Original Article

Expression of Brain-derived Neurotrophic Factor and Tyrosine Kinase B in Cerebellum of Poststroke Depression Rat Model

Yun Li[1], Chun Peng[2], Xu Guo[1], Jun-Jie You[1], Harishankar Prasad Yadav[1]

[1]Department of Neurology, The Affiliated Hospital of Dali University, Dali, Yunnan 671000, China
[2]The Institute of Criminal Science and Technology, The Public Security Bureau of Dali Prefecture, Dali, Yunnan 671000, China

Abstract

Background: The pathophysiology of poststroke depression (PSD) remains elusive because of its proposed multifactorial nature. Accumulating evidence suggests that brain-derived neurotrophic factor (BDNF) plays a key role in the pathophysiology of depression and PSD. And the cerebellar dysfunction may be important in the etiology of depression; it is not clear whether it also has a major effect on the risk of PSD. This study aimed to explore the expression of BDNF and high-affinity receptors tyrosine kinase B (TrkB) in the cerebellum of rats with PSD.

Methods: The rat models with focal cerebral ischemic were made using a thread embolization method. PSD rat models were established with comprehensive separate breeding and unpredicted chronic mild stress (UCMS) on this basis. A normal control group, depression group, and a stroke group were used to compare with the PSD group. Thirteen rats were used in each group. Immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) for detecting the expression of BDNF and TrkB protein and mRNA in the cerebellum were used at the 29th day following the UCMS.

Results: Compared with the normal control group and the stroke group, the number of BDNF immunoreactive (IR) positive neurons was less in the PSD group ($P < 0.05$). Furthermore, the number of TrkB IR positive cells was significantly less in the PSD group than that in the normal control group ($P < 0.05$). The gene expression of BDNF and TrkB in the cerebellum of PSD rats also decreased compared to the normal control group ($P < 0.05$).

Conclusions: These findings suggested a possible association between expression of BDNF and TrkB in the cerebellum and the pathogenesis of PSD.

Key words: Brain-derived Neurotrophic Factor; Cerebellum, Poststroke Depression; Tyrosine Kinase B

Introduction

Poststroke depression (PSD) is the most common mood disorder following a stroke, and also is the main factor limiting recovery and rehabilitation in stroke patients. In addition, it may increase mortality by up to 10 times.[1] PSD has become a prominent negative factor in stroke recovery. However, the pathogenesis of the PSD is not unclear.

In the past 10 years, an emerging hypothesis suggested that the pathogenesis and treatment of depression are likely to involve a plasticity of neuronal pathways.[2,3] Brain-derived neurotrophic factor (BDNF), the most abundant neurotrophin in the brain,[4,5] is a member of the neurotrophin growth factor family. BDNF has been known to be involved in synaptic plasticity.[6,7] BDNF plays a critical role in the development and plasticity of the central nervous system (CNS)[8,9] and has been implicated in the biology of psychiatric disorders as well as in learning and memory.[10] There have been many documents proved that decrease of BDNF was associated with PSD.[11,12]

The cerebellum has traditionally been looked upon as a brain area primarily involved in motor behavior. The last decade has however heralded the cerebellum as a brain region of renewed interest for neuropsychiatric disorders. The relationship

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Address for correspondence: Dr. Yun Li, Department of Neurology, The Affiliated Hospital of Dali University, Dali, Yunnan 671000, China
E-Mail: liyun_neuron@163.com

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between the cerebellum and neuropsychiatric disorders is becoming clearer. Recent data suggested that cerebellar dysfunction may be common, especially in depression. Other studies have also suggested that cerebellar dysfunction is associated with poor mood.[13] Whereas the relationship between the expression of BDNF in the cerebellum and PSD is still unclear. In this study, we sought to test whether a change of BDNF and tyrosine kinase B (TrkB) in the cerebellum is associated with the occurrence of PSD.

Methods

Animals and grouping

Fifty-two adult, young female Sprague-Dawley rats weighing 250–300 g obtained from Experimental Animal Center of Kunming Medical University were used in this study. The experimental procedures were done in accordance with the Care and Use of Laboratory Animals published by the National Institute of Health Guide (1996). All efforts were made to minimize animal suffering and reduce the number of animals used. The rats were randomly divided into four experimental groups. Group I was a normal control in which the rats did not receive any treatment and were maintained in standard laboratory conditions (normal control group). The rats in Group II were subjected to the thread embolization method to occlude the middle cerebral artery (MCA) (stroke group). The rats in Group III were placed in small, individual cages and subjected to unpredicted chronic mild stress (UCMS) (depression group). The rats in Group IV received comprehensive separate breeding and were subjected to UCMS after thread embolization (PSD group). Altogether 13 rats were used in each group. All the animals were allowed to survive 4 weeks before they were killed for various laboratory procedures. Behavioral tests for cognition and mood functions were done once a week during the survival period.

Middle cerebral artery thread occlusion procedures

The rats were deeply anesthetized with intraperitoneal injection of 3.6% chloral hydrate ($CCl_3CH(OH)_2$) (1 ml/100 g). Then, middle cerebral artery occlusion (MCAO) was carried out using an intraluminal thread introduced via the common carotid artery up to the origin of the MCA according to the document.[14] During surgery, the rats were kept on a heating pad to maintain the body temperature at 37.0°C. Then, they were placed in temperature and humidity controlled incubation chambers until they woke up. They were transferred to cages. All the rats received an intraperitoneal injection of penicillin (20,000 U/d) for 3 days. Neurological function evaluation was performed 24 h after the induction of ischemia and scored on a 5-point scale, as proposed by Longa *et al.*,[14] and the baseline preference was also performed. Then the UCMS regimen began.

Unpredictable chronic mild stress

The UCMS regimen contained 9 different stressors randomly arranged during 18 consecutive days: 20 h food and water deprivation, 18 h water deprivation, 17 h of 45° cage tilt, overnight illumination, 21 h wet cage, 5 min swimming in water at 4°C, 30 min on a 160 Hz rocking bed, 1 min tail pinch, and 2 h immobilization.[15,16] The behavioral tests were employed 7 days after ischemia.

Behavioral assessment of the experimental rat

Open-field test

The basal locomotor activity of rats was evaluated with an automatic-recording (man-made) open-field working station stereotypic movements as well as resting over a time of 30 min.[17] The field consisted of a wooden box (75 cm^2 chamber, 40 cm high walls) with black walls and a white floor, which was divided by 1-cm wide black lines into 25 (565) equal squares. The open-field test was used to evaluate general locomotor and rearing activities of the animals. Locomotor activity was defined as at least three paws in a quadrant and rearing behavior defined as the animal standing upright on its hind legs. These two activities were tallied. Both locomotor and rearing activities were manually recorded by trained observers who were blind to the condition of the animals over a 5-min period. Three observers blinded to the animals' identity conducted the evaluation. And the scores were averaged.

The sucrose preference test

The measurement of sucrose preference was performed according to the document described.[18] The rats were trained to adapt to a 1% sucrose solution (w/v) for 48 h at the beginning of the experiment, during which two bottles of 1% sucrose solution were placed in each cage. The test subjects were allowed to consume water and 1% sucrose solution for 1 h after 24 h of food and water deprivation. The position of the 2 bottles (left/right sides of the cages) varied randomly between trials. For each trial, the position of both bottles was counterbalanced across the rats in each group. At the end of 1 h, sucrose and water consumption (in milliliters) was measured, and sucrose preference (%) was calculated as the ratio of sucrose consumption to sucrose plus water consumption. The test was carried out once a week until the 29th day following the UCMS.

Body weight evaluation

The body weight of each animal was evaluated weekly until the end of the UCMS procedure.

Morris water maze test

The Morris water maze test was performed as described previously and used to characterize the spatial learning performance of rats.[19,20] Rats were allowed up to 60 s to locate the escape platform. If an animal failed to find the platform within the period, then it was gently guided to the platform and allowed to stay on it for 30 s.

Immunohistochemical evaluation of brain-derived neurotrophic factor and tyrosine kinase B expression in the cerebellum

Tissue preparation

At the end of the behavioral tests, all of the reserved rats were anesthetized with 3.6% chloral hydrate as described earlier, and 1000 ml of 0.1 mmol/L phosphate buffer saline

solution (PBS, pH 7.2–7.3, room temperature) was introduced into the left ventricle for 20–30 min, followed by 1000 ml of 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.2–7.3, 4°C for 1 h). The cerebellum was taken out and immersed in the same fixative at 4°C for 24 h and then transferred to a solution of PBS containing 30% sucrose before cryosectioning. Serial sections of the cerebellum segments were sliced at a thickness of 10 μm (some sagitally and others coronally) in a cryomicrotome. They were collected and mounted on gelatin-pretreated slides for the following laboratory procedures.

Histological evaluation of the host cerebellum

Immunohistochemistry, using anti-BDNF and anti-TrkB, were employed to observe the BDNF and TrkB positive cells in the cerebellum. In each section group, the sections were labeled A, B, C, and D. All labeled sections were processed for BDNF and TrkB immunohistochemistry, respectively. BDNF (1:200, Santa Cruz, USA) or trkB (1:200, Santa Cruz) antibodies and goat-anti-rabbit IgG (1:200, Santa Cruz) were used. The immunohistochemical procedure followed the Avidin-Biotin Complex method was shown in the previous report. The sections were stained by 3,3'-diaminobenzidine. The specificity of the antibodies had been tested by the western blot analysis. Control experiments in which PBS was substituted for the primary antibody were also performed. The numbers of BDNF and trkB immunoreactive (IR) cells in each section were counted using the HPIAS-1000 Image Program (Olympus, Japan) from 5 sections. Counting in each section was done under a high power field (original magnification ×400).

Reverse transcription-polymerase chain reaction of brain-derived neurotrophic factor and tyrosine kinase B expression in the cerebellum

Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the gene expression level of BDNF and TrkB in the cerebellum. Total RNA was isolated from the cerebellum sample (weighing 100 mg) using Trizol (Invitrogen) according to the manufacturer's instruction. The concentration was measured using a Nanodrop spectropho-tometer (ND-1000) (Bio-Rad, USA), and the total RNA was subjected to reverse transcription procedure. Complementary DNA (cDNA) was generated using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas Company, USA). The first strand of cDNAs was synthesized by adding 2 μg of total RNA, using reverse transcriptase I (Fermentas International Inc., Ontario, Canada). For PCR, 1 μl of cDNA was used to amplify specific sequences for the BDNF, TrkB, and internal control GAPDH using rat specific primers shown in Table 1. The PCR procedures followed the protocol for the kit (Fermentas Company, USA). The procedure consisted of an initial step (94°C, 5 min), then 35 cycles of melting (95°C, 1 min), annealing (the appropriate temperature of different neurotrophic factors, 1 min), extension (72°C, 1 min), and the final step (72°C, 10 min). The separation was completed by using 1% agarose gel and the products were stained with 0.05% ethidium bromide. Band intensities were scanned with Bio-Gel imagery apparatus (Bio-Rad, USA) with GAPDH used as an internal control. The primers and the anneal temperature of the BDNF and TrkB are shown in Table 1.

Statistical analysis

In all quantification procedures, observers were blind to the nature of the experimental design. SPSS version 13.5 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Data are presented as mean ± standard deviation (SD). Multiple group comparisons were made by one-way analysis of variance (ANOVA) test. Statistical significance was set at $P < 0.05$.

Results

Behavioral tests

Table 2 shows open-field activities, weight, and relative

Table 1: The primer of BDNF and TrkB

Factor	Primer	Production length (bp)	Anneal temperature (°C)
BDNF	Forward: 5'-CGAAGAGCTGCTGGATGAG-3"	366	52
	Reverse: 5'-ATGGGATTACACTTGGTCTCG-3'		
TrkB	Forward: 5'-CCTCCACGGATGTTGCTGA-3'	315	56
	Reverse: 5'-GGCTGTTGGTGATACCGAAGTA-3'		
GAPDH	Forward: 5'-CCGTATCGGACGCCTGGTTA-3'	512	57
	Reverse: 5'-GGCTGTTGGTGATACCGAAGTA-3'		

BDNF: Brain-derived neurotrophic factor; TrkB: tyrosine kinase B.

Table 2: Open-field activity, relative sucrose intake and weight of all groups at 29-day after UCMS (n = 13/each group, mean ± SD)

Items	Normal control group	Stroke group	Depression group	PSD group
Locomotor activity	56.40 ± 11.10	20.20 ± 8.64*	11.40 ± 4.98*	3.40 ± 3.05*
Rearing activity	18.40 ± 5.60	9.00 ± 2.55*	3.00 ± 0.71*,†	1.40 ± 2.19*,†
Sucrose preference (ml)	24.60 ± 5.41	20.60 ± 8.91	5.80 ± 2.39*,†	4.80 ± 0.84*,†
Weight (g)	286.20 ± 1.92	274.20 ± 10.43	253.80 ± 13.70*	246.80 ± 15.17*,†

*P<0.05, compared with normal control group; †P<0.05, compared with stroke group. SD: Standard deviation; PSD: Poststroke depression; UCMS: Unpredicted chronic mild stress.

sucrose intake preference at 29-day after UCMS. Results from one-way ANOVA indicated that the locomotor activity and the frequency of rearing in PSD group significantly reduced and the weight of PSD rats decreased significantly compared with the normal control group (F = 121.313, F = 75.383, and F = 36.225, respectively, all P < 0.05). Furthermore, the sucrose intake preference in the PSD rats was also reduced significantly compared with the stroke group and normal control group (F = 47.875, F = 17.843, respectively, all P < 0.05). The current MCAO + UCMS protocol induced behavioral changes in ischemic animals, including reduced sucrose preference and decreased activity. Reduced sucrose preference indicated desensitization of the brain reward mechanism, which was consistent with the behavioral correlates of depressive-like symptoms in poststroke humans. All of these behavioral results provided the evidence that the PSD model has been established successfully.

Expression of brain-derived neurotrophic factor and tyrosine kinase B in the cerebellum

Immunochemical staining of brain-derived neurotrophic factor and tyrosine kinase B in the cerebellum

Immunohistochemical staining with anti-BDNF [Figure 1a-1d] and anti-TrkB [Figure 1e-1h] evaluated the number of the BDNF and TrkB IR positive neurons in the cerebellum. There were no positively stained cells in the negative control. Compared with the normal control group and the stroke group, the number of BDNF IR positive neurons was the least in the PSD rats (F = 25.998, P < 0.05). And the number of BDNF IR positive neurons was also less in rats of depression group, compared to the normal control group (F = 11.613, P < 0.05). There was no significant difference for BDNF IR positive neurons between the PSD group and the depression group (F = 0.314, P > 0.05). Furthermore, the number of TrkB IR positive cells was significantly less in the PSD group than that in the normal control group (F = 4.715, P < 0.05). A similar difference for the number of TrkB IR positive cells was also seen between the depression group and normal control group (F = 6.132, P < 0.05); however, there was no significantly difference between the PSD group and the depression group (F = 1.315, P > 0.05) [Figure 2].

mRNA expression of brain-derived neurotrophic factor and tyrosine kinase B in the cerebellum

RT-PCR displayed mRNA expression of BDNF and TrkB in the cerebellum of all groups. Electrophoresis picture of BDNF, TrkB, and GAPDH PCR productions by 1% agarose gel [Figure 3]. The results showed the gene expression of BDNF and TrkB in the cerebellum of PSD group decreased compared to the normal control group (F = 4.300, F = 3.710, respectively, all P < 0.05). Whereas, compared with the other groups (stroke group and depression group), the expression of BDNF mRNA and TrkB mRNA showed no statistical difference in PSD group (F = 0.503, F = 0.170, respectively, all P > 0.05, Figure 2). The experimental results of immunohistochemical staining and the RT-PCR did not match exactly. Control reactions were negative for the amplification products, demonstrating that the PCR method and reagents used yielded specific amplified products only when a cDNA source was included.

Discussion

Stroke is the third most common cause of death in developed countries, following only coronary heart diseases and cancer.[21] Depression is a common neuropsychiatric consequence of stroke and has been reported to negatively affect functional and cognitive recovery.[22] Since then, research on depression after stroke has gained momentum.[23]

An emerging hypothesis suggested that the pathogenesis and treatment of depression are likely to involve a plasticity

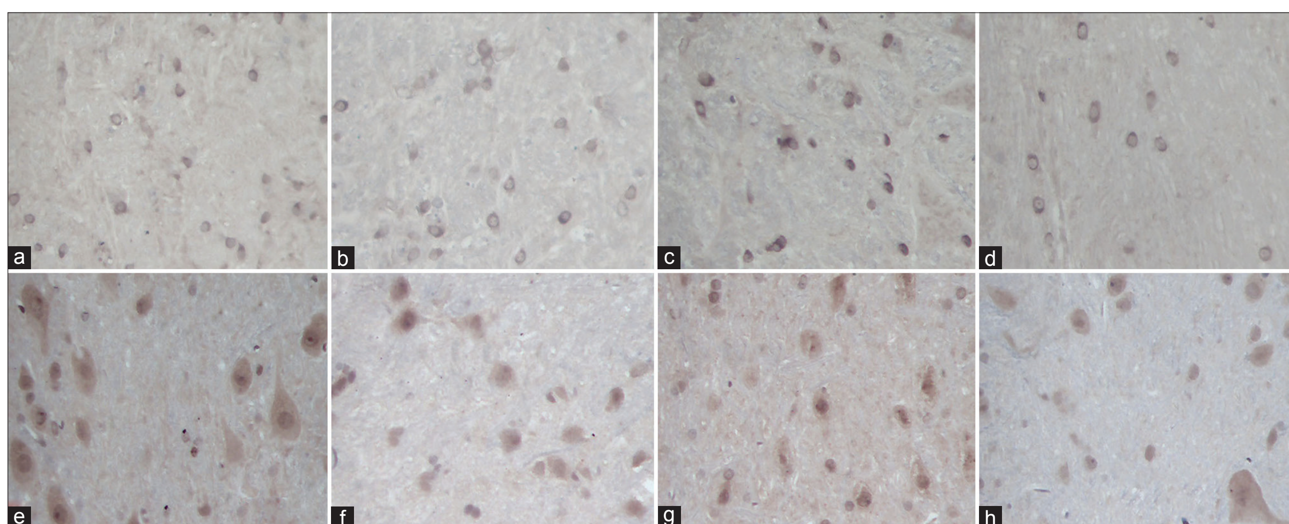

Figure 1: The morphology of BDNF-IR (a-d), TrkB-IR (e-h) in the cerebellum among group rats (immunohistochemical staining). (a) BDNF immunopositive neurons in normal control group; (b) BDNF immunopositive neurons in stroke group; (c) BDNF immunopositive in depressed group; (d) BDNF immunopositive in PSD group; (e) TrkB immunopositive neurons in normal control group; (f) TrkB immunopositive neurons in stroke group; (g) TrkB immunopositive neurons in depressed group; (h) TrkB immunopositive neurons PSD group ([a-h] magnification ×400). BDNF: Brain-derived neurotrophic factor; IR: Immunoreactive; TrkB: Tyrosine kinase B; PSD: Poststroke depression.

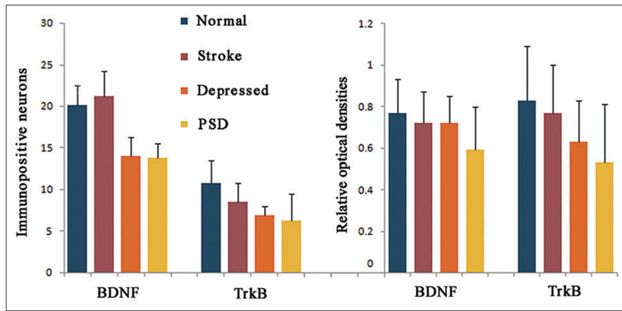

Figure 2: The histogram shows the expression of the immune-positive cells and the genes for BDNF and TrkB. Horizontal axis marked BDNF and TrkB. And vertical axis labeled the number of immune positive cells and the relative optical density. The left side showed the expression of the immune-positive cells for BDNF and TrkB. The right side showed the genes expression of BDNF and TrkB. BDNF: Brain-derived neurotrophic factor; TrkB: Tyrosine kinase B; PSD: Poststroke depression.

of neuronal pathways.[2,3,23] BDNF, the most abundant neurotrophin in the brain,[4,5] is known to exert a powerful influence on the development, survival, maintenance and plasticity of neurons within the immature and adult nervous system, and has recently been shown to elicit rapid action potentials thus influencing neuronal excitability.[24,25] Many documents supported the notion that BDNF plays a key role in the pathophysiology of PSD at the acute stage of ischemic stroke.[11,26] This study first investigated the expressions of BDNF and TrkB in the cerebellum of PSD rats. Our findings demonstrated that the mRNA and protein decrease of BDNF in the cerebellum has an adverse effect on the course and pathogenesis of PSD.

The role of the cerebellum has traditionally been seen as limited to the coordination of voluntary movement, gait, posture, speech, and motor functions.[27] The increasing evidences suggested that the cerebellum is not only connected with motor pathways but also with other cortical and association areas involved in superior mental functions, suggesting the involvement of the cerebellum in cognition[28] and the pathophysiology of psychiatric disorders. Studies on an association between the cerebellum and mental disorders revealed that the cerebellum may play an important role in patients with PSD.[13] This study demonstrated that the expressions of BDNF and TrkB in the cerebellum of PSD rats were lower than those in the cerebellum of the other groups. Our findings provided new insight into the cerebellum to play an important role in the pathogenesis of PSD, probably by the decrease of the expression of BDNF and TrkB.

Neurotrophic factors mediate their effects on cellular function and plasticity through the stimulation of specific tyrosine kinase coupled receptors, which signal through mitogen-activated protein kinase signaling cascades. BDNF and its high-affinity receptor TrkB are widely expressed in the CNS and play a crucial role in regulating synaptic transmission and plasticity during development and adulthood.[14,29-31] In this study, the gene expression of BDNF and TrkB as well as BDNF and TrkB immunostained positive cells was the lower in the cerebellum of the PSD

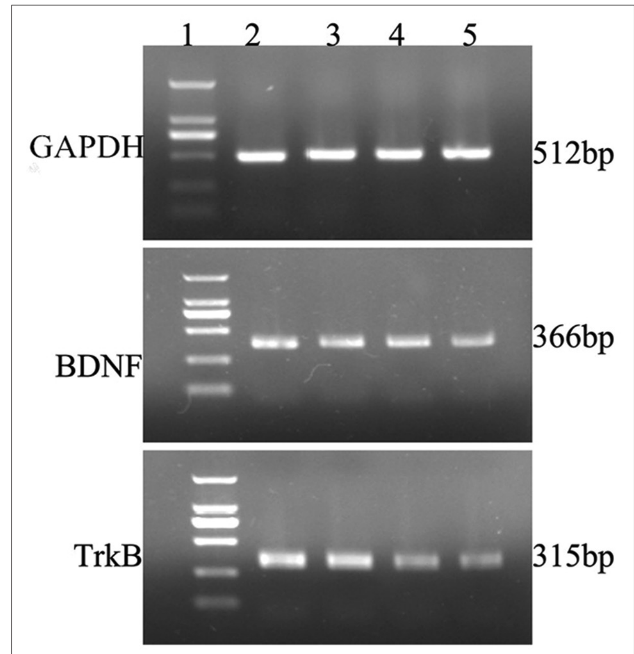

Figure 3: Electrophoresis picture of BDNF, TrkB, and GAPDH PCR productions among groups by 1% agarose gel. GAPDH was used as control. Marker in the left side is shown in Lane 1; normal control group is shown in Lane 2, while depressed group, stroke group and PSD group are shown in Lane 3–5 respectively. The left showed different gene and the right showed the genes molecular weight. BDNF: Brain-derived neurotrophic factor; TrkB: Tyrosine kinase B; PSD: Poststroke depression; PCR: Polymerase chain reaction.

rats as compared to the normal control rats. There were some differences between the expressions of gene and protein in the present experience. These discrepancies between mRNA and protein levels under basal conditions and following brain insults were probably to a large extent due to translational and/or posttranslational regulation of protein synthesis, or release and transport of the BDNF protein.[32] BDNF may function as a classical target-derived trophic factor, but probably also act locally through autocrine or paracrine mechanisms.[33,34]

In summary, the present findings indicated that cerebellar dysfunction may be responsible for the pathogenesis of PSD. BDNF, as a very important member of the neurotrophin growth factor family, mediates its effects on cellular function and plasticity through the stimulation of specific tyrosine kinase coupled receptors, TrkB. The protein and mRNA expression of BDNF and TrkB reduction in the cerebellum may play an important role in the pathogenesis of PSD.

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Conflicts of interest

There are no conflicts of interest.

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