NEUROPEPTIDE Y ATTENUATES NEURONAL APOPTOSIS AFTER EPILEPTIFORM DISCHARGE IN HIPPOCAMPAL NEURONS BY REGULATING AMPA RECEPTOR

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ABSTRACT

Objective*: To explore the effect of neuropeptide Y (NPY) on neuronal apoptosis after epileptiform discharge of hippocampal neurons by regulating amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor and its mechanism.*

Methods: *In this study, 34 newborn Sprague Dawley (SD) rats within 24 h of clean grade were selected. Their heads were severed, their brains were collected, and each bilateral hippocampus was isolated to obtain the purified neurons. After 12 d, the neurons were treated with magnesium-free extracellular fluid prepared in advance and were removed after 3h. Hippocampal neurons were randomly divided into a control group, a model group, and an NPY group. The hippocampal neurons in the control group were treated with normal extracellular fluid for 3 h, and those in the model group were treated with magnesium-free extracellular fluid for 3 h. Next, those in the NPY group were treated with 1 μmol/L of NPY for 30min and then cultured with magnesium-free extracellular fluid for 3 h. After the hippocampal neurons in each group were returned to a normal extracellular fluid culture for 12 h, further studies were conducted. Finally, the current densities, apoptotic rates, and protein and mRNA expression levels of glutamic acid receptor 2 (GluR2) subunit and caspase-3 of the hippocampal neurons in each group were compared.*

Results: *The current densities of hippocampal neurons in the model group and the NYP group were significantly lower than that of the control group (P<0.05). The current density of hippocampal neurons in the NYP group was remarkably higher than that of the model group (P<0.05). The GluR2 subunit mRNA expression levels of hippocampal neurons in the model group and the NPY group were dramatically lower than that of the control group (P<0.05). While the GluR2 subunit mRNA expression level of hippocampal neurons in the NPY group was higher than that of the control group (P<0.05). There was no statistically significant difference in GluR2 subunit protein expression levels among the hippocampal neurons in each group (P>0.05). The apoptosis rates of hippocampal neurons in the model group and the NPY group were markedly higher than that of the control group. The apoptosis rate of hippocampal neurons in the NPY group was significantly lower than that of the control group. The expression levels of caspase-3 protein and mRNA in the hippocampal neurons of model group and NPY group were remarkably higher than that of the control group (P<0.05). The expression levels of caspase-3 protein and mRNA in the hippocampal neurons of NPY group were significantly lower than that of the control group (P<0.05).*

Conclusion: *NPY can inhibit the apoptosis of hippocampal neurons after epileptiform discharge, and its mechanism may be realized by regulating the expression level of the GluR2 subunit of the AMAP receptor.*

Keywords: *Neuropeptide Y (NPY), AMPA, epilepsy, GluR2.*

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Introduction

Epilepsy is a disease that can lead to transient central nervous dysfunction. At present, the incidence of epilepsy in China is about 4%, with about 300,000 new cases every year. Most of the patients have good prognoses, but still more than 20% of the patients fail to improve after long-term drug treatment(1). Epilepsy is characterized by chronic, acquired character and repeated abnormal discharges, which are the problematic focus of domestic and foreign scholars at present. Previous reports have shown that a large number of cytokines can be secreted during epileptic episodes. These cytokines can act on nerve cells and accelerate the production of excitatory or inhibitory neurotransmitters. The generated

neurotransmitters can bind to the receptors to change the activity of ion channels in the cell membrane, thereby causing changes of ion current, affecting cell excitability and causing the onset of epilepsy[2, 3]. Neuropeptide Y (NPY) can be involved in the secretion of inflammatory factors and anti-oxidative stress. In addition, it plays an anti-apoptotic role in many cell tissues, but its specific mechanism has not been elucidated[4]. An amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor is an ionic channel on the synaptic membrane, which can be involved in rapid excitatory synaptic transmission in the nervous system.

Previous reports have shown that changes in the number and function of AMPA receptors on the synaptic membrane can significantly affect the synaptic transmission efficiency.

In addition, changes in synaptic transmission efficiency can serve as the physiological and pathological bases for neurological diseases such as epilepsy[5]. However, there are few reports on the relationship between AMPA receptors and epilepsy. Therefore, this study aims to explore the effect of NPY on the apoptosis of hippocampal neurons after epileptiform discharge by regulating the AMPA receptor and further exploring its mechanism.

Materials and methods

Experimental materials

34 newborn Sprague Dawley (SD) rats within 24 h of clean grade were purchased from Beijing Jiamei Zhenyuan Biotechnology Co., Ltd., with production license number of SCXK (Jing) 2018-2-15. Next, experiments on 34 SD rats were all conducted in accordance with the "Guidelines on Treating Experimental Animals Well" issued by the Ministry of Science and Technology.

Main reagents and instruments

Reagents

The trypsin was purchased from Hefei Bomei Biotechnology Co., Ltd. Neuropeptide Y was purchased from Shenzhen Haodi Huatuo Biotechnology Co., Ltd. The RT-PCR reverse transcription kit was purchased from Guangzhou Dongsheng Biotechnology Co., Ltd. The PCR amplification kit was purchased from Huake Jianlian Gene Technology (Beijing) Co., Ltd. The random primers were purchased from Beijing Kangrun Chengye Biotechnology Co., Ltd. The goat anti-actin polyclonal antibody was purchased from Abbkine (Wuhan) Biotechnology Co., Ltd. The TUNE kit was purchased from Beijing Jiamei Niunuo Biotechnology Co., Ltd. Rabbit anti-Capase-3 polyclonal antibody was purchased from Wuhan Huamei Bioengineering Co., Ltd. Rabbit anti-GluR2 antibody was purchased from Beijing Mairibo Biotechnology Co., Ltd.

Instruments

A super clean workbench was purchased from Jinan Bohang Scientific Instrument Co., Ltd. The desktop high-speed centrifuge was purchased from Wuxi Lifes Biological Laboratory Equipment Co., Ltd. An electrophoretic apparatus was purchased from Hangzhou Bigfish Biotechnology Co., Ltd. The real-time quantitative PCR instrument was purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. The microplate reader was purchased from Shenzhen HiSean Biotechnology Co., Ltd. A constant temperature incubator was purchased from Beijing Qinye Yongwei Technology Co., Ltd. Finally, an optical microscope was purchased from Guangzhou Dahui Biotechnology Co., Ltd.

Methods

The heads of the newborn rats were severed, their brains were collected, each bilateral hippocampus was isolated, and their meninges were removed and cut into pieces. Purified neurons were obtained after routine digestion with 0.25% trypsin and other steps. After 12 days of continuous culture, the neurons were treated with a magnesium-free extracellular fluid prepared in advance and were removed after 3 h. Hippocampal neurons were randomly divided into a control group, a model group, and an NPY group. The hippocampal neurons in the control group were treated with normal extracellular fluid for 3h, those in the model group were treated with magnesium-free extracellular fluid for 3h, and those in the NPY group were treated with 1 μmol/L of NPY for 30min. Next, they were cultured with a magnesium-free extracellular fluid for 3 h. After the hippocampal neurons in each group were returned to the normal extracellular fluid culture for 12 h, further studies were conducted.

A standard whole patch clamp technique was used to detect AMPA receptor-induced current density of hippocampal neurons in each group.

Western blot and real-time fluorescent quantitative PCR assay were used to detect the protein and mRNA expression levels of GluR2 subunits of AMAP receptors after the epileptiform discharge

of hippocampal neurons. Apoptosis of hippocampal neurons, caspase-3 protein, and mRNA expression were detected by TUNEL assay, western blot, and real-time fluorescence quantitative PCR, respectively.

Statistical methods

All of the statistical data were analyzed using a SPSS22.0 software package. Measurement data such as the current density, GluR2 subunit protein, and mRNA expression level of each group were expressed as ($\bar{x}\pm s$). The T test was used for comparison between two groups, and the one-way analysis of variance (ANOVA) was used for multi-group comparison. Finally, $P<0.05$ represented the difference when it was statistically significant.

Results

Comparison of current density induced by AMPA receptor in hippocampal neurons of each group

The current densities of hippocampal neurons in the model group and the NYP group were significantly lower than that of the control group ($P<0.05$). The current density of hippocampal neurons in the NYP group was remarkably higher than that that of the model group ($P<0.05$). The results are shown in Table 1 and Figure 1.

Group	Current density (pA/pF)
Control group	1.16±0.05
Model group	0.75±0.02[a]
NPY group	0.96±0.02[ab]

Table 1: Comparison of AMPA induced current density of primary cultured neurons in each group ($\bar{x}\pm s$).
Notes: Compared with the control group, [a]P<0.05; compared with the model group, [b]P<0.05.

Comparison of protein and mRNA expression levels of GluR2 subunit of AMPA receptor after epileptiform discharges of hippocampal neurons of each group

The GluR2 subunit mRNA expression levels of hippocampal neurons in the model group and the NPY group were dramatically lower than that of the control group ($P<0.05$). While the GluR2 subunit mRNA expression levels of hippocampal neurons in the NPY group were higher than that of the control group ($P<0.05$). There was no statistically significant difference in GluR2 subunit protein expression levels among the hippocampal neurons in each group ($P>0.05$). The results are shown in Table 2 and Figure 1.

Group	GluR2 protein	GluR2 mRNA
Control group	0.73±0.206	1.13±0.22
Model group	0.72±0.36	0.29±0.27[a]
NPY group	0.75±0.29	0.82±0.09[ab]

Table 2: Comparison of GluR2 subunit protein and mRNA expression levels in each group ($\bar{x}\pm s$).
Notes: Compared with the control group, [a]P<0.05; compared with the model group, [b]P<0.05.

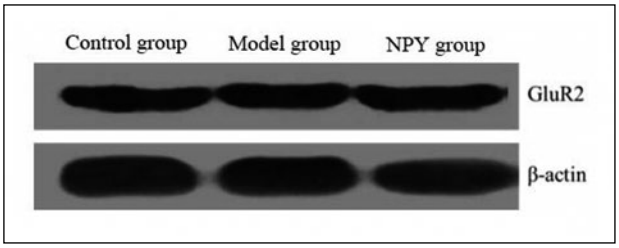

Figure 1: Comparison of GluR2 subunit protein and mRNA expression levels in each group.

Comparison of apoptosis of hippocampal neurons in each group

The apoptosis rates of hippocampal neurons in the model group and NPY group were markedly higher than that of the control group.

The apoptosis rate of hippocampal neurons in the NPY group was significantly lower than that of the control group. The results are shown in Table 3 and Figure 2.

Group	Apoptosis rate (%)
Control group	8.66±1.33
Model group	38.97±3.30[a]
NPY group	22.00±4.80[ab]

Table 3: Comparison of apoptosis of hippocampal neurons in each ($\bar{x}\pm s$).
Notes: Compared with the control group, [a]P<0.05; compared with the model group, [b]P<0.05.

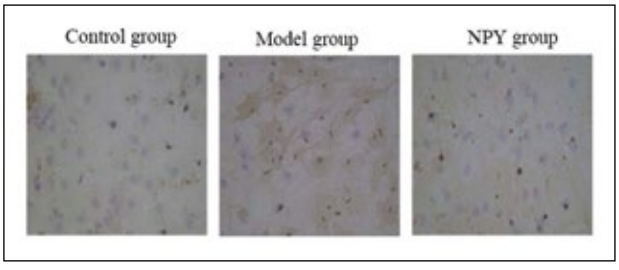

Figure 2: Comparison of apoptosis of hippocampal neurons in each group.

Comparison of the expression levels of caspase-3 protein and mRNA in the hippocampal neurons of each group

The expression levels of caspase-3 protein and mRNA in the hippocampal neurons of model group

and NPY group were remarkably higher than that of the control group (P<0.05). The expression levels of caspase-3 protein and mRNA in the hippocampal neurons of the NPY group were significantly lower than that of the control group (P<0.05). The results are shown in Table 4 and Figure 3.

Group	Caspase-3 protein	Caspase-3 mRNA
Control group	0.32±0.22	1.12±0.23
Model group	0.89±0.28[a]	1.87±0.30[a]
NPY group	0.51±0.18[ab]	1.50±0.19[ab]

Table 4: Comparison of the expression levels of caspase-3 protein and mRNA in hippocampal neurons of each group ($\bar{x}\pm s$).
Notes: Compared with the control group, [a]P<0.05; compared with the model group, [b]P<0.05.

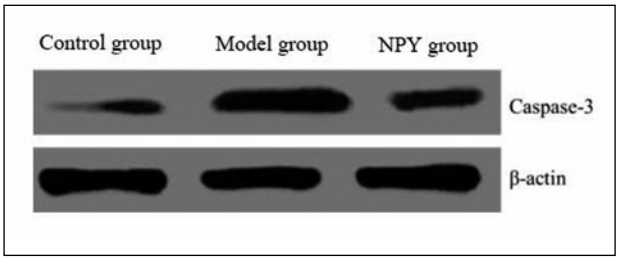

Figure 3: Comparison of the expression levels of caspase-3 protein and mRNA in hippocampal neurons of each group.

Discussion

Epilepsy is a common disease of the nervous system, and medically refractory epilepsy is a relatively difficult type to clinically treat. This variation accounts for about 25% of the total cases of epilepsy and about 70% of the patients are temporal lobe epilepsy[6]. Therefore, the research on the pathogenesis and treatment of this type of epilepsy was the focus of this study.

As glutamate receptors, AMAP receptors are mainly assembled in pairs of GluR1, GluR2, GluR3, and GluR4. These are widely distributed in the nerve center, and their concentration in the hippocampus is second only to that in the cerebral cortex[7]. Among them, the relative content of GluR2 in the receptor plays a decisive role in its functional characteristics. A decrease in the GluR2 protein expression level can act as a molecular switch. Inhibition or reduction of the GluR2 protein expression level can induce the production AMAP receptor with calcium ion permeability, improve the influx of calcium ions, and increase the excitatory toxicity of endogenous glutamic acid[8]. Clinical reports have suggested that GluR2 can be highly expressed in many neurons. Under normal conditions, there would be a large amount of GluR2 at the synapse. However, hypoxia and other injuries can lead to a significant decrease in the GluR2 content of the surface of neural membranes. This can lead to a significant decrease in the mechanism of rapid Ca^{2+} influx, an increase in positive charge in the membrane, and a large amount of Ca^{2+} influx leading to calcium overload in neurons, which ultimately activates enzymes closely related to cytotoxicity[9-10].

NPY is extracted from pig brains, and its concentration in the hippocampus is the highest in the central nervous system, which is closely related to epilepsy, learning, and other traits[11]. A number of studies have shown that NPY plays an important role in the treatment of epilepsy[12, 13]. Temporal lobe epilepsy is a common type of epilepsy, one that displays strong drug resistance. The model of temporal lobe epileptic rats was established, and they were treated with NPY antibody, and it was found that the antibody could trigger epilepsy and improve the onset of chronic spontaneous epilepsy[14]. Other studies have shown that the protective effect of NPY on the central nervous system is achieved by binding to the NPY receptor on the synapse, reducing the release of glutamic acid and improving excitatory injury of neurons[15].

The results of this study showed that the current densities of hippocampal neurons in the model group and the NYP group were significantly lower than those in the control group (P<0.05), and the current density of hippocampal neurons in the NYP group was remarkably higher than that of the model group (P<0.05), suggesting that the NPY could block the epileptiform discharge of hippocampal neurons, which confirmed that NYP could inhibit epileptic seizures. The GluR2 subunit mRNA expression levels of hippocampal neurons in the model group and the NPY group were dramatically lower than those of the control group (P<0.05). While the GluR2 subunit mRNA expression level of hippocampal neurons in the NPY group was higher than that of the control group (P<0.05).

Therefore, we believe that the decrease of AMAP containing the GluR2 subunit on the neuronal membrane surface may be due to the absence of protein content reduction on the neuronal membrane surface subunits. In addition, the change of AMAP function may lead to the increase of Ca^{2+} influx and eventually lead to a series of neuronal death or apoptosis changes. The apoptosis rates of

hippocampal neurons in the model group and the NPY group were markedly higher than that of the control group. On the other hand, the apoptosis rate of hippocampal neurons in the NPY group was significantly lower than that of the control group. These results suggested that NPY could significantly inhibit the apoptosis of hippocampal neurons and play a protective role. The expression levels of caspase-3 protein and mRNA in the hippocampal neurons of the model group and the NPY group were remarkably higher than that of the control group ($P<0.05$). The expression levels of caspase-3 protein and mRNA in the hippocampal neurons of the NPY group were significantly lower than that of the control group ($P<0.05$). These results suggested that NPY may ultimately exert its neuroprotective role through AMAP receptor function.

To conclude, NPY can inhibit the apoptosis of hippocampal neurons after epileptiform discharge, and its mechanism may be realized by regulating the expression level of the GluR2 subunit of the AMAP receptor.

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