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Research Paper

Effect of behavior training on learning, memory and the expression of NR2B, GluR1 in hippocampus of rats offspring with fetal growth restriction ☆

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Abstract

Objective: To study effects of behavior training on learning, memory and the expression of NR2B, GluR1 in hippocampus of rat's offspring with fetal growth restriction(FGR). **Methods:** The rat model of FGR was established by passive smoking method. The rats offspring were divided into the FGR group and the control group, then randomly divided into the trained and untrained group, respectively. Morris water maze test was proceeded on postnatal month(PM2/4) as a behavior training method, then the learning-memory of rats was detected through dark-avoidance and step-down tests. The expressions of NR2B and GluR1 subunits in hippocampal CA1 and CA3 areas were detected by immunohistochemical method. **Results:** In the dark-avoidance and step-down tests, the performance record of rats with FGR was worse than that of control rats, and the behavior-trained rats was better than the untrained rats, when the FGR model and training factors were analyzed singly. The model factor and training factor had significant interaction($P < 0.05$). The expressions of NR2B and GluR1 subunits in hippocampal CA1 and CA3 areas of rats with FGR reduced. In contrast, the expressions of GluR1 and NR2B subunits in CA1 area of behavior-trained rats increased, when the FGR model and training factors were analyzed singly. **Conclusion:** These findings suggested that the effect of behavior training on the expressions of NR2B and GluR1 subunits in CA1 area should be the mechanistic basis for the training-induced improvement in learning-memory abilities.

Key words: FGR; learning and memory; behavior training; NR2B; GluR1

INTRODUCTION

Fetal growth restriction(FGR) is one of the serious complications during perinatal period, and the offspring not only appear as low birth weight, but also as deficits of intelligence and behavior because of disorders in the nervous system[1-3]. Animal experimental results indicated that behavior training could elevate rats' learning-memory abilities, and the rehabilitative training could hasten the functional recovery of patients with infarction cerebrum, but there have been no data about the effect of behavior training on learning-memory abilities of FGR offspring and its mechanism. In order to seek the theoretical basis of postnatal education for FGR offspring, the learning and memory ability of rats were tested, and the expressions of NR2B(N-methyl-D-aspartate receptor subunit2B, NR2B) and GluR1 (Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunit 1) in hippocampus CA1 and CA3 areas of rats with FGR were detected.

MATERIALS AND METHODS

Modeling and grouping

Rat FGR model was established by passive smoking method[4-5], adopting maturate unmated Sprague-Dawley rats(Experimental Animal Center of Xi' an Jiaotong University). The diagnostic criteria of FGR was that the birth weight of rat offspring was lower than the 10th

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percentile of the normal birth weight without smoking intervention during the pregnancy period. FGR rat offspring with smoking intervention were as the FGR group, and non-FGR rat offspring without smoking intervention were as the control group. Then the FGR and control groups were randomly subdivided into the trained and untrained groups. So there were four groups, with 10 arrheno-rats per group.

Behavior training

Behavioral training were conducted by a Morris water maze(150 cm in diameter and 55 cm in height) in a dim room illuminated by a carefully positioned lamp whose reflections from pool water were not perceived by the video camera. The pool was filled with water to a depth of 45 cm, with a temperature of 22 ± 12℃. The position and orientation of the pool in the testing room remained unchanged throughout the study. Moreover, both geometric and landmark cues were maintained constant. The pool was divided into four quadrants. A round transparent platform(8 cm in diameter) was placed at the center of a designated quadrant and submerged 1~1.5 cm beneath the water surface. On the first day, rats swam in the pool without the platform for about 2 min, and on the next day begun the formal training. The formal training on each rat was conducted for 4.5 successive days. Two training sessions were performed in each day, each session composed of four trials, with an inter-trial interval of 60 s. The inter-session interval within a single day was 2 h. In each trial, the rat was placed into the pool at the middle site of the circular edge in a randomly selected quadrant, with the head facing the pool edge. The rat was allowed to find the hidden platform within 120 s. If a rat failed to find the platform within 120 s, it was placed on the platform for 5 s by the experimenter, and its performance score (latency) was marked as 120 s. After each training, rats were removed from the pool, dried and placed in a heated dry cage.

Evaluation of learning-memory ability

Dark-avoidance test

Dark-avoidance testing was conducted using a device composed of two rooms made of opacus material, with shock installations on the bottom and a hole between the two rooms. There was a bright valve for lighting in one room. Rats were placed into the room with lighting to accommodate for 3 min without the shock installation, then rats will enter into the dark room automatically because of the skototaxis, If not, they will be driven to the dark room. On the first day, rats were placed into the bright room with the head backing the hole, and meanwhile the shock installation(25V, alternating current) of the dark room was switched on. We read the time(latency) when rats' fore-legs were both in the dark room for the first time, and counted the frequency of rats' entering into the dark room except for the first time within 5 min. Both the latency and frequency were marked as the learning performance score. The test was repeated 24 h later, and the latency and frequency were remarked as the memory performance score. If rats failed to enter the dark room within 5 min, the frequency was recorded as 5 min.

Step-down test

Step-down device is changed from the dark-avoidance device with the hole shut and a platform placed in one corner of the bright room. Rats were placed into the bright room to accommodate for 3 min without the shock installation working. On the first day, rats were placed in the bright room with the head backing the platform, and meanwhile the shock installation(25V, alternating current) in the bright room was switched on. Read the time(latency) when rats were stepping up on the platform for the first time, and counted the frequency of rats' stepping down from the platform within 5 min. Both the latency and frequency were remarked as learning performance score. Rats were placed on the platform 24 h later, and meanwhile the shock installation was switched on. Read the latency when rats were stepping down from the platform for the first time and the frequency of rats' stepping down from the platform except for the first time. The latency and frequency were remarked as the memory performance score. If rats failed to step down the platform within 5min, the frequency was recorded as 5 min.

Tissue preparation

All animals were anesthetized and intracardiacly perfused with 120 ml 0.9% saline, followed by 500 ml 4% paraformaldehyde for 4~6 h. The brains were removed and postfixed in 4% paraformaldehyde overnight at 4℃. Then the brains were dehydrated by ethanol from 75% to 100%, embedded in paraffin at 60℃, cut into 5- μm coronal sections and mounted onto slides. Brain sections were dried for 2 weeks at 56℃.

Immunohistochemical stain

SABC methods was used. Brain sections were deparaffinaged by dimethylbenzene from ethanol to H_2O, cooled to room temperature naturally after having been fixed in boiled citrate buffer for 15min, fixed in 3% H_2O_2 for 20 min and in 1%TritonX-100 for 20 min, with washed 3 times in PBS buffer for 5min after every step. Normal goat-sourced blood serum was added to the sections for 30 min at 37℃, followed by appropriate diluted first antibody overnight at 4℃[1:1500 NR2B antibody(developed in rabbit, BOSHIDE Company, China) or 1:2000 GluR1 antibody(developed in rabbit,

SIGMA Company, US.)]. Second antibody(developed in goat, BOSHIDE Company, China) labeled with biotin was added to the sections for 30 min at 37℃. SABC compound(BOSHIDE Company, China) was add to the sections for 30 min at 37℃, and the sections were washed 3 times in PBS buffer for 5 min after every step. Sections were stained with DAB developer till satisfied coloration coming, washed 3 times with distilled water for 5 min. Then the brain sections were dehydrated with ethanol, made transparent with dimethylbenzene, and mounted by neutral balsam.

Test on grey scale of immunohistochemical stain and statistical analysis

The grey scale of immunohistochemical stain in CA1 and CA3 areas was analyzed with image analysis(Q550CW, Leica Company, Germany). Six animals were chosen in each group, and 3 same focus were chosen and measured in the same area in different brains, then the grey scale of the 3 focus was averaged. Grey scale and the expression strength become inverse ratio, the higher grey scale enunciates the lower expression strength. The results were present as $\bar{x} \pm s$, SPSS 13.0 factorial analysis of two factors was used to analyze the results. The P value < 0.05 was considered statistical significance.

RESULTS

Valuation of learning-memory ability.

Compared with the control group, the learning-memory ability of rats with FGR descended, especially on postnatal month 2($P < 0.001$), and could be elevated by behavior training with Morris water maze, especially on postnatal month 2($P < 0.001$).

The expressions of NR2B and GluR1 in CA1 and CA3 areas of rats offspring

NR2B and GluR1 in CA1 and CA3 areas of rats with FGR were reduced, NR2B and GluR1 in CA1 area of trained rats were increased(***Table 1,2*** and ***Fig.1~6***).

DISCUSSION

Brain structure is very sensitive to hypoxia, and the hippocampus is one of the most sensitive areas[6]. Once the hippocampus is damaged, there are hippocampus dependent deficits in learning and memory[7-8]. The present study showed that the performance scores of rats with FGR in dark-avoidance test and step-down test were worse than that of control rats on PM2 and PM4, which indicated that FGR rat offspring had descended learning-memory ability, and there might be a functional disorder of the brain. Antenatal ischemic and hypoxia caused by factors(such as smoking) that result in FGR in the gestational period have effects on the formation and development of the nervous system, which may cause offspring with brain damages in perinatal period and with deficits of learning and memory in postnatal period. This may be the mechanism of descended learning-memory of rats with FGR.

In the development of the FGR brain there is a requirement for functional remodeling under environmental conditions in the postnatal period. Both environmental enrichment and telic behavior training in postnatal period could reverse the effects of adverse environment in neonatal period. This kind of reverse has effects on intensification and steadiness of synapse in development process. These data indicates that effects of adverse environments on brain structure and cognition in earlier period of life could be antagonized by manipulating the environment in postnatal period. There are data demonstrating that swimming training can elevate learning-memory ability of rats[9-10], and other telic behavior training can elevate experimental animals' learning-memory ability. Van[11] and his colleagues found that performance score of rats trained with running experiment was better than that of untrained rats, and there was more nerve ending in dentate gyrus of trained rats. Running partly enhanced long-term

Table 1 **Expressions of NR2B andGluR1 in rat offspring of normal and FGR group** (grey scale)

Groups	NR2B				GluR1			
	2 month		4 month		2 month		4 month	
	CA1	CA3	CA1	CA3	CA1	CA3	CA1	CA3
normal	196.58 ± 7.67	191.23 ± 20.41	192.87 ± 4.80	193.84 ± 2.65	187.30 ± 3.87	185.70 ± 0.46	183.87 ± 6.03	181.95 ± 2.32
FGR	187.47 ± 8.55*	194.09 ± 3.02	186.51 ± 11.94*	192.77 ± 9.39	179.90 ± 0.98**	176.89 ± 1.41**	177.84 ± 1.41*	177.68 ± 3.47**

Compare with the normal group, $^{*}P < 0.05$; compare with the normal group, $^{**}P < 0.01$. The expression of NR2B and GluR1 in FGR rats offspring were decreased, especially that of GluR1.

Table 2 **Expressions of NR2B and GluR1 in untrained and trained group of FGR offspring** (grey scale)

Groups	NR2B				GluR1			
	2 month		4 month		2 month		4 month	
	CA1	CA3	CA1	CA3	CA1	CA3	CA1	CA3
untrained	185.82 ± 5.53	186.23 ± 9.12	190.96 ± 2.77	193.52 ± 6.57	184.28 ± 1.02	185.51 ± 7.75	179.32 ± 7.09	180.05 ± 5.67
trained	196.58 ± 7.67**	191.23 ± 20.41	192.87 ± 4.80	193.84 ± 2.65	187.30 ± 3.87**	185.70 ± 0.46	183.87 ± 6.03*	181.95 ± 2.32

Compare with the untrained group,$^{*}P<0.05$; compare with the untrained group, $^{**}P<0.01$. The expression of NR2B and GluR1 in the trained group of FGR offspring were increased, especially that in CA1 area of the trained rats offspring.

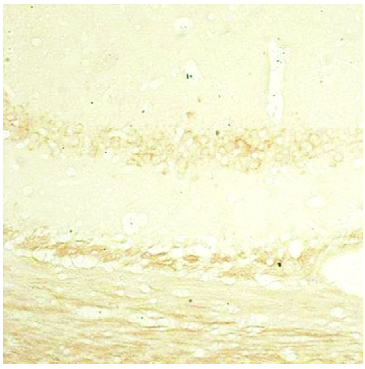

Fig. 1 The expression of NR2B in CA1 area of rats in FGR untrained group on PM2(IHE,× 200)

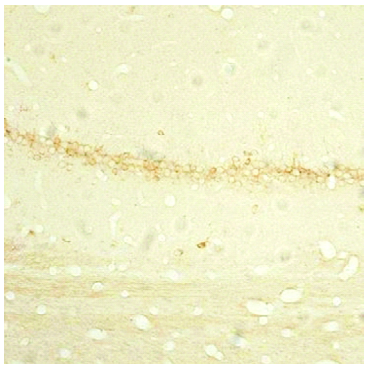

Fig. 2 The expression of NR2B in CA1 area of rats in FGR trained group on PM2(IHE,× 200)

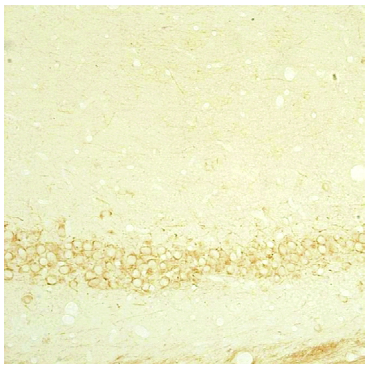

Fig. 3 The expression of GluR1 in CA1 area of rats in FGR untrained group on PM2(IHE,× 200)

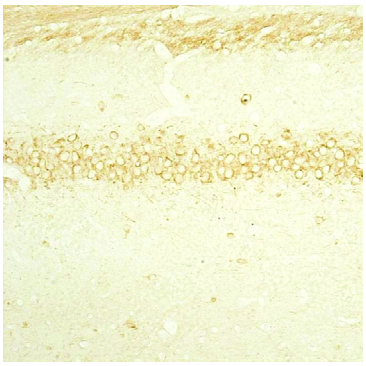

Fig. 4 The expression of GluR1 in CA1 area of rats in FGR trained group on PM2(IHE,× 200)

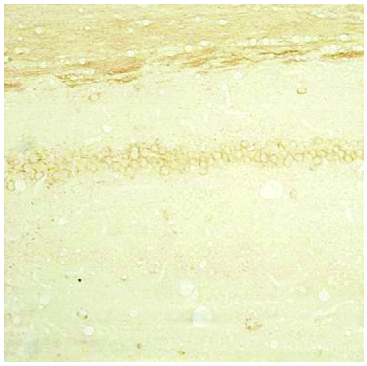

Fig. 5 The expression of GluR1 in CA3 area of rats in FGR untrained group on PM2(IHE,× 200)

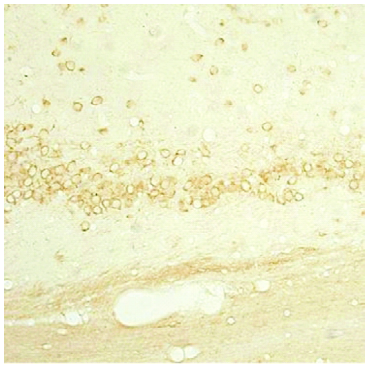

Fig. 6 The expression of GluR1 in CA3 area of rats in FGR trained group on PM2(IHE,× 200)

potentiation in the dentate gyrus. Pan-HJ[12] and her colleagues found Morris water maze training could improve learning-memory ability of rats with infarction in hippocampus. Through this experiment in which Morris water maze test was used as a behavior training method, the performance scores of trained rats were found better than that of untrained rats on PM2 and PM4, especially for rat's offspring with FGR. These findings demonstrated that Morris water maze training could elevate learning-memory ability of rat with FGR on PM2 and PM4. We also found the learning-memory ability of rats with FGR descended, especially on postnatal month 2($P < 0.001$), suggested that FGR rat offspring had descended learning-memory ability. Otherwise our research indicated that learning-memory ability could be elevated by behavior training with Morris water maze, especially on postnatal month 2($P < 0.001$). These results were consistent with past reports.

NMDAR and AMPAR, which are two critical glutamic acid receptors in the central nervous system, are correlated with learning and memory. NMDAR are composed of NR1 and NR2(A-D) subunits, NR2B is more important in neural plasticity and learning and memory than any other NR2 subunits. Increase of NR2B in CA1 area could strengthen learning-memory ability by lengthening coexcitation time of ante-synapse and post-synapse and strengthening activation of NMDAR. Mice with NR2B subunit over-expressed in procerbrum cellular membrane became more smart, because of long-term potentiation and attenuation of NMDAR channel current was lengthened[13-15]. NR2B gene was named "smart gene".

AMPARs are tetramers or pentamers containing various combinations of subunits(GluR1-4). Its effect on nervous activities is to mediate most of the synaptic transmission. Unpolarization of AMPAR excitation is faster than that of NMDAR. AMPAR usually cooperates with NMDAR to activate the postsynaptic neurons. Many studies confirmed that the neurons named "silent synapse" only expressed with NMDAR and without AMPAR. "Silent synapse" could be activated by gaining functional AMPARs with formation of long-term potentiation[16-18]. GluR1 subunit is a critical ingredient of AMPAR, and it is more important in learning and memory than any other AMPAR subunits. Some findings demonstrated that long-term potentiation couldn't be induced in rats with GluR1 gene knocked out, and there was redistribution of AMPAR containing GluR1 subunit after long-term potentiation[19]. Phosphorylation of GluR1 was involved in LTP process, too[20].

Our studies found that NR2B and GluR1 in CA1 and CA3 areas of rats with FGR were reduced on postnatal month 2 and month 4($P < 0.05$) indicated that the brain of FGR offspring was damaged in perinatal period.

The present study showed that NR2B and GluR1 in CA1 and CA3 areas of rats with FGR reduced on PM2 and PM4. The function of receptor channel will be descended following the reduction, then the formation of synaptic plasticity, and the transmission and storage of information will be affected. Moreover, the learning-memory ability of rats with FGR descended. The expressions of NR2B, GluR1 in CA1 area of the trained rats was stronger than those in untrained rats on PM2 and PM4. It was said that NR2B and GluR1 were critical for neural plasticity, learning and memory. The results indicated that, on one hand, the behavior training increased the net number of GluR1 in synapse by enhancing the expression of GluR1 in cellular membrane in CA1 area, then activated the "silent synapse"; on the other hand, it enhanced the function of NMDAR channel by enhancing the expression of NR2B in CA1 area. Their cooperation activated the fibers between synapses, precipitated formation of long-term potentiation, then strengthened hippocampus synaptic plasticity, and improved learning-memory ability of rats with FGR. Our research also proved that training could improve learning-memory ability of FGR offspring, and suggested training could increase NR2B and GluR1 in CA1 and CA3 areas of rats with FGR.

This experiment suggested that behavior training could effectively improve learning-memory ability of rats with FGR, which may be associated with the enhancement of NR2B and GluR1 subunits in CA1 area. The study provided important information for exploring new methods of diagnosis and treatment of FGR, and provided theoretical basis of postnatal education for FGR offspring. It is believed that an effective treatment method of deficits in learning and memory in FGR offspring will be found in the future, which is helpful for improving the qualification of the population and pushing the progress of perinatology.

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