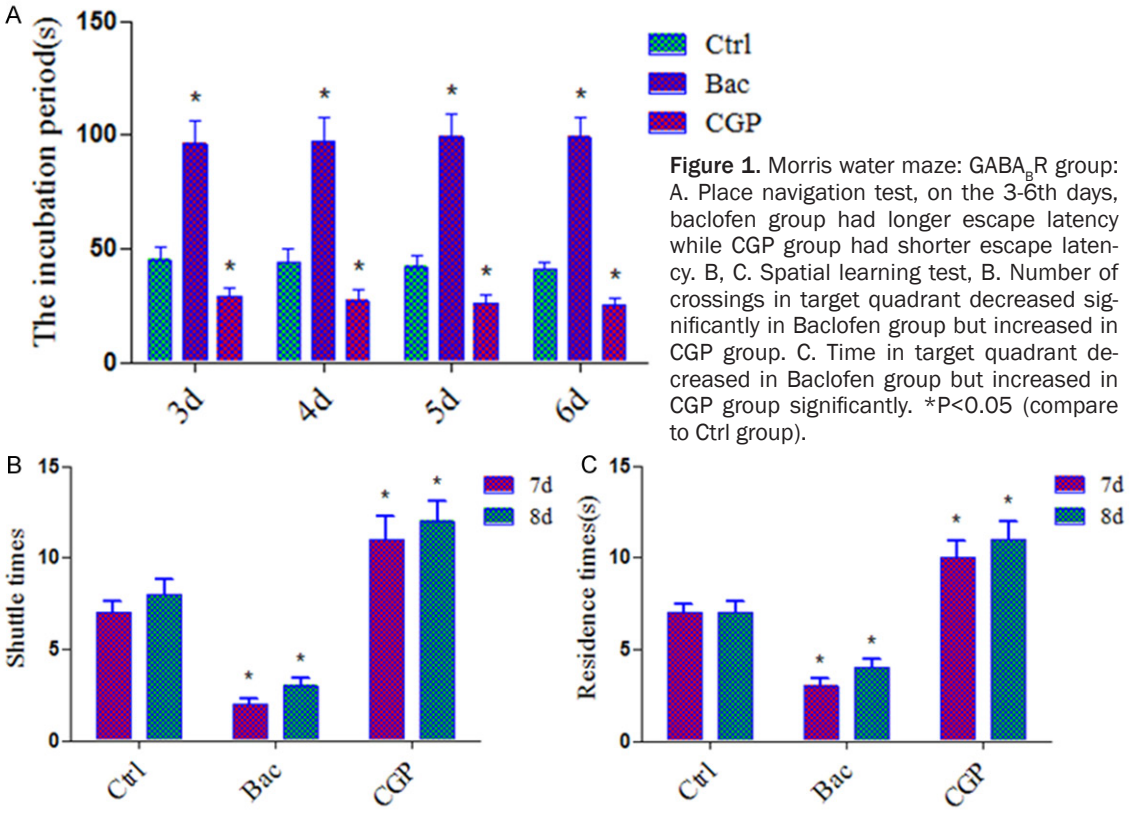

Figure 1. Morris water maze: $GABA_BR$ group: A. Place navigation test, on the 3-6th days, baclofen group had longer escape latency while CGP group had shorter escape latency. B, C. Spatial learning test, B. Number of crossings in target quadrant decreased significantly in Baclofen group but increased in CGP group. C. Time in target quadrant decreased in Baclofen group but increased in CGP group significantly. *P<0.05 (compare to Ctrl group).

These results demonstrated that Baclofen suppressed spatial learning ability. In the contrast, CGP35348 enhanced spatial learning ability of the epileptic rats. For Arc group, number of crossings in target quadrant decreased significantly in Virus group compared to the other two groups (**Figure 2B** and **2C**). There was significant difference between Virus group and Shame operation group (P<0.05), suggesting that Arc siRNA could inhibit spatial learning ability of epileptic rats.

Passive avoidance performance test (step through test)

For $GABA_BR$ group, there were statistical differences in shuttle times and retention time from each other (P<0.05, compared to Ctrl group). Baclofen group revealed the least shuttle times and the longest retention time, while CGP showed the most shuttle times and the shortest retention time (**Figure 3A** and **3B**). From these data we concluded that Baclofen disrupted but CGP35348 improved memory formation of epileptic rats. For Arc group, shuttle times of Virus group decreased significantly and latency to enter the dark compartment (retention time) became longer compared to the other two groups (P<0.05, comparing with shame operation group). No differences (P>0.05) were found between Shame operation group and Empty vector group (**Figure 4A** and **4B**). Therefore, Arc siRNA could induce memory dysfunction according to these results.

Immunohistochemistry

Arc/Arg3.1 positive cells showed brown in cytoplasm. For $GABA_BR$ group, there were statistical differences in Arc/Arg3.1 positive ratio from each other (P<0.05). Baclofen group possessed the least Arc/Arg3.1 positive cells while CGP group possessed the most. GB_1 and GB_2 positive cells showed brown in cytomembrane. Also there were statistical differences in GB_1 and GB_2 positive ratio (P<0.05). Baclofen group presented the most GB_1 and GB_2 positive cells whereas CGP group presented the least (**Figure 5**). For Arc group, Arc/Arg3.1 positive ratio of Virus group decreased compared to the other two groups, difference is statistically significant (P<0.05). However, there was no significant difference between Sham operation group and Empty vector group.

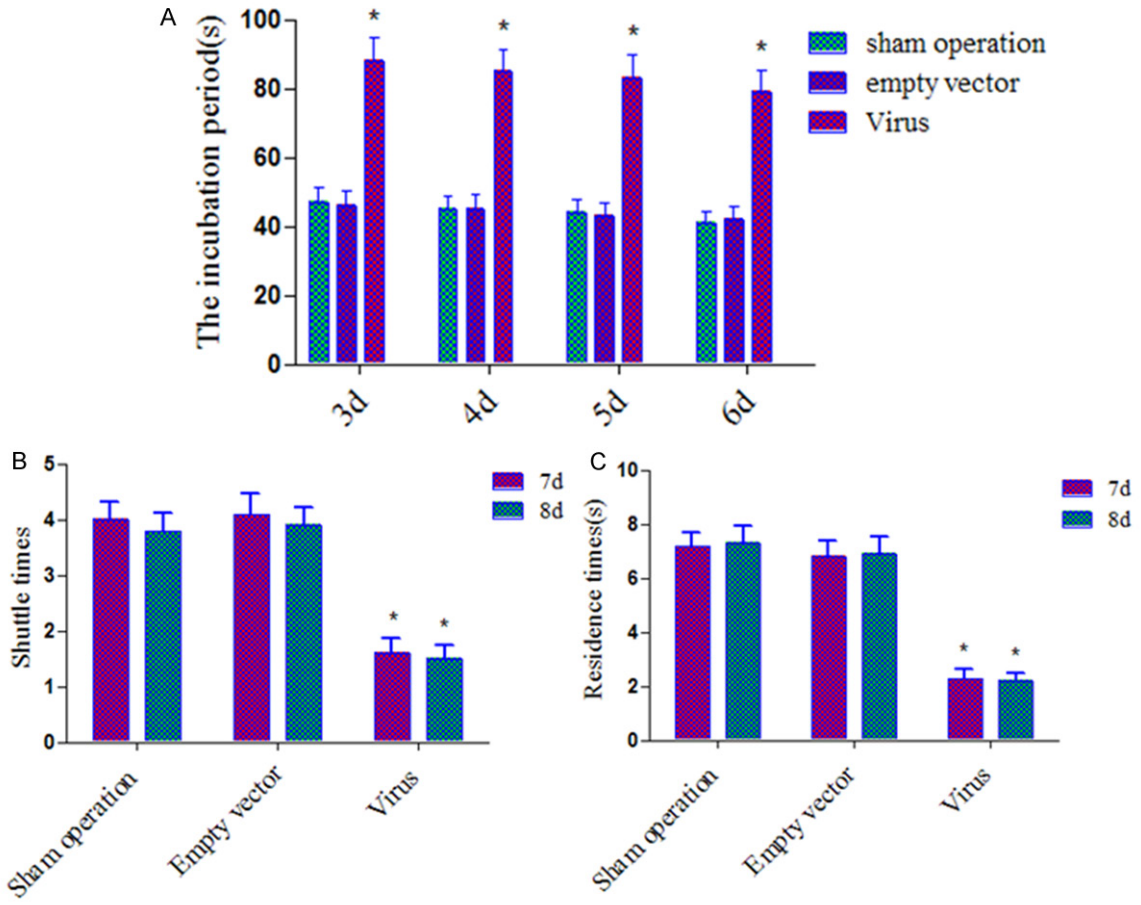

Figure 2. Morris water maze: Arc group: A. Place navigation test, on the 3-6th days, Virus group had longer escape latency compared to other two groups (*P<0.05, compared to Sham operation group), but there is no significant difference between Sham operation group and Empty vector group. B, C. Spatial learning test, B. Number of crossings in target quadrant significantly decreased in Virus group (*P<0.05, compared to Sham operation group). No significant difference showed between the other two groups. C. Time in target quadrant decreased in Virus group (*P<0.05, compared to Sham operation group). No significant difference showed between the other two groups.

In addition, no significant difference was shown in comparison of these three groups (**Figure 6**). (P>0.05).

RT-PCR

For $GABA_BR$ group, Arc/Arg3.1 was downregulated in Baclofen group and upregulated in CGP group significantly compared to the Ctrl group at mRNA level (P<0.05). The expression of GB_1 and GB_2 was much higher in Baclofen group (P<0.05) but lower in CGP group (P<0.05) compared to the Ctrl group (**Figure 7**). For Arc group, the expression of Arc/Arg3.1 decreased dramatically in virus group (P<0.05) while the expression of GB_1 and GB_2 were not changed (P>0.05). There were no differences in mRNA expression of Arc/Arg3.1, GB_1 and GB_2 between Shame operation group and Empty vector group (**Figure 8**).

Western blotting

For $GABA_BR$ group, Arc/Arg3.1 was downregulated in Baclofen group and upregulated in CGP group significantly compared to the Ctrl group at protein level (P<0.05). The expression of GB_1 and GB_2 was much higher in Baclofen group (P<0.05) but lower in CGP group (P<0.05) to the ctrl group (**Figure 9**). For Arc group, the protein expression of Arc/Arg3.1 decreased significantly in Virus group (P<0.05) but not changed in Shame operation group and Empty vector group (P>0.05). There were no differences in the expression of GB_1 and GB_2 in protein level form each other (**Figure 10**).

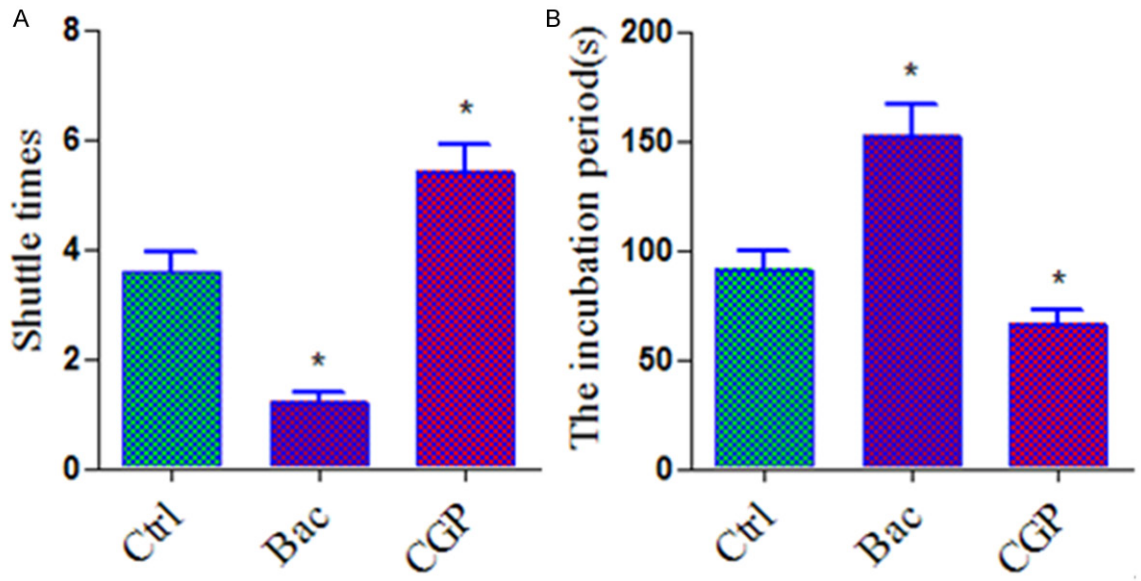

Figure 3. Passive avoidance performance test: $GABA_BR$ group, A. Shuttle times, B. Retention time. Baclofen group had fewer shuttle times and longer retention time while CGP group was in opposite situation. *P<0.05, Dunnett-t test.

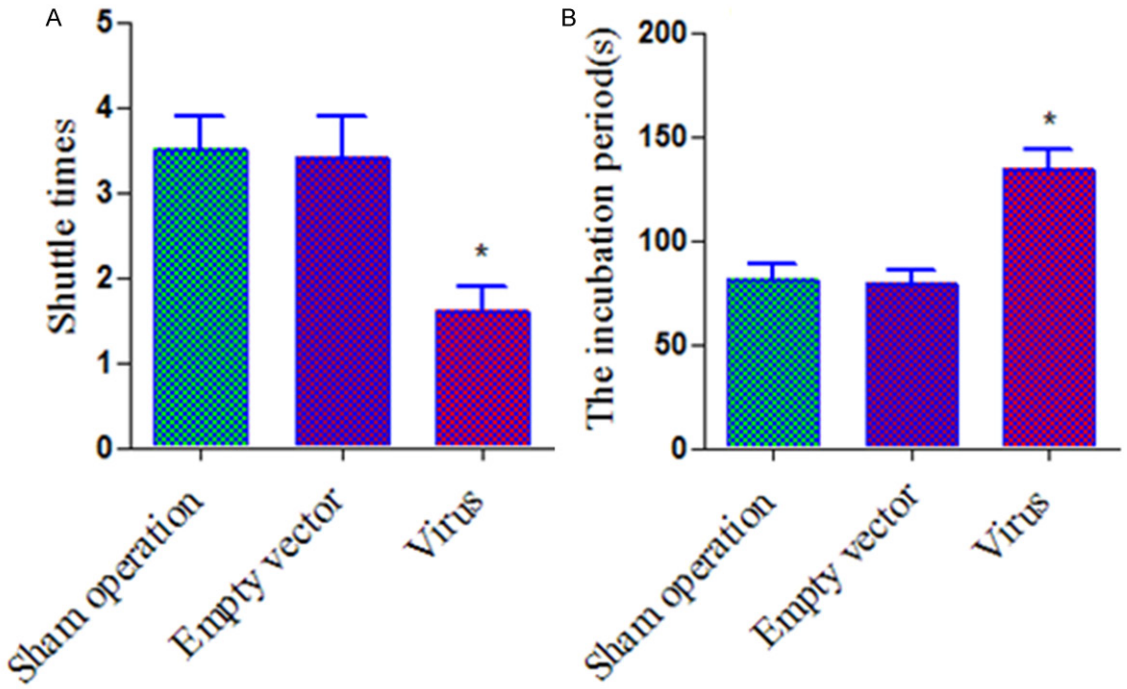

Figure 4. Passive avoidance performance test: Arc group: A. Shuttle times, B. Retention time. Shuttle times decreased and retention time increased in Virus group (*P<0.05, compared to Sham operation group); There was no significant change in Empty vector group. Dunnett-t test.

Discussion

$GABA_BR$ is the mainly metabotropic receptor of inhibitory neurotransmitter γ-aminobutyric acid B in the central nervous system. $GABA_BRs$ are widely expressed both in presynaptic and postsynaptic elements of neuron. Meanwhile, it is one member of G-protein coupled receptor

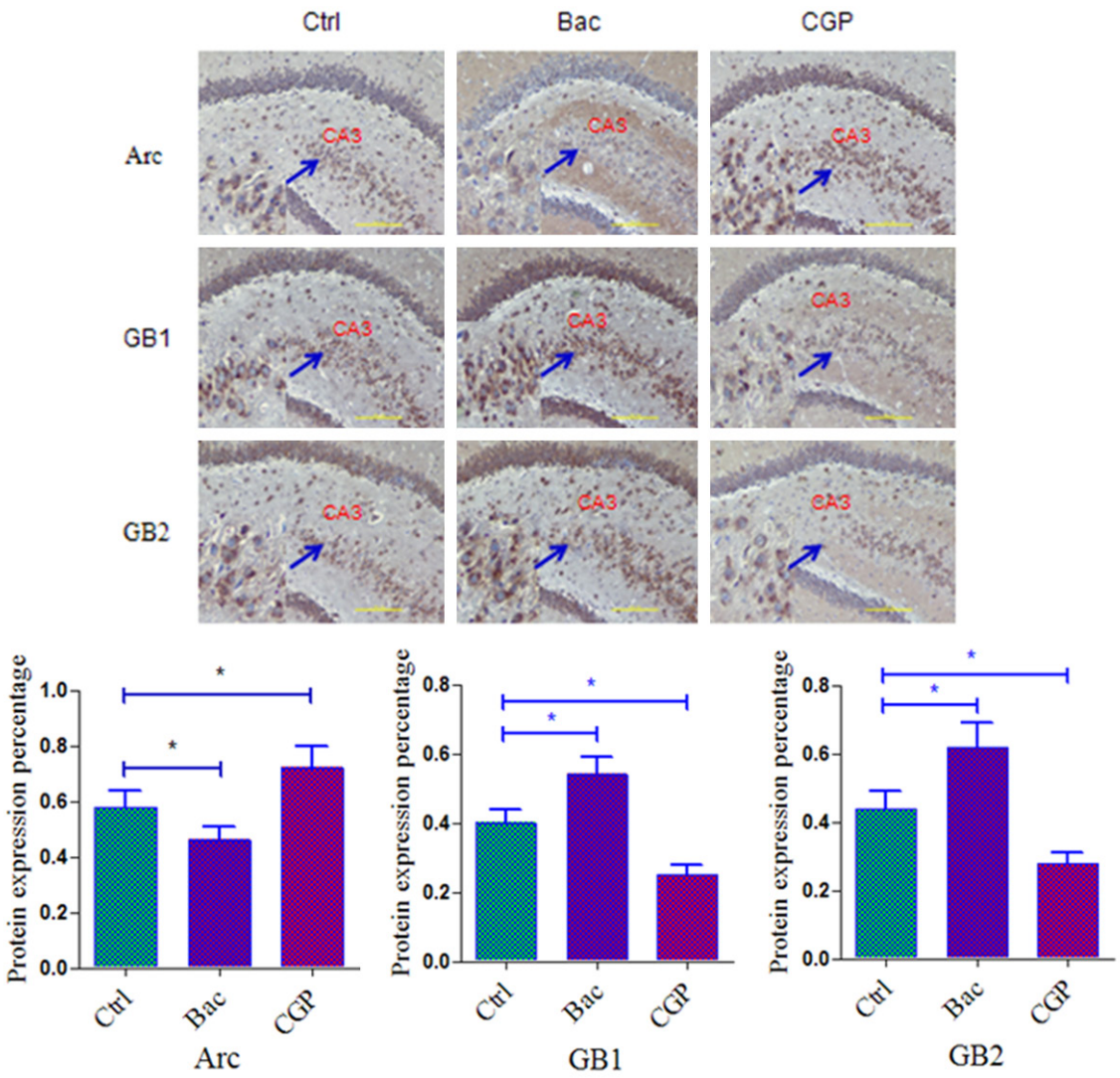

Figure 5. Immunohistochemistry analysis: $GABA_BR$ group: Arc positive ratio of hippocampal CA3 areas significantly decreased in Baclofen group but increased in CGP group; GB_1 and GB_2 positive ratio of hippocampal CA3 areas increased in Baclofen group but decreased in CGP group. (*P<0.05, Dunnett t-test).

(GPCR) associated with Gi/o family, which can mediate the persistent action of synapses. Dysfunction of $GABA_BR$ is closely related to various neurological diseases including epilepsy, anxiety, depression, algogenia, drug addiction, and cognition impairment [3, 8].

Productions of new genes and proteins are required for long-term memory formation. Activity regulated cytoskeletal protein (Arc) is the only immediate-early gene that rapidly activated to be transcribed and the Arc mRNA remains diffusely distributed [9]. The combination of neuron activity with new protein synthesis and synaptic plasticity revealed the cellular mechanism of synaptic plasticity dependent on gene transcription and protein translation. Also, Arc has been used as a marker for plastic changes in the brain [10]. The newly synthesized Arc mRNA expresses near strongly activated synapses. Then the protein productions accumulate around the activated synapses and interact with neuronal cytoskeleton to influence synaptic plasticity. Many researches show that Arc plays a key role in memory retention. Arc is one of the key proteins for regulating memory consolidation [11]. Arc/Arg3.1 knockout mice fail to form long-lasting memories, despite of intact short-term memory [12, 13]. In addition, excitatory neuronal architec-

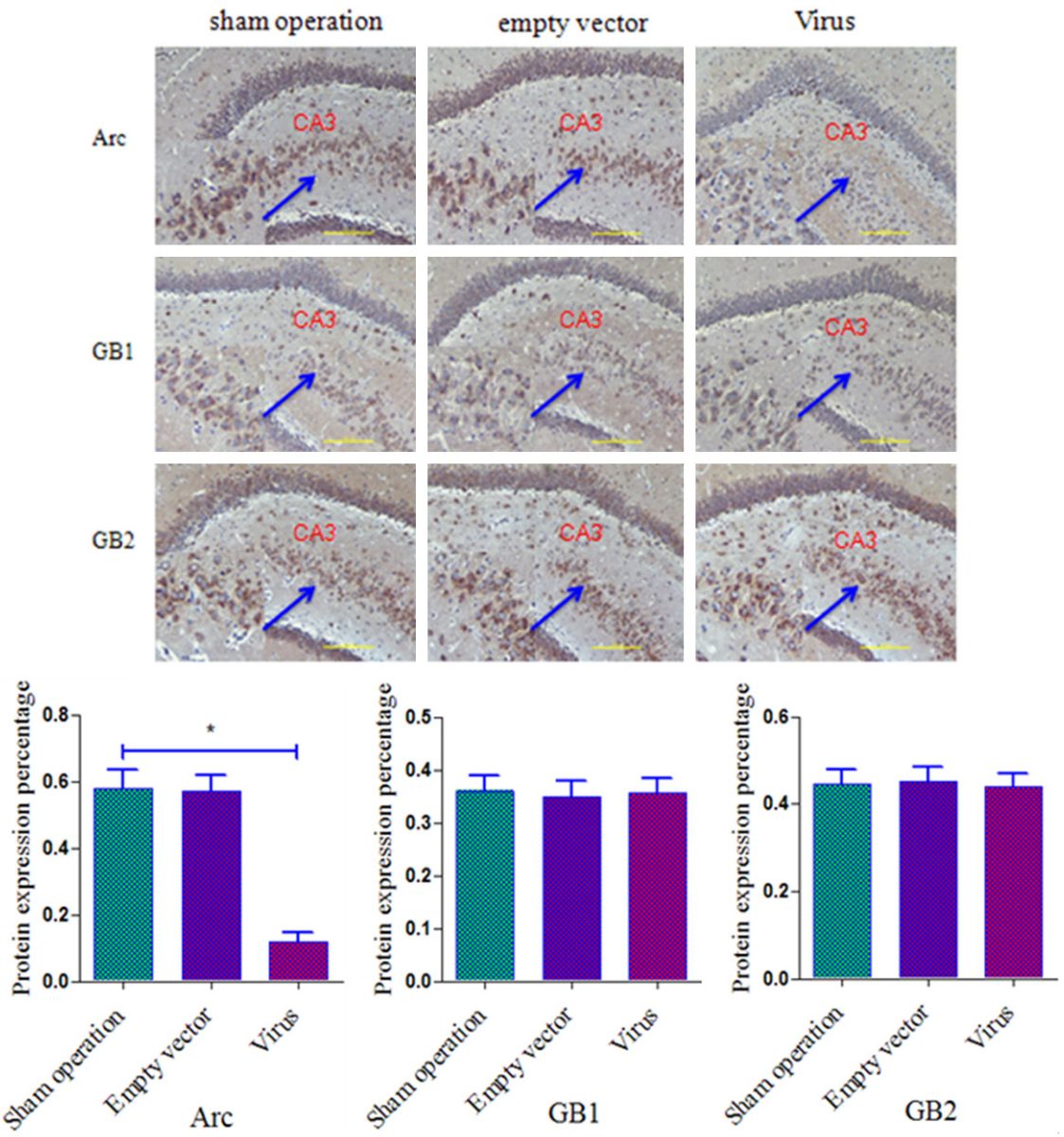

Figure 6. Immunohistochemistry analysis: For Arc group: Arc positive ratio of hippocampal CA3 areas significantly decreased in Virus group (*P<0.05), but there was no significant difference between Sham operation group and Empty vector group (P>0.05). GB_1 and GB_2 positive ratio of hippocampal CA3 areas showed no difference in comparison of these three groups. (*P>0.05).

ture and spatial memory are determined by $GABA_BR$ via regulating Arc [14]. Therefore, it is a key issue to study the function and modulating mechanism of Arc and $GABA_BR$.

Both Arc and $GABA_BR$ are crucial for neuronal activity modulation and long-term memory formation [4, 5], but the action of mechanism in epilepsy-induced cognitive dysfunction remains unknown. Our evidences confirmed that $GABA_BR$ selective agonist baclofen could prolong escape latency and decrease number of crossings in target quadrant of epileptic rats in Morris water-maze test, demonstrating that baclofen inhibited spatial learning and memory ability, and disrupted memory retention. On the contrary, $GABA_BR$ specific antagonist CGP35348 could shorten escape latency and increase number of crossings in target quadrant of epileptic rats. It was reported that

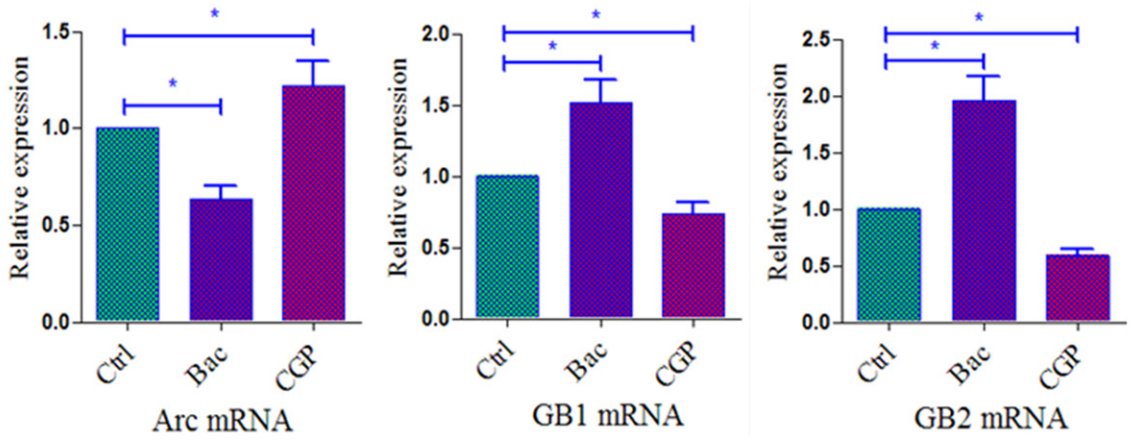

Figure 7. RT-PCR: Relative expression of Arc at mRNA level, for $GABA_BR$ group, Arc downregulated significantly in Baclofen group and upregulated in CGP group compared to Control group; Relative expression of GB_1 and GB_2 at mRNA level, GB_1 and GB_2 upregulated in Baclofen group and downregulated in CGP group. *P<0.05.

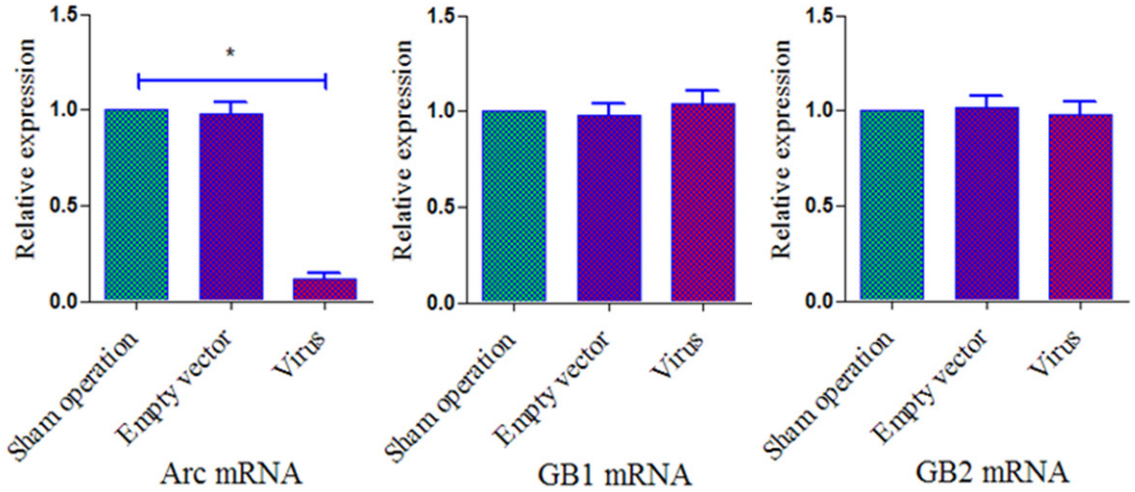

Figure 8. RT-PCR: Relative expression of Arc at mRNA level, for Arc group, Arc downregulated significantly in Virus group compared to the other two groups (*P<0.005), but there is no significant difference between Empty vector group and Sham operation group. Relative expression of GB_1 and GB_2 at mRNA level, there is no significant difference between each group (*P>0.05).

baclofen suppressed spatial learning ability by inhibiting $GABA_BR$ via activating TREK-$2K^+$ channel [15], while CGP35348 can suppress inhibitory postsynaptic potential (IPSP) and enhance the activation efficiency of $GABA_BR$ to improve the memory-formation process [16]. These phenomena were consistent with the function of $GABA_BR$ in cognitive function of normal rats [15, 17]. Downregulation of Arc by siRNA could increase escape latencies and decrease number of crossings in target quadrant, indicated that the learning and spatial memory abilities of epileptic rats were inhibited. However, $GABA_BR$ was not regulated by Arc in our study. Taken together, memory retention was influenced by both Arc and $GABA_BR$.

Method using epileptic rat model induced by LiCl-pilocarpine is one of the ideal model for studying human temporal lobe epilepsy. It can damage GABA interneurons and regulate the expression of $GABA_AR$ and $GABA_BR$. $GABA_BR$ is a heterodimer composed of GB_1 and GB_2 subunits. GABA or $GABA_BR$ agonists bind to the extracellular domain of GB_1 subunit and the intracellular domain of GB_2 combine to the

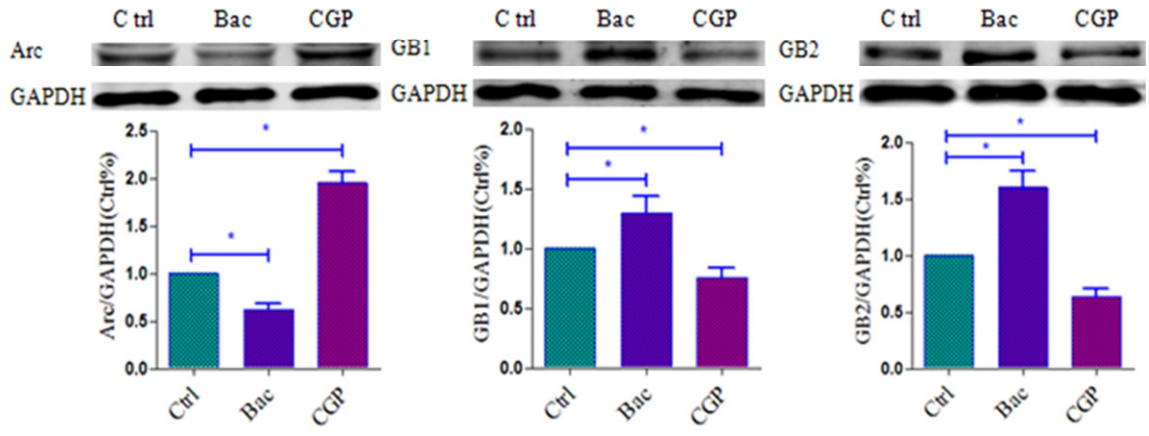

Figure 9. Western Blotting: For $GABA_BR$ group, relative expression of Arc at protein level decreased in Baclofen group but increased in CGP group significantly; relative expression of GB_1 and GB_2 at protein level increased in Baclofen group but decreased in CGP group. *P<0.05.

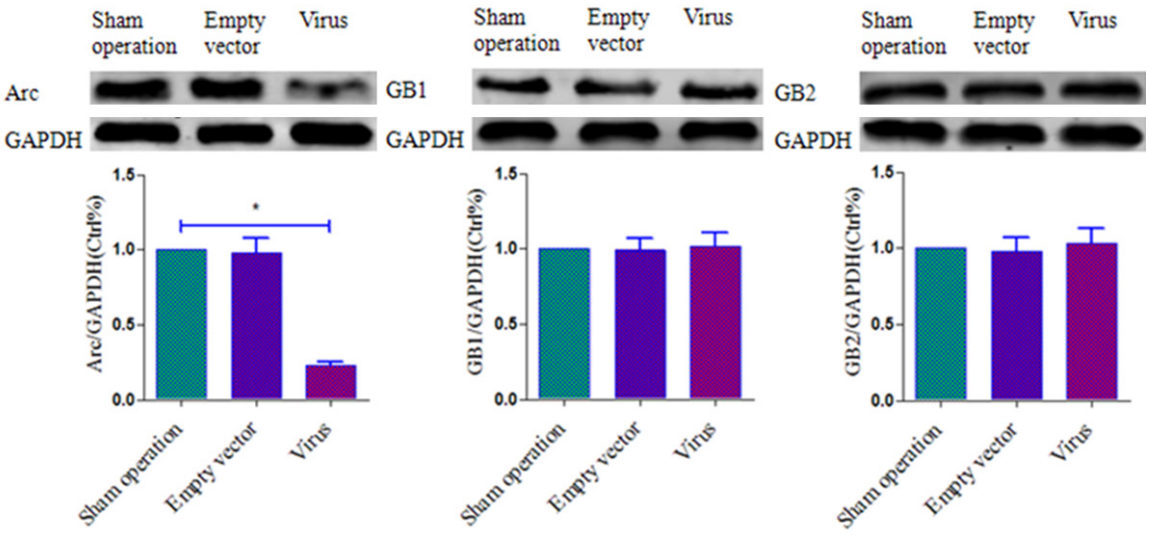

Figure 10. Western Blotting: for Arc group, relative expression of Arc at protein level decreased in Virus group significantly (*P<0.05), but there was no significant difference between Sham operation group and Empty vector group; relative expression of GB_1 and GB_2 at protein level didn't change statistically. (*P>0.05).

G-protein, resulting in G-protein activation [3, 18, 19]. Our study showed that baclofen could upregulate GB_1 and GB_2 expression of epileptic rats, in contrast CGP35348 downregulated the expression of these subunits, especially GB_2 expression. Baclofen and CGP35348 interfered with the inhibition of hippocampal synapses by regulating the expression of GB_1 and GB_2. In addition, we found that Arc siRNA downregulated Arc expression by interfering with Arc transcription and translation.

Spatial learning and background reconstruction mainly depend on the regulation of hippocampal neuron activity and structural alteration. It was reported that Arc/Arg3.1 was one of complementary "genomic timer" of neural activity and indispensable for hippocampus-dependent long-term memory consolidation [7]. In addition, dysregulation of Arc/Arg3.1 was related to cognitive disorders induced by many kinds of neurological diseases [11]. Terunuma et al reported that $GABA_BR$ activity was essential in spatial memory capacity via regulating Arc/Arg3.1 [14]. The present data in our study revealed that Baclofen could downregulate Arc/Arg3.1 expression of hippocampus in epileptic rats while CGP35348 upregu-

lated Arc/Arg3.1 expression significantly. Moreover, Arc siRNA downregulated Arc/Arg3.1 expression of hippocampus in epileptic rats but the expression of $GABA_BR$ (GB_1 and GB_2) was not changed. Based on the results of behavioral experiments, it was demonstrated that $GABA_BR$ activity influenced memory formation of epileptic rats via regulating Arc/Arg3.1 expression.

In conclusion, our study demonstrated that upregulation of $GABA_BR$ or downregulation of Arc could damage the capacity of memory retention and spatial learning memory. On the contrary, downregulation of $GABA_BR$ improved learning and memory functions. We also found that $GABA_BR$ regulated Arc/Arg3.1 expression but Arc activity has no effect on $GABA_BR$ regulation. However, both $GABA_BR$ and Arc/Arg3.1 had a significant role in spatial learning and memory retention.

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Disclosure of conflict of interest

None.

Address correspondence to: Feng Wang, Department of Neurosurgery, General Hospital of Ningxia Medical University, Yinchuan, Ningxia, China. Tel: +86-951-6743247; E-mail: wangfengsnowhappy@163.com

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