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Original Article

Role of gap junction protein 36 in pentetrazole-induced acute epilepsy in rats

Jia Lu[1], Mingyue Yue[2], Zhen Lin[2], Su Lue[2], Hao Zhang[2], Hang Zhao[2], Yanyan Xu[2], Haiyan Liu[2], Ping Ren[3]

[1]Department of Ophthalmology, China-Japan Union Hospital of Jilin University, Changchun, People's Republic of China; [2]Department of Anatomy, College of Basic Medicine Sciences, Jilin University, Changchun, People's Republic of China; [3]Department of Thoracic Surgery, The First Hospital of Jilin University, Changchun, People's Republic of China

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Abstract: Gap junction communication is an important aspect of synchronized discharge from neurons during epileptic seizure. This study surveyed the role of connexin (Cx) 36 in this process and the effect of carbenoxolone (CBX) intervention using a rat model of pentetrazole (PTZ)-induced acute epilepsy. Rats were divided into control, PTZ, and PTZ+CBX (hereafter CBX) groups. Expression of Cx36, caspase-3, B cell lymphoma (Bcl)-2, and Bcl-2-associated X protein (Bax) at different time points (2, 4, 8, 12, and 24 h) after the occurrence of seizure was evaluated by hematoxylin and eosin staining, immunohistochemistry, and Western blotting. Apoptotic cells were detected by DAPI staining. Cx36 expression increased starting 2 h after seizure and persisted for up to 24 h. Cx36 levels in the PTZ and CBX groups were similar and higher than those in the control group. After 24 h, caspase-3, and Bax expression increased but Bcl-2 expression decreased. Caspase-3 and Bax levels were downregulated, whereas Bcl-2 levels were upregulated in the CBX group as compared to the PTZ group. The data show that Cx36 is involved in PTZ-induced epilepsy in rats. CBX inhibits seizure-induced neuronal death by modulating expression of caspase-3, Bcl-2, and Bax.

Keywords: Epilepsy, connexin (Cx) 36, pentetrazole (PTZ), carbenoxolone (CBX)

Introduction

Epilepsy is a chronic disorder characterized by abnormal, synchronized discharge from neurons of the central nervous system. Approximately 25% of patients with epilepsy do not respond to anti-epilepsy drugs, most of which control the occurrence of seizures but do not improve secondary effects such as sleep disturbance and cognitive deficits. As such, more effective treatments are needed for epilepsy management. Clarifying the molecular mechanisms underlying epilepsy could potentially yield new pharmacological targets [1]. Cell-cell communication through gap junctions underlies the synchronized discharge of neurons during epileptic seizure [2]. The gap junction protein connexin (Cx) 36 is widely expressed in the brain [3]; however, its role in seizures is unclear. We addressed this aspect in the present study using a rat model of pentetrazole (PTZ)-induced acute epilepsy by examining expression of Cx36 as well as that of the apoptosis-associated factors caspase-3, B cell lymphoma (Bcl)-2, and Bcl-2-associated X protein (Bax) after the onset of seizures.

Materials and methods

Materials

Healthy adult male Wistar rats (n = 101; weight: 200 ± 20 g) were used for experiments. Rat polyclonal antibodies against the following proteins were used for analyses: rat Cx36 (Boster Biological Technology, Pleasanton, CA, USA) and rat caspase-3, Bcl-2, and Bax (Sigma-Aldrich, St. Louis, MO, USA). Carbenoxolone (CBX) was purchased from Yongning Pharma (Taizhou, China).

Rat model of PTZ-induced epilepsy

Rats were randomly divided into control (n = 11), PTZ (n = 45), and PTZ+CBX (hereafter CBX, n = 45) groups. Control animals were given an intraperitoneal (i.p.) injection of 0.5 ml of saline. Epilepsy was induced in the remaining animals by i.p. injection of PTZ (50 mg/kg).

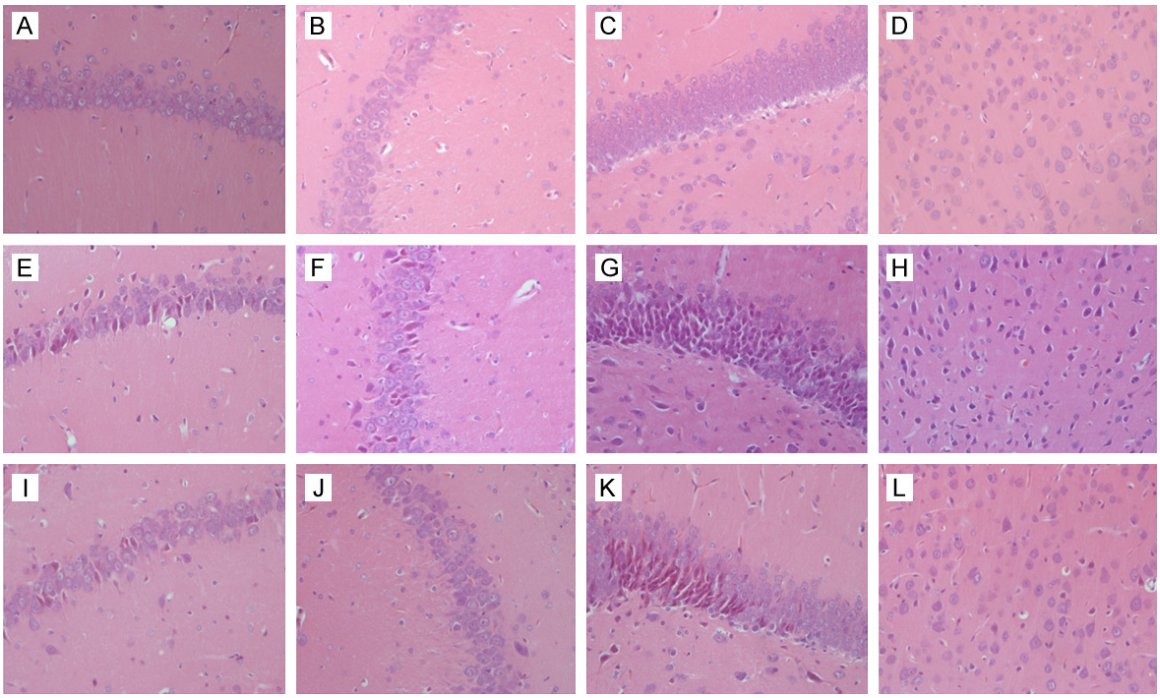

Figure 1. A-D. Hematoxylin and eosin staining of the dentate gyrus, parietal lobe, and CA1-CA3 regions of the hippocampus in the control group. E-H. Hematoxylin and eosin staining of the dentate gyrus, parietal lobe, and CA1-CA3 regions of the hippocampus in the PTZ group 24 h after the induction of seizures. I-L. Hematoxylin and eosin staining of the dentate gyrus, parietal lobe, and CA1-CA3 regions of the hippocampus in the CBX group 24 h after the induction of seizures. The magnifications are all 400×.

Animals in the CBX group received i.p. injection of CBX (300 mg/kg) 30 min prior to PTZ administration. Epileptic seizures were scored according to the Racine scale [4]. The model was considered as successful when a score of V was attained. One rat per group was selected for electroencephalogram (EEG) recordings and the others were sacrificed at 2, 4, 8, 12, and 24 h after PTZ treatment for immunohistochemical and Western blot analyses.

Histology and immunohistochemistry

Rats were perfusion-fixed at the indicated times and the hippocampus was removed. After paraffin embedding and sectioning at a thickness of 6 μm, 20 sections per specimen were used for hematoxylin and eosin and DAPI staining. Sections were treated with hydrogen peroxide, blocked, and incubated overnight at 4°C with primary antibodies. Immunoreactivity was visualized using diaminobenzidine.

Western blotting

Rats were anesthetized and perfused at the predetermined time points; brain tissue was removed and placed on ice and the hippocampus was dissected. Proteins were extracted from the tissue using conventional methods and separated by electrophoresis, transferred to a nitrocellulose membrane that was blocked with 5% skim milk powder, and incubated overnight at 4°C with primary antibodies against Cx36 (1:200), caspase-3 (1:1000), Bcl-2 (1:1000), and Bax (1:1000). After incubation for 2 h at room temperature with the appropriate secondary antibodies, immunoreactivity was visualized by enhanced chemiluminescence.

Statistical analysis

Data were analyzed using SPSS v.16.0 software. Results are expressed as the mean ± standard deviation. Group means were compared with the t test, and differences with $P < 0.05$ were considered significant. Protein band intensity in Western blots was analyzed with the Tanon GIS image analysis system (Shanghai, China) and processed using Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

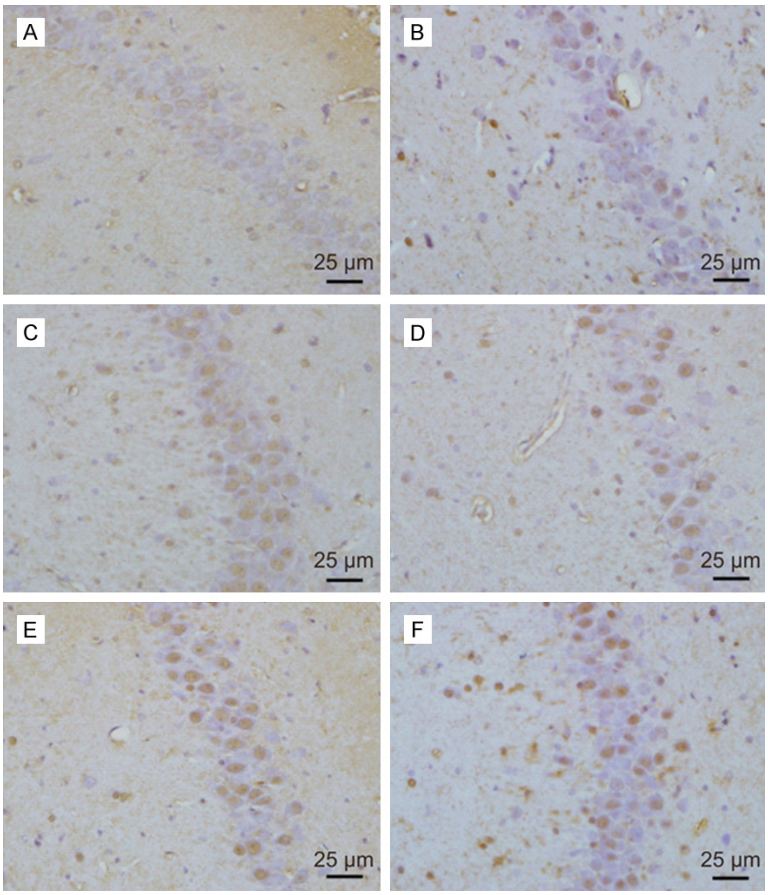

Figure 2. Immunohistochemical staining of Cx36 protein expression in the hippocampal CA3 region of the PTZ group at different time points after epilepsy was detected. A. NC group. B-F. At 2, 4, 8, 12, and 24 h after the induction of seizures. Scale bar = 25 μm. The magnifications are all 400×.

Results

Histological analysis

Hematoxylin and eosin staining was performed at 24 h after seizure and revealed an ordered arrangement and intact morphology of pyramidal cells and dendritic granulosa cells in the hippocampal CA1-CA3 region in the control group (**Figure 1**). In contrast, neurons in these regions in the PTZ group were in disarray and showed morphological abnormalities, including a perturbed structure. Additionally, cytoplasmic vacuoles were observed in some neurons, whereas others appeared swollen. The spacing between neurons was increased, and the cytoplasm was darkly stained, with evidence of nuclear condensation. In the CBX group, most pyramidal neurons showed normal morphology, although some exhibited chromatin condensation and irregular contours. Survival of CA3 neurons was significantly increased relative to those in the PTZ group ($P < 0.05$).

Immunohistochemical detection of Cx36 expression

Immunohistochemical analysis of Cx36 expression was performed on brain tissue at 2, 4, 8, 12, and 24 h after induction of seizures (**Figures 2**, **3** and **Table 1**). In the control group, a small number of Cx36-positive cells was present in the cerebral cortex and hippocampus. In the PTZ group, more Cx36-positive cells were observed in the cortex and hippocampus relative to control animals ($P < 0.05$) starting from 2 h after PTZ-induced seizure, with the number increasing up to 24 h. Similar patterns for Cx36 immunoreactivity were observed in the CBX group, which also had more Cx36-positive neurons than control animals ($P < 0.05$). There was no difference in Cx36 expression between the PTZ and CBX groups in the hippocampal CA1 and CA3 regions.

Detection of apoptotic cells by DAPI staining

In the hippocampus of control rats, nuclei showed uniform chromatin distribution (**Figure 4**), whereas increased chromatin condensation along with granules and nuclear fragmentation was observed in the PTZ group, and this effect was attenuated in the CBX group.

Western blot analysis of the time course of Cx36 expression and apoptosis-related protein levels

Cx36 protein was expressed in all animals. However, although expression in the control group was stable, levels in the PTZ and CBX groups began to increase 2 h after the onset of seizures, continuing through 8 and 12 h, and reaching a peak at 24 h ($P < 0.05$ at each time point) (**Figure 5**). The Bcl-2 expression level was decreased in the hippocampus 24 h after seizure induction in both the PTZ and CBX groups

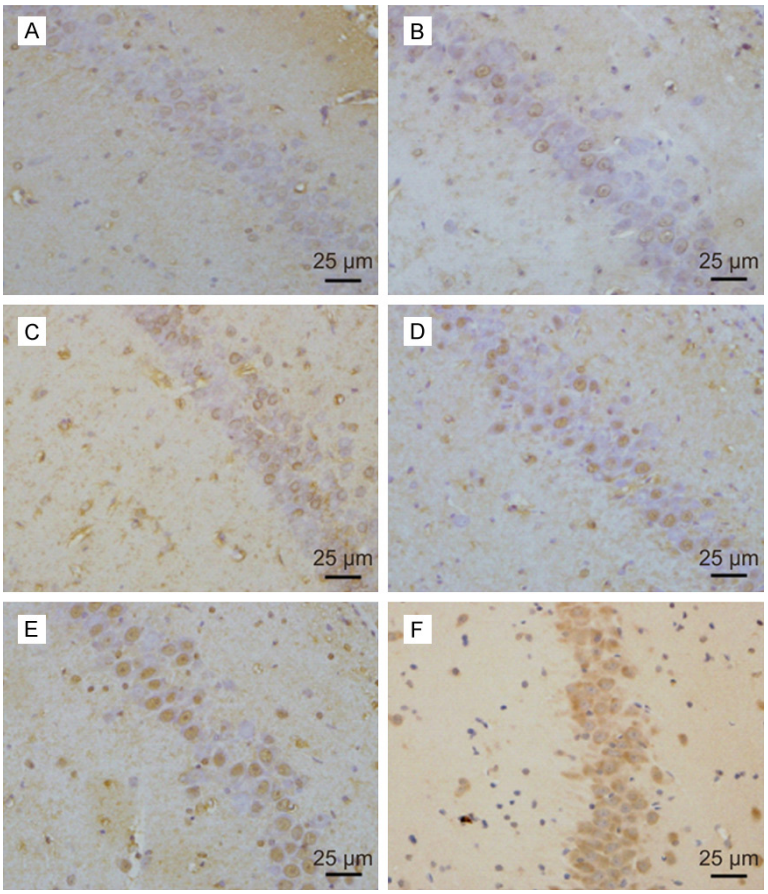

Figure 3. Immunohistochemical staining of Cx36 protein expression in the hippocampal CA3 region of the CBX group at different time points after epilepsy was detected. A. NC group. B-F. at 2, 4, 8, 12, and 24 h after the induction of seizures. Scale bar = 25 μm. The magnifications are all 400×.

Table 1. OD values of Cx36 immunoreactive cells in the hippocampal CA3 region ($\bar{x}$ ± SD)

Time	NS	PTZ	CBX
2 h	20.47 ± 1.64	35.46 ± 2.27*	33.46 ± 1.97[&]
4 h	21.24 ± 1.78	40.14 ± 2.37*	42.14 ± 2.07[&]
8 h	22.16 ± 1.98	56.15 ± 2.02*	54.17 ± 2.02[&]
12 h	23.04 ± 2.21	66.47 ± 2.58*	67.47 ± 1.88[&]
24 h	22.97 ± 2.11	74.23 ± 2.77*	70.29 ± 1.97[&]

*P < 0.05 vs NC group; [&]P < 0.05 vs NC group; There was no statistically significant difference between the PTZ and CBX groups.

relative to control animals (P < 0.05), with a higher level observed in the CBX than in the PTZ group. Compared to control animals, Bax and caspase-3 protein levels were upregulated in the PTZ and CBX groups, although levels were lower in the latter group (P < 0.05; **Figure 6**).

Discussion

In this study, we used a rat model of PTZ-induced epilepsy because of its pathological similarity to human seizures. We confirmed successful establishment of the model in 86% of animals by EEG recordings of rats with a score of V on the Racine scale.

Cx36 has been linked to the pathogenesis of epilepsy [5]. Our immunohistochemical and Western blot analyses showed that Cx36 is expressed at low levels in the CA1 and CA3 regions of the hippocampus of control animals, but is upregulated in PTZ-treated rats 2 h after seizure. This effect persisted for 24 h, suggesting that Cx36 modulates seizures. CBX can bind directly to and block gap junctions without affecting the number and expression of connexins at synapses [6]. CBX has been reported to inhibit the function of Cx32 and Cx43 [7] as well as Cx36 [8]. We found here that Cx36 expression in the CBX group occurred primarily in the CA1 and CA3 regions of the hippocampus and showed similar trends to the PTZ group. These results indicate that CBX affects the activity but not the expression or abundance of gap junctions and connexins.

Apoptosis is mediated by death receptors (extrinsic pathway) or mitochondria (intrinsic pathway), both of which require activation of caspases 8, 1, and 3 [9]. Bcl-2 and Bax are components of the mitochondrial apoptotic pathway. Bcl-2 is localized in the mitochondrial outer membrane and inhibits the release of cytochrome C. Bax is located in the cytoplasm and is translocated to mitochondria upon apoptosis signaling where it promotes the release of cytochrome C, which in turn induces cleavage and activation of caspase-3, resulting in apoptosis [10]. During brain development, caspase-3 promotes neuronal apoptosis [11] and

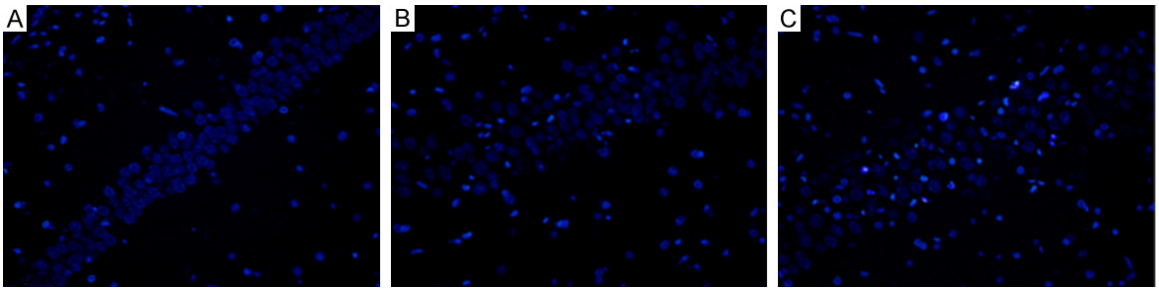

Figure 4. DAPI staining to detect cell apoptosis in the CA3 regions of the hippocampus after the induction of seizures A. NC group. B. CBX group. C. PTZ group. The magnifications are all 400×.

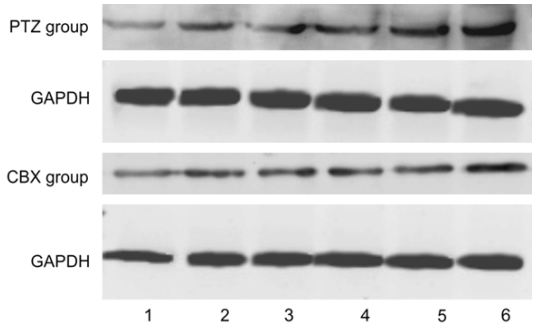

Figure 5. Western blot to detect Cx36 expression in the hippocampus of epileptic rats. 1-6: NC group and at 2, 4, 8, 12, and 24 h after the induction of seizures.

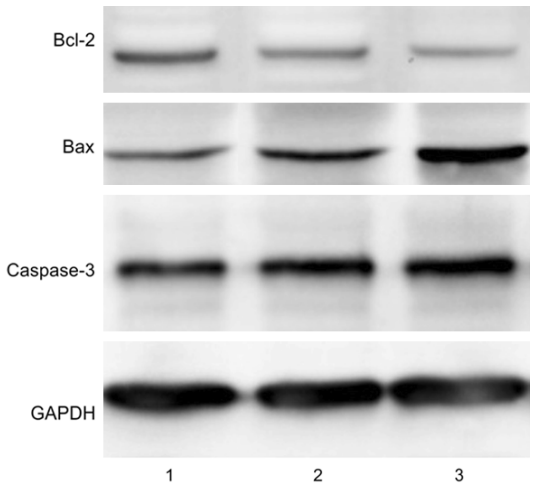

Figure 6. Western blot to detect caspase-3 expression in the hippocampus of epileptic rats.

as such, its expression level can serve a marker of the extent of apoptosis.

In this study, we found that expression of caspase-3, Bcl-2, and Bax was higher in PTZ-treated animals with or without CBX treatment relative to the control group 24 h after the occurrence of seizure. DAPI staining of brain tissue revealed nuclear shrinkage (pyknosis) and fragmentation (karyorrhexis) in hippocampal neurons. These results demonstrate that epilepsy promotes neuronal apoptosis. We also found that Bcl-2 levels were higher in the CBX group than in the PTZ group, whereas the opposite was true for Bax and caspase-3 expression, implying that pro-apoptotic activity of the latter two factors was suppressed, whereas anti-apoptotic effect of Bcl-2 was increased in the presence of CBX. This resulted in suppression of the mitochondrial apoptotic pathway and its downstream effector caspase-3, thereby protecting neurons against epilepsy-induced apoptosis. This was confirmed by quantitative analysis of DAPI-stained apoptotic cells and by Western blotting.

In conclusion, our results provide evidence that Cx36 is involved in the pathogenesis of epilepsy and may modulate apoptosis of epileptic neurons via regulation of Bcl-2, Bax, and caspase-3. The gap junction blocker CBX was shown to inhibit neuronal apoptosis induced by epilepsy without altering Cx36 levels, suggesting that it could be an effective therapeutic intervention for epileptic seizures.

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Disclosure of conflict of interest

None

Address correspondence to: Haiyan Liu, Department of Anatomy, College of Basic Medical Sciences, Jilin

University, 126 Xinmin Street, Changchun 130021, Jilin Province, People's Republic of China. Tel: +86-13944850567; Fax: +8643185619185; E-mail: haiyan@jlu.edu.cn; Ping Ren, Department of Thoracic Surgery, The First Hospital of Jilin University, 71 Xinmin Street, Changchun 130021, Jilin Province, People's Republic of China. E-mail: rpemail@163.com

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