DOI: 10.5137/1019-5149.JTN.13533-14.1
Received: 11.12.2014 / Accepted: 27.01.2015
Published Online: 11.07.2016

Original Investigation

Analysis of the Expressions of TrkB and GLT-1 in Brain Tissues on a Rat Pentylenetetrazol Kindling Model of Chronic Epilepsy

Jinping LIU[1,2], Yi ZENG[1], Chao YOU[2], Zhong YANG[3]

[1]Sichuan Provincial People's Hospital, Department of Neurosurgery, First Ring Road, Chengdu, Sichuan, 610072, China
[2]Sichuan University, West China Hospital, Department of Neurosurgery, Chengdu, Sichuan, 610041, China
[3]The Third Military Medical University, Department of Neurobiology, Shapingba District, Chongqing, 400038, China

ABSTRACT

AIM: Tyrosine kinase receptor-B (TrkB), a high-affinity receptor for brain-derived neurotrophic factor (BDNF), functions via specific binding with the BDNF. Although studies have shown that BDNF promotes the expression of glutamate transporter-1 (GLT-1) in the glial cells during neurodegeneration, evidence is lacking regarding whether BDNF/TrkB pathway is associated with the abnormal function of GLT-1.The aim of this study is to analyze the expressions of TrkB and GLT-1 in the brain of pentylenetetrazol (PTZ) kindling model of chronic epilepsy in rats, and to provide evidence for the correlation between the BDNF/TrkB pathway and GLT-1 and clues for the prevention and treatment targets of epilepsy.

MATERIAL and METHODS: In total, 40 Sprague-Dawley rats were randomly classified as model (n= 30) and control (n= 10) groups respectively. Western blotting was used for analyzing the expression of TrkB and GLT-1, and double immunofluorescence staining was used for detecting the cells positive for both glial fibrillary acidic protein (GFAP) and TrkB in the hippocampus and temporal cortex 7 days after establishing the kindling model.

RESULTS: Compared with the control group, protein level expression of TrkB and GLT-1, and mean optical density (OD) value of the cells positive for both GFAP and TrkB in the hippocampus and temporal cortex were significantly increased after the onset of epilepsy ($P<0.05$).

CONCLUSION: The BDNF/TrkB pathway may participate in the epileptogenesis through modulating the biological effect of GLT-1 in the glial cells of PTZ kindling rats, though the specific mechanisms require further investigations.

KEYWORDS: Epilepsy, TrkB, GLT-1, Pentylenetetrazol (PTZ), Astrocyte

INTRODUCTION

Cell pathological phenomena such as neuron loss, reactive gliosis, and nerve fiber sprouting are common during epilepsy, and are closely related to the alterations of expressions and functional activities of neurotrophic factors in the central nervous system (CNS) (26). As a high-affinity functional receptor for brain-derived neurotrophic factor (BDNF) (33), tyrosine kinase receptor-B (TrkB) functions via the specific binding with BDNF(2), then affects synaptic transmission, induces axonal sprouting, and triggers reconstruction of neuroplasticity and synaptic remodeling, (2,7) which would enhance the neuronal excitability and result in the production of hyperexcitable re-entrant circuits in the brain.These processes will further increase the susceptibility to epileptic seizures or the onsets of recurrent epilepsy, indicating that BDNF/TrkB pathway is involved in epileptogenesis (9,13,19).

Existing documents reported that abnormal expression and function of glutamate transporter-1 (GLT-1) existed in the brain tissues of animals and humans with different forms of epilepsy (41). Study on hereditary epilepsy using a rat model of Genetic Absence Epilepsy Rat from Strasbourg indicated that decrease in the expression of GLT-1 existed before the onsets

Corresponding author: Zhong YANG
E-mail: zhongyang_cq@163.com

of epilepsy (31), and blocking of glutamate transporter would result in *in vivo* epileptic discharges of neurons (1), but the epileptic pathogenesis resulting from the altered expression and function of glutamate transporter in the glial cells is still unclear.

In the present study, a Sprague-Dawley rat pentylenetetrazol (PTZ) kindling model of chronic epilepsy was established to analyze the expression of TrkB and GLT-1 in the hippocampus and temporal cortex tissue, so as to provide experimental basis for the effect of BDNF/TrkB pathway as well as GLT-1 in epileptogenesis.

MATERIAL and METHODS

Specific pathogen free (SPF) Sprague-Dawley (SD) rats (weight,250±10 g) were provided by the Experimental Animal Center of the Third Military Medical University (Chongqing, China) [SCXK (Chongqing) 20070003]; PTZ was purchased from the Sigma Company(USA); rabbit anti-rat GLT-1 and glial fibrillary acidic protein (GFAP) antibody, and goat anti-mouse fluorescein isothiocyanate (FITC)-labeled immunofluorescence secondary antibodies (green) were purchased from Chemicon Company (USA), rabbit anti-rat phosphorylated-TrkB (p-Trk) antibody was purchased from Cell Signaling Technology Biological Reagents Company Limited (Shanghai China), mouse anti-rat GFAP antibody and goat anti-rabbit fluorescence-labeled secondary antibodies (Cy3, red) were purchased from Wuhan Boster Biological Technology (Wuhan, China), biotinylated secondary antibody (immunoglobulin G), peroxidase-labeled streptavidin, and diaminobenzidine (DAB) developing kit were purchased from the ZSGB-Bio Company (Beijing, China).

Animal Grouping and Establishment of Model for Chronic Epilepsy

The study protocol was evaluated and approved by the Animal Ethics Review Committee at the Third Military Medical University. In total, 40 healthy SPF adult male SD rats (6~8 weeks, 250±10 g) were randomized into two groups: 10 for the control group and 30 for the model group. As it was required in the experiment, the model was established 7days after the kindling (45). The specific procedures were as follows: PTZ 35 mg/kg/day (a subthreshold dose) was given to the rats via intraperitoneal injection for 15-23 days, while a same dose of saline solution was injected intraperitoneally for the rats in the control group; injections were administered between 8:00 am and 10:00 am every day, and the rats were observed for 30-60 min after injections. The Racine's scoring system was used for grading the motor seizures (27): level 0, no seizure; level I, rhythmic mouth and facial tics; level II, clonuses of the forelimb, but without orthostatic tremor; level III, clonuses of the forelimb, with orthostatic tremor; level IV, tonic-clonic seizures; level V, generalized tonic seizures associated with tumbling and falling. Kindling criteria: level IV or level V seizures were observed after intraperitoneal injections of PTZ to the rats.

Sample Collection

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (39). The hearts of the anesthetized rats from the model and control groups were perfused by the heparinized saline for 15min, followed by the perfusion of 4% paraformaldehyde for 30 min. After perfusion, the brain tissues were removed and soaked in 4% paraformaldehyde overnight. The 30% sucrose-paraformaldehyde solution was used for dehydration for 2 days, and later the brain tissues were sliced coronally at a thickness of 30 μm on a freezing microtome. Two consecutive slices were used at an interval of 60 μm.

Double-Labeling Immunofluorescence

Methods mentioned by Burbach et al. (4) were referred. Three animals from the control group and four animals from the model group were used. The primary antibodies were rabbit anti-rat TrkB (1:100) and mouse anti-rat GFAP (1:100). Endogenous peroxidase activity of the sections was terminated after treatment with 0.3% hydrogen peroxide for 30 min; after blocking by 10% calf serum under room temperature for 1 h, rabbit anti-rat TrkB was added for incubation at room temperature for 2h and incubated overnight in refrigerator at the 4°C, and the sections were washed thrice (10 min per wash) by the 0.01M phosphate-buffered solution (PBS). Later the goat anti-rabbit Cy3-labeled immunofluorescence secondary antibody (1:100) was added for incubation at 37°C in an incubator for another 2 h. After being washed by the 0.01 M PBS thrice (10 min per wash), mouse anti-rat GFAP antibody was added for incubation at room temperature for 2 h and incubated overnight at 4°C in the refrigerator, and the sections were washed thrice (10 min per wash) by the 0.01 M PBS. Later the goat anti-mouse FITC-labeled immunofluorescence secondary antibody (1:200) was added for incubation at 37°C in an incubator for another 2 h. Then the sections were washed by the 0.01 M PBS thrice (10 min per wash) and sealed by the glycerin-PBS (1:1). The sections were observed and photographed under the fluorescence microscopy.

Western Blot

The methods described by Ueda et al. (41) were referred. Two animals from the control group and four from the model group were used. After decapitation, the cerebral cortex and hippocampus of the rats were immediately harvested on ice. After homogenization and centrifugation, the supernatants of the brain tissues were collected. Protein samples and loading buffers were mixed at a volume ratio of 1:1 before the 10 min boiling water bath; after the protein mixture cooled down, they were loaded (15 μL mixture was used) for electrophoresis; after being transferred to a membrane, the protein was blocked by the 5% skimmed milk for 2 h; incubations with the primary antibodies (TrkB 1:1000 or GLT-1 1:1000) lasted overnight, and the durations of incubations with the secondary biotinylated antibody (under 37°C) and peroxidase-labeled streptavidin were for 2 h each, and finally DAB was used for coloring. β-Actin was used as an internal control (45).

Image Analysis

Three sections were randomly selected for observation under the confocal microscopy (Zeiss LSM 510); three

nonoverlapping fields were randomly selected for analysis; the images were scanned and analyzed by the Zeiss LSM 5 Image Browser software; optical densities (ODs) of the GFAP/TrkB-positive cells in each field were measured and the mean values were used in the statistical analysis. Results of Western blot hybridization were quantitatively analyzed using the Image Pro plus 5.0 image analysis software, and the data were expressed as relative OD (GFAP/β-actin, TrkB/β-actin, or GLT-1/β-actin).

Expressions of the TrkB and GLT-1 in the Glial Cells Treated by BDNF

Concentration of the BDNF used was 100 ng/L; the modified McCarthy's method was referred for the isolation and purification of the glial cells. Cells with good shape and medium density were selected for the addition of BDNF (with a final concentration of 100 ng/L), and the cells were harvested 1, 3, 7, and 14 days after culturing. Protein lysates for eukaryotic cells were added during the sampling process (total protein concentration: 1-2 mg/mL; BioDev-Tech. Scientific & Technical Co., Ltd, Beijing, China); after a 30 min ice bath, fluids inside the flasks were collected; supernatants were collected after centrifugation; protein samples and loading buffers were mixed at a volume ratio of 1:1 before the 10 min boiling water bath; after the protein mixture cooled down, they were loaded (15 μL mixture was used) for electrophoresis; after being transferred to a membrane, the protein was blocked for 2 h by the 5% skimmed milk; incubations with the primary antibodies (TrkB 1:1000 or GLT-1 1:1000) lasted overnight, and the durations of incubations with the secondary biotinylated antibody (under 37°C) and peroxidase-labeled streptavidin were for 2 h each, and finally DAB was used for coloring. β-Actin was used as an internal control.

Statistical Analysis

Data were expressed as mean±standard deviation and analyzed by the Student's t-test using the Statistical Package for Social Sciences (SPSS) 12.0 software. $P<0.05$ indicated statistical significance.

RESULTS

Animal Model

Rats in the kindling model gradually developed symptoms such as gazing, nodding, and head and facial twitching (level I or level II) 2 weeks after the injections of PTZ (subthreshold dose, repeated every day). The seizures leveled up gradually as the injection process going on, with symptoms of myoclonus, falls, incontinence, salivation, and eventually the generalized tonic-clonic seizures. After 23 days, with the exception of the four rats that died, all the remaining 26 rats developed level IV or level V seizures and achieved the ignition status. In the present study, the fatality rate of the rats was 10%, which met the standard requirements for the study.

Expression of TrkB and GLT-1 (Western Blot)

Protein level expression of TrkB and GLT-1 of the hippocampus and temporal cortex in the model group were significantly higher than that in the control (Figures 1, 2; Table I) ($p<0.05$).

Analysis of Double-Labeling Immunofluorescence of GFAP/TrkB

All GFAP-positive cells in the model and control groups were astrocytes with typical morphology. A majority of the TrkB-positive cells in the temporal cortex and hippocampus of the rats from the control group were neuron-like cells and minor positive cells were glial-like cells, and the number of dual positive (GFAP/TrkB) cells was less (Figure 3). Both TrkB-

Table I: Western Blot Results of TrkB and GLT-1 Express in PTZ-Induced Epileptic Rats

Groups	Trk B		GLT-1	
	Cortex	Hippocampus	Cortex	Hippocampus
Model	0.66±0.02*	0.92±0.02*	0.73±0.01*	0.86±0.02*
Control	0.46±0.02	0.39±0.02	0.42±0.01	0.46±0.02

TrkB: *Tyrosine kinase receptor B;* ***GLT-1:*** *Glutamate transporter-1;* ***PTZ:*** *Pentylenetetrazol; *p<0.05 vs control.*

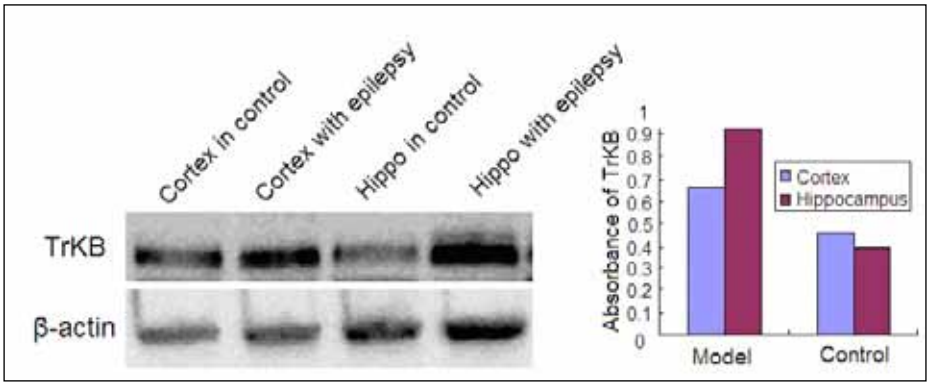

Figure 1: Western blot analysis of TrkB in hippocampus and cortex of temporal lobe in rats showed that the protein expressions of TrkB in the model group were significantly higher than the control group (Day 7).

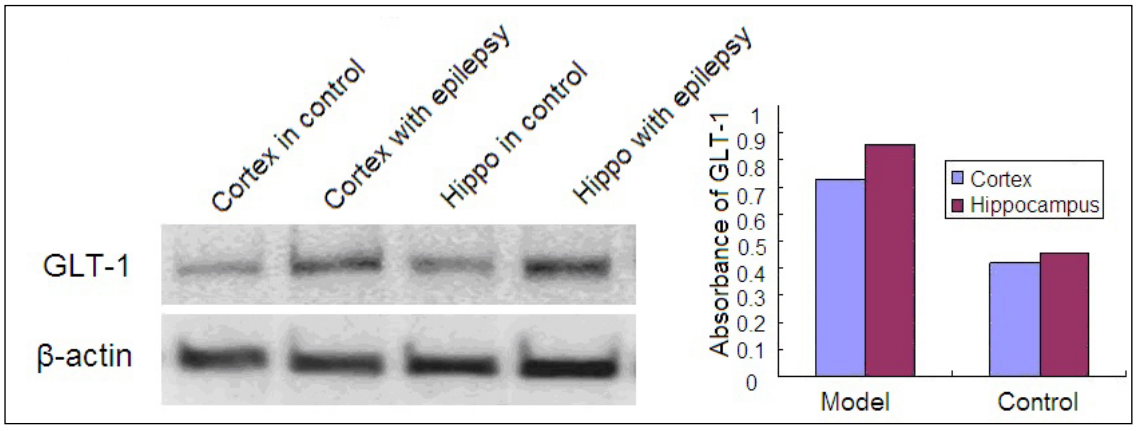

Figure 2: Western blot analysis of GLT-1 in hippocampus and cortex of temporal lobe in rats showed that the protein expressions of GLT-1 in the model group were significantly higher than the control group (Day 7).

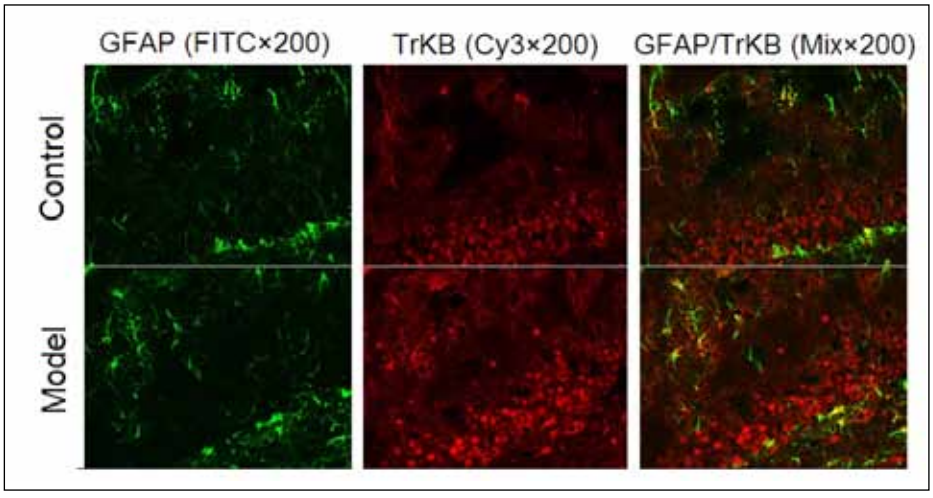

Figure 3: Immunofluorescence double staining of GFAP and TrkB in PTZ-induced epileptic rats showed that dual positive (GFAP/TrkB) cells in the temporal cortex and hippocampus of the rats from the model group were significantly increased (Day 7).

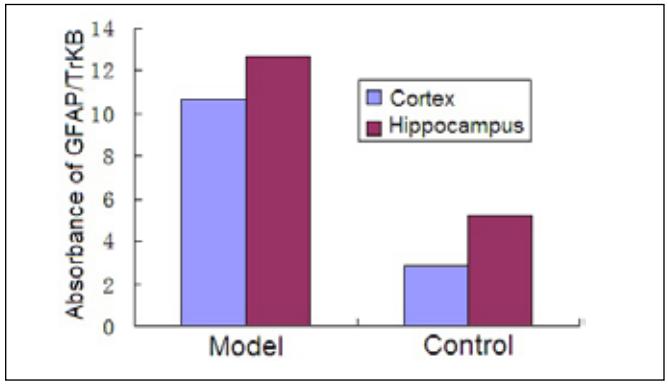

Figure 4: Absorbance of GFAP/TrkB positive cells in hippocampus and cortex of temporal lobe in model group was higher than that in the control group (Day 7).

positive cells (most were neuron-like cells) and dual positive (GFAP/TrkB) cells in the temporal cortex and hippocampus of the rats from the model group increased significantly (Figure 3), and their mean OD value was notably higher than that in the control group ($p<0.05$) (Figure 4, Table II).

Expression of the TrkB and GLT-1 in the Glial Cells Treated with BDNF (Western blot)

Expression of TrkB in cultured astrocytes increased 3 days after treatment with 100ng/L BDNF. The increase (versus the control group) was significant ($P<0.05$) at 7 days and the expression of TrkB still maintained at a high level 14 days after the treatment with BDNF (Figure 5, Table III). Expression of GLT-1 peaked at 7 days after treatment with 100 ng/L BDNF, and the elevated level maintained till 14 days after treatment (Figure 6, Table IV).

■ DISCUSSION

TrkB is a high-affinity receptor for BDNF in the CNS (2). As the main CNS neurotrophic factor, BDNF could potentiate or facilitate synaptic transmission (17,40). Evidences for the involvement of the BDNF/TrkB pathway in epilepsy are as follows: 1) Occurrence or pathogenesis of epilepsy is often associated with upregulated expression of TrkB. In epileptic models induced by electrical stimulation or kainic acid, the expression of TrkB is significantly upregulated (3,12,22,44); timescale of the temporal expression of TrkB is usually consistent with that of BDNF, and its increase is frequently more significant than BDNF (9,19). 2) Overexpression of TrkB protein promotes epileptogenesis, and inhibition or knockout

of the TrkB receptor prevents epileptogenesis. Heinrich et al. (14) reported that overexpression of the intact TrkB protein in the hippocampus of transgenic mice would promote epileptogenesis via enhancing TrkB signaling. Kotloski and McNamara (18) used tamoxifen inducible mice(Act-CreERTrkB$^{flox/flox}$) to specifically downregulate the expression of TrkB in the hippocampal region, then found it could inhibit epileptogenesis induced by the stimulation of hippocampus or amygdala. Intrahippocampal infusion of BDNF and transgenic overexpression of BDNF or TrkB increased the susceptibility or severity of seizure,while conditional knockout of TrkB eliminated epileptogenesis altogether in the kindling model (22). Wang et al. (42) revealed that cyclothiazide (CTZ) could induce epileptiform discharges in the hippocampal neurons both in vivo and in vitro, and pre-injection of a receptor tyrosine kinase inhibitor K252a or a specific antibody for TrkB receptors before intracerebroventricular injection of CTZ significantly suppressed the epileptiform activity induced by CTZ; similarly, in cultured hippocampal pyramidal neurons, pretreatment with CTZ together with K252a or TrkB receptor antibody also inhibited the induction of epileptiform activity by CTZ. 3) Neuroanatomical evidence: a large number of studies noted that when expression of BDNF/TrkB is disturbed by epilepsy, abnormal mossy fiber sprouting often appears in epileptic areas (21,25,36,37). Xie et al. (43) established a kainic acid-induced (injected via the right lateral ventricle) epileptic model in which simvastatin was administered to the rats continuously from 0.5 h after epileptogenesis to 14 h during status epilepticus, they found that mossy fiber sprouting was significantly restrained and epilepsy was alleviated.

Table II: Absorbance of GFAP/TrkB Positive Cells in PTZ-Induced Epileptic Rats

Group	Cortex	Hippocampus
Model	10.68±0.73*	12.71±1.21*
Control	2.88±0.39	5.17±0.74

GFAP: *Glial fibrillary acidic protein;* ***TrKB****: Tyrosine kinase receptor B;* ***PTZ:*** *Pentylenetetrazol;* ****:*** *P<0.01 vs. control.*

In PTZ-induced epilepsy, expression of p-Trk in the temporal cortex and hippocampus of the SD rats is significantly upregulated, indicating the activation of BDNF/TrkB pathway. Phosphorylation of TrkB is a transmembrane signal transduction process following BDNF binding (22), which would potentiate or facilitate synaptic transmission, enhance neuronal excitability, or cause the establishment of hyperexcit-

Table III: Expression of TrkB in Astrocytes at Different Time Points (Treated with 100 ng/L BDNF)

	1d	3d	7d	14d
BDNF	0.347±0.023	0.512±0.029	0.731±0.027*	0.798±0.028*
Control	0.301±0.017	0.382±0.021	0.416±0.014	0.436±0.025

: *p<0.05 vs. Control.*

Table IV: Expression (OD Value) of GLT-1 in Astrocytes at Different Time Points (Treated with 100 ng/L BDNF)

	1d	3d	7d	14d
BDNF	0.351±0.019	0.658±0.023*	0.687±0.013*	0.694±0.019*
Control	0.345±0.017	0.383±0.018	0.430±0.017	0.481±0.019

: *p<0.05 vs. Control.*

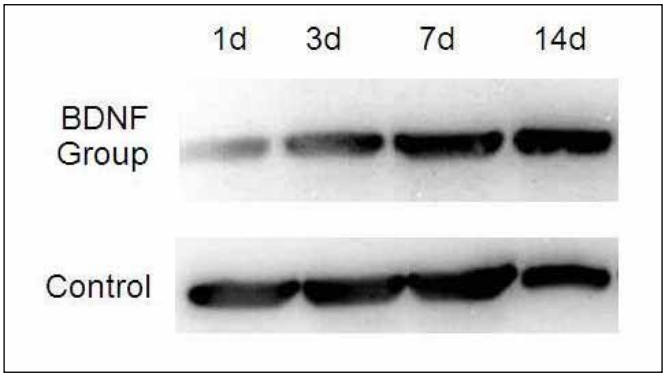

Figure 5: Expression of TrkB in astrocytes at different time points. Expression of TrkB in astrocytes treated with 100 ng/L BDNF increased (versus the control group) significantly (P<0.05) at 7 days after the treatment with BDNF and the expression of TrkB was still maintained at a high level till 14 days after the treatment with BDNF.

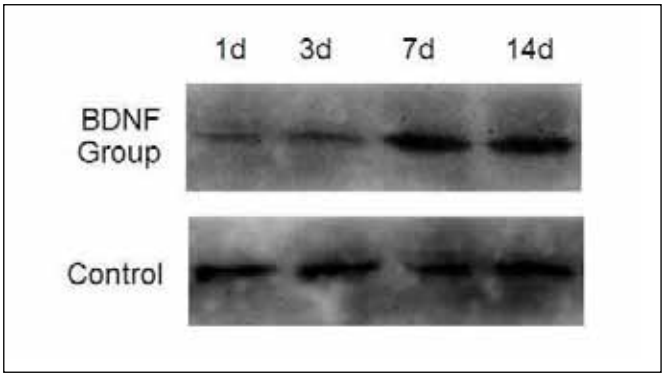

Figure 6: Expression of GLT-1 in astrocytes at different time points. Expression of GLT-1 in astrocytes treated with 100 ng/L BDNF increased (versus the control group) significantly (P<0.05) at 7 days after the treatment with BDNF and the elevated level could be maintained till 14 days.

able re-entrant circuits (13,30). The expression of p-TrkB is significantly upregulated in the epilepsy models induced by electrical stimulation and kainic acid (3,12,22,44). Koyama et al. found that BDNF-induced establishment of hyperexcitable re-entrant circuits in the dentate gyrus were dependent on the phosphorylation of TrkB (20). Deleting BDNF and TrkB off the brains of mice through the synapsin-Cre system, they reported that electric kindling-induced epileptic behaviors and electrophysiological processes in the $TrkB^{-/-}$ mice were terminated, while epileptogenesis still occurred in the $BDNF^{-/-}$ mice, indicating that the inhibition of TrkB and its downstream pathway may be an effective way for the prevention and disturbance of epileptogenesis (5,10,13). In the present study, we found that the OD value of dual positive GFAP/TrkB cells in the model group was significantly higher than that of the control, indicating that activation and hyperplasia of the astroglial cells after epileptic seizure are the main contribution for upregulated expression of TrkB. Meanwhile, glial hyperplasia and highly expressed levels of TrkB in the astrocytes may also potentiate epileptogenesis in the PTZ-induced SD rats.

As a neurotoxin, glutamic acid is closely correlated with epileptogenesis (5). Extracellular glutamate concentration in the hippocampus of the spontaneously epileptic rats was 2-3 times more than that in the normal (16). GLT-1 is mainly expressed in the glial cells (6). Under physiological state, GLT-1 plays important roles in the termination of glutamatergic neurotransmission, maintenance of extracellular glutamate concentrations of cells and prevention of excitotoxicity (29). GLT-1 is critical in epileptogenesis (6,34) and rats with mutations of GLT-1 may suffer from spontaneous epilepsy (38).

Previous studies have reported that the expression of GLT-1 demonstrated a decreasing tendency in the kindling models (24,32), while the results of the Western blot from the present study indicated that the expression of GLT-1 was significantly increased in the hippocampus and temporal cortex areas of pentylenetetrazol kindling group. It is assumed that two main reasons for the difference in the expression of GLT-1 are as follows: 1) Different kindling models. Acute epileptic kindling is the major model used in the previous studies, and significant differences exist in many aspects between acute and chronic kindling models. 2) Different selection of time points. In the previous studies, the major examined times were among within a few hours and several days after epileptic seizures, during which the rats were still under a state of epileptic seizures (8,11,20,35,42). In the present study, the time point of 7 days after epileptic seizures (which was a chronic kindling model) was chosen, a time during which it is assumed that the rats are already in a compensatory state. Therefore, the upregulation of expression of GLT-1 might be a compensatory response, in which the uptake of the epilepsy-caused elevated extracellular glutamate is enhanced and the epilepsy-induced neural network hyperexcitability and epileptogenesis inhibited.

In summary, activation of the BDNF/TrkB pathway may constitute the molecular basis for epileptogenesis, while GLT-1 is involved in modulating this process. As a BDNF receptor, TrkB exerts biological effects in promoting epileptogenesis via increased neuronal excitability in the lesioned areas after activation. GLT-1 is a glutamate transporter expressed only in the astrocytes, which bears the major assignment of uptakes of the CNS glutamate neurotransmitter and plays an important role in the maintenance of the balance of excitatory neural networks (24). Functional disorder of GLT-1 is closely correlated with epileptogenesis. Epidermal growth factor could alter the expression of GLT-1 in the astrocytes (4,9). BDNF could promote the expression of GLT-1 in astrocytes during neurodegeneration (15,28).

CONCLUSION

The results of the present study indicated that after epileptic seizures, the expression of TrkB and GLT-1 in the hippocampus and temporal cortex of the SD rats is significantly increased in the kindling model than that in the control group ($p<0.05$), which reminds us of the two important uncorrelated pathological phenomena, i.e., upregulation of BDNF/TrkB and altered expression of GLT-1. The BDNF/TrkB pathway may affect or participate in the epileptogenesis by altering the biological effects of GLT-1 in the glial cells of the SD rats under the PTZ kindling model, though the specific mechanism requires further investigations.

ACKNOWLEDGEMENT

This study was supported by Sichuan Province Science and Technology Key Project (No:05SG022-013).

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