Original Research Article

Effect of simvastatin on brain-derived neurotrophic factor (BDNF)/TrkB pathway in hippocampus of autism rat model

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Abstract

Purpose: *To study the effect of simvastatin on behavioral performance in a rat model of autism, and its effect on hippocampal brain-derived BDNF-TrkB pathway.*
Methods: *Twelve rats with valproic acid (VPA)-induced autism were randomly divided into model group and simvastatin group, while six healthy rats served as normal control group. Rats in the simvastatin group received the drug (5 mg/kg) via i.p. route, while rats in model group and normal control group were injected with equivalent volume of normal saline in place of simvastatin. Capacity for interaction and repetitive stereotyped behavior, as well as results of Morris water maze test were determined for each group. The expressions of BDNF-TrkB proteins were assayed with immunoblotting.*
Results: *The frequencies of sniffing normal saline, alcohol and rat urine were significantly higher in model and simvastatin rats than in normal rats, but they were significantly lower in simvastatin-treated rats than in model rats (p < 0.05). There was higher duration of turning, jumping and grooming in the model group and simvastatin group than in the normal rats, but the duration was significantly reduced in simvastatin rats, relative to model rats. Escape latency times was significantly longer in model and simvastatin rats than in controls, but number of target quadrant crossings was significantly reduced. However, escape latency time was lower in simvastatin rats than in model rats, but number of target quadrant crossings was significantly higher. The model and simvastatin rats had down-regulated levels of BDNF and TrkB protein, relative to control rats, but there were markedly higher levels of these proteins in simvastatin-treated rats than in model rats.*
Conclusion: *Simvastatin improves the behavioral performance of autistic rats by regulating BDNF/TrkB signal axis. This finding may be useful in the development of new drugs for treating autism.*

Keywords: Simvastatin, Autism, Brain-derived BDNF TrkB pathway, Behavior

INTRODUCTION

Autism spectrum disorder (ASD) refers to syndrome that features sustained deficits in socialization and interactive behavior, stereotyped behavior, and limited concerns [1]. Nowadays, autism has become a serious health issue of public concern. It has been reported that the ASD cases in USA have risen from 1 in 68 people in 2012, to 1 in 59 in 2018 [2]. At present, not much is clearly known about the pathogenesis of ASD. Although research suggests that it is due to the negative effects of factors such as environment and gene on

neurodevelopment [3], the specific mechanism is still unclear. Research findings indicate that ASD results in hippocampal lesions due to disappearance of some neurons and granular cells, decreased Purkinje cell count and cerebellar atrophy, physiological changes in the temporal lobe, delayed sensorimotor development, and alterations in prefrontal cortical synaptic linkages [4]. These lesions predispose to ASD and impairment of cognition. Recent evidence suggests that distortion of the balance in excitatory and antagonistic transmissions at the synapses may be involved in the behavioral manifestations associated with ASD [5]. Overall, the imbalance between excitatory and antagonistic synaptic transmissions may be implicated in the etiology of ASD. Brain-derived neurotrophic factor (BDNF) plays an important role in the survival, growth and differentiation of neuronal cells, while tyrosine kinase receptor B (TrkB) is the main receptor of BDNF which performs a regulatory role in downstream signaling pathways after binding to BDNF and activating its biological effects. Studies have shown alterations in the expressions of BDNF and TrkB in the hippocampus of autism mouse model, indicating that these proteins are associated with autism [6]. In addition, it was found that simvastatin improved the behavioral performance of VPA-induced autism model in mice [7]. Moreover, simvastatin significantly regulated the activities of BDNF and TrkB in rats with Parkinson's disease [8]. However, it is not clear whether simvastatin improves the behavioral performance of autistic rats by affecting the BDNF/TrkB pathway in the hippocampus. Therefore, this study was carried out to investigate the effect of simvastatin on protein expressions of BDNF/TrkB pathway in the hippocampus of VPA-induced autistic rats, and its effect on behavioral performance of rats.

EXPERIMENTAL

Establishment of rat model of autism, and animal grouping

A total of 20 healthy female and 10 healthy male adult Wistar rats were used in this study. The animals were raised and fed in a clean environment. At 7 pm, the male and female rats were caged in the ratio of 2:1, and vaginal smears were collected on the following day at 7 am. If sperm was observed in the smears, that day was marked as the first day of pregnancy. At 12½ days of gestation, 10 pregnant rats were randomly injected intraperitoneally with VPA at a dose of 600 mg/kg. The resultant neonatal rats (offspring of pregnant rats) were used as the autism rat model. A total of 87 neonates of

autism model were delivered. Two pregnant rats were randomly selected and intraperitoneally injected with equivalent dose of normal saline, and the offspring of these pregnant rats served as normal control. The number of offspring of the normal control group delivered was 23. Six autistic neonatal rats chosen at random, were given simvastatin. They received intraperitoneal injection of simvastatin (5 mg/kg) for 2 weeks. Another set of 6 neonatal rats were randomly selected as the model group, and they were given an equivalent amount of normal saline via intraperitoneal injection for 14 days. Six young rats in the normal control group served as the control group, and the same amount of normal saline was injected intraperitoneally for 14 days.

Ethical approval

This research was approved by the Animal Ethical Committee of Yangzhou University (approval no. 20220102), and was conducted according to the guidelines of "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [9].

Evaluation of behavior

Social communication test

The rats were placed in a clean and odor-free observation box with dimensions 60cm x 60cm x 40cm. Cotton swabs stained with normal saline, rat urine and alcohol were placed at a distance of 10cm from the bottom of the box. The rats were monitored to see how often they sniffed the cotton swabs. Replacement of the cotton buds was done three times during the test period, and the test was repeated three times. The number of times a rat sniffed each cotton swab was recorded.

Determination of repeated stereotyped behavior

The rats were placed in observation boxes of dimensions 48cm x 24cm x 20 cm. The inner side of each observation box was equipped with an infrared generator and detector. Prior to recording rat activity, the infrared ray was blocked, and the surrounding environment was kept completely noise-free. Then, the cumulative frequencies of jumping, turning and grooming of rats were recorded within 30min.

Morris water maze test

The test included an acquisition phase in the first 6 days, and an assessment test on the seventh day. The platform navigation test was conducted

for 6 days, and each test time lasted for 60 sec. Each rat was put in the water at 1 of the 4 quadrants, and it was permitted to locate the platform without assistance. The test was done four times daily, and rats had 60 sec to locate the platform. If a rat was successful in locating the platform, it was permitted to tarry on it for 20 sec. If it was unable to locate the platform within 60 sec, the rat was guided to it and permitted to stay on it for 20 sec. Escape latency was the time spent finding the hidden platform. It was accurately determined with a video tracking appliance. On day 7 of the test, the space detection test involving removal of the platform was carried out. The rats were tested with respect to their ability to locate the previous position of the platform. Four trials were conducted. The number of crossings of the target quadrant was recorded using a video tracking device as indicated before.

Determination of protein expressions

Total protein was extracted from hippocampal tissues using RIPA, and determined using Western blotting. The protein lysates were centrifuged, and the protein content of each lysate was determined using BCA method. Thereafter, equal amounts of protein (50-µg samples) were resolved on 10% SDS-polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) electrophoresis and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with TBST containing 5 % non-fat milk solution in 0.05 % Tween 20 for 1 h at room temperature. This was followed by overnight incubation with specific primary antibodies at 4 °C. Then, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. The immuno-positive bands were scanned using ImageJ software, while amounts of different proteins were computed with densitometric analysis.

Statistical analysis

Data analysis was done with SPSS 23.0 software package. Measurement data are expressed as mean ± SD, comparison amongst multiple groups was carried out with one-way ANOVA, while LSD-t test was used for pairwise comparison. Statistical significance was assumed at $p < 0.05$.

RESULTS

Interactive ability of rats

The durations of sniffing normal saline, alcohol and rat urine were markedly higher in model and simvastatin rats than in normal rats, but they were significantly lower in the simvastatin group than in the model group ($p < 0.05$). These results are presented in Table 1.

Repetitive stereotyped behavior of rats

The frequency of turning, jumping and grooming for rats was significantly higher in model and simvastatin rats than in normal rats, but was significantly lower in the simvastatin group than in the model group ($p < 0.05$; Table 2).

Learning and memory abilities of rats

The escape latency times of model and simvastatin rats were significantly higher than that of normal rats, while the number of crossings of the target quadrant was significantly lower than that in the control group ($p < 0.05$). In contrast, the escape latency time of rats in simvastatin group was significantly lower than that of model rats, while the number of crossings of the target quadrant was significantly higher than that in the model group ($p < 0.05$). These results are shown in Table 3.

Table 1: Comparison of interactive ability of rats amongst the groups (mean ± SD, n = 6)

Group	Normal saline (s)	Alcohol (s)	Rat urine (s)
Control	8.12 ± 1.42	5.26 ± 1.24	11.84 ± 2.59
Model	12.41 ± 1.73[a]	7.83 ± 1.86[a]	22.58 ± 2.96[a]
Simvastatin	10.53 ± 1.50[ab]	6.03 ± 1.42[ab]	16.37 ± 2.60[ab]

[a]$P < 0.05$, compared with normal group; [b]$p < 0.05$, compared with model group

Table 2: Frequencies of repetitive stereotyped behaviors of rats in each group (mean ± SD, n = 6)

Group	Turning	Jumping	Grooming
Control	172.65 ± 6.84	173.37 ± 8.29	176.85 ± 8.16
Model	241.86 ± 7.89[a]	268.48 ± 10.27[a]	284.34 ± 11.87[a]
Simvastatin	213.76 ± 7012[ab]	235.24 ± 9.85[ab]	226.85 ± 10.21[ab]

[a]$P < 0.05$, vs normal rats; [b]$p < 0.05$, vs model rats

Table 3: Learning and memory abilities of rats in each group (mean ± SD, n = 6)

Group	Escape latency time (s)	Frequency of crossing of target quadrant
Control	13.28 ± 2.38	6.24 ± 2.18
Model	22.73 ± 2.97 [a]	2.27 ± 1.05 [a]
Simvastatin	18.67 ± 2.65 [ab]	3.85 ± 1.37 [ab]

[a]$P < 0.05$, compared with the normal group; [b]$p < 0.05$, compared with model group

Protein expression levels of BDNF and TrkB

The protein expression levels of BDNF and TrkB were markedly down-regulated in the model and simvastatin rats, relative to control rats, but they were markedly higher in simvastatin-treated rats than in model rats ($p < 0.05$). These results are shown in Table 4.

Table 4: Protein expression levels of BDNF and TrkB in each group (mean ± SD, n = 6)

Group	BDNF	Trkb
Control	1.16 ± 0.26	0.86 ± 0.13
Model	0.65 ± 0.17 a	0.57 ± 0.08 a
Simvastatin	0.89 ± 0.21 ab	0.71 ± 0.14 ab

[a]$P < 0.05$, vs normal rats; [b]$p < 0.05$, vs model rats

DISCUSSION

Many investigations have suggested a causal link between environment/gene and autism. Therefore, the establishment of animal models of autism is essential in autism research. It has been reported that *in utero* administration of VPA led to neuro-developmental abnormalities consistent with the clinical features of ASD [10]. In this study, the neonatal rats delivered by pregnant rats after prenatal injection of VPA served as model for research on ASD. Previous research found that microanatomy and macroanatomy are associated with variations in brains of ASD subjects [11]. Hippocampal structural changes that regulate perception of emotion are associated with ASD-like behavior [12]. Moreover, alterations in neurochemistry due to impairment of metabolic pathways of catecholamines were reported in ASD patients, and also in propionate-mediated autistic rats [13]. However, the exact mechanism involved is not yet clear.

It is known that BDNF is a crucial cell survival element associated with a variety of brain diseases [14]. During development, it is vital for neuronal survival and differentiation, maturation of dendritic spine, and neuritic branching and connections [15]. Moreover, BDNF is involved in vital processes such as synaptic plasticity and long-term potentiation, as well as in attention, learning and memory [16]. It has been reported that serum or brain BDNF levels are low in schizophrenia [17], major depression [18], and Alzheimer's disease [19]. In the model of major depression, based on the region of the brain acted on, BDNF brings about pro- and anti-depressant, and stress-sensitive and stress-resilient outcomes. More importantly, when BDNF acts on hippocampal tissues, it decreases negative emotional status and stress sensitivity.

Plasticity-enhancing effect of BDNF is induced via activation of tyrosine kinase B (TrkB) receptor which initiates specific intracellular signaling pathways [20]. Tyrosine kinase B (TrkB) is expressed by a gene, and it exists as a full-length (TrkB) and truncated isoform in cells. The Trkb.t1 isoform is an anti-plastic receptor because of absence of Trk domain. Indeed, it inhibits TrkB signal transduction by trapping BDNF, thereby reducing the expressions of downstream signal route proteins [21]. The BDNF/TrkB signal route is a well-established pathway in dendritic growth and differentiation processes, e.g., spine development. In addition, the TrkB downstream effectors, i.e., Akt, GSK-3 and mTOR are involved in neurite and growth cone regulation. Patients with ASD may have *de novo* genetic changes in TrkB [22]. Moreover, ASD patients may have abnormal cortical, striatal, hippocampal, and serum levels of BDNF. These findings indicate the association between ASD and BDNF/TrkB system [23]. Previous studies have found that dorsal striatal BDNF/TrkB are essential for the major features of ASD. This finding is supported by the report that vector-induced dorsal striatal BDNF expression in mice alleviated autism-like behavior [24]. Simvastatin is considered the most effective neuroprotective statin [25]. Studies have demonstrated the efficacy and safety of simvastatin in the treatment of neurofibromatosis and autistic children [26]. Studies have shown that simvastatin exerts specific effects on brain regions, and these effects have been shown to be highly correlated with social disorders and psychopathology of autism [27]. Simvastatin downregulates the role of Ras pathway in NF1 animal model, thereby reducing GABA, improving synaptic long-term potentiation and rescuing behavioral phenotypes. At the same time, simvastatin affects myelination, regional axon and astrocyte integrity in NF1. Statins provide neuroprotection against various cognitive and neurological disorders [28]. Upregulation of BDNF has been reported in a mouse model treated with simvastatin after brain injury and spinal cord injury [29]. This investigation has

demonstrated that protein expressions of BDNF and TrkB were markedly down-regulated in model and simvastatin groups, relative to control rats, but were markedly more up-regulated in simvastatin-treated rats than in model rats. Therefore, simvastatin regulated the protein expressions of BDNF and TrkB in the hippocampus.

CONCLUSION

Simvastatin improves the behavioral performance of autistic rats through regulation of protein expressions of BDNF-TrkB axis. This finding may be useful in the development of new drugs for treating autism.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Yingrui Chen conceived and designed the study, Linqian Cai and Yuxian Xu collected and analysed the data, Yingrui Chen wrote the manuscript.

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