Correlation between Nurr1 expression and drug resistance in the brain of rats with epilepsy

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Abstract. – OBJECTIVE: To investigate the correlation between nuclear receptor related 1 (Nurr1) expression and drug resistance in the brain of rats with epilepsy.

MATERIALS AND METHODS: A total of 60 adult male Sprague-Dawley rats were selected, and the animal model of epilepsy was established by electrical stimulation. These rats were randomly divided into the control group and the drug-resistant group. The model of drug-resistant epilepsy was screened with phenytoin (PHT) and phenobarbital (PB); the hippocampus and temporal lobe cortex tissues were isolated from rats; the messenger ribonucleic acid (mRNA) and protein levels of Nurr1 in the hippocampus and cortex tissues of the two groups of rats were detected by reverse transcription polymerase chain reaction (RT-PCR), Western blotting and immunohistochemistry, respectively. The budding tissue marker growth-associated protein-43 (GAP43) in hippocampus tissues were labeled by immunofluorescence staining; the mRNA and protein levels of indicators related to drug-resistant epilepsy, including leukemia-associated phenotype (LAP), multi-drug resistance protein (MRP), P-glycoprotein (P-gp) and monocyte chemoattractant protein-1 (MCP-1), were further detected. Pearson correlation analysis was used to analyze the correlations of the protein level of Nurr1 with drug-resistant indicators.

RESULTS: The mRNA and protein levels of Nurr1 in hippocampus tissues of the drug-resistant group were significantly increased compared with those of the control group (p < 0.05). In temporal lobe cortex tissues, there was no significant difference in the mRNA level of Nurr1 between the control group and the drug-resistant group (p > 0.05). The immunohistochemistry results showed that the fluorescence intensity of GAP3 in hippocampus tissues of the drug-resistant group was significantly higher than that of the control group. The mRNA and protein levels of epilepsy-related indicators, LAP, MRP, P-gp and MCP-1 in hippocampus tissues of drug-resistant group were significantly up-regulated compared with those of the control group (p < 0.05). Pearson correlation analysis indicated that the protein level of Nurr1 in hippocampus tissues was positively correlated with those of LAP, MRP, P-gp and MCP-1 in the corresponding regions (p < 0.05).

CONCLUSIONS: Nurr1 enhances the drug resistance of epilepsy in rats by up-regulating the expression of proteins related to drug resistance.

Key Words: Epilepsy, Drug resistance, Nurr1.

Introduction

Epilepsy is a common and devastating neurological disorder mainly characterized by recurrent epileptic seizures induced by no factors. By 2015, there were approximately 50 million patients worldwide^{1,2}. Currently, epileptic seizures have not been adequately controlled by antiepileptic drugs, leading to poor curative effects in some patients³. Temporal lobe epilepsy is one of the most common forms of epilepsy, and the treatment effectiveness of at least 30% of patients with epilepsy is limited by adverse reactions or multidrug resistance. The resistance to antiepileptic drugs (AEDs) is one of the most serious clinical problems, leading to shortened life expectancy, excessive body injury, mental disorders and increased social burden^{4,5}. Enhancing awareness of the underlying mechanisms of drug resistance is a prerequisite for improving drug treatment by preventing or reversing drug resistance.

Nuclear receptor related 1 protein (Nurr1) is an orphan nuclear receptor, and its ligand has not been found so far. It activates deoxyribonucleic acid (DNA) transcription by binding to the corresponding DNA sequence or retinal X receptor into a heterodimer in the form of a monomer⁶. A study⁷ has shown that Nurrl is closely related to the growth, development and survival of midbrain dopaminergic neurons. A recent report⁸ has revealed that Nurrl may be involved in the pathophysiological process of drug-resistant epilepsy.

Therefore, this investigation aimed to provide a new perspective for the difficult treatment of multi-drug-resistant epilepsy by exploring the physiological mechanism of Nurr1 participating in drug-resistant epilepsy.

Materials and Methods

Experimental Animals

A total of 60 adult male Sprague-Dawley rats (8 weeks old, 20-22 g) were purchased from the Experimental Animal Center of Yantai Yuhuangding Hospital. These rats were housed in a 12-hour light/dark cycle with normal access to food and water. They were bred for 1 week to adapt to the environment, and then the experiment was conducted. All protocols were approved by the Animal Experimental Ethics Committee of Yantai Yuhuangding Hospital, and the experiment was conducted in accordance with international standards.

Establishment of Animal Model of Epilepsy

The electrodes were stereo-tactically implanted into the right anterior basolateral amygdala (BLA) under anesthesia and served for electrical stimulation and electroencephalography (EEG) recording. About 2 weeks after electrode implantation, 60 rats were electrically stimulated by BLA electrodes to induce their self-maintenance. Stimulation was performed using an Accupulser A310C stimulator connected to the stimulation isolator A365 (World Precision Instruments, Berlin, Germany). Stimulus parameters: a 100 ms electrode and millisecond positive and negative square wave pulses. Pulse frequency within the electrode was 50/s, pulse peak intensity was 700 μ A, and stimulation endured 25 min. At 4 h after stimulation, diazepam (10 mg/kg i.p.) was used to stop it. If necessary, diazepam at the same dose should be repeatedly used. 4 weeks later, rats began to be monitored in EEG/video

recording for about 2 weeks until self-sustained epileptic seizures occurred in rats; then, further experiments were performed.

Drug Screening of Drug-resistant Epilepsy Model

Forty epilepsy model rats were randomly divided into the normal saline group and the drug-resistant group. Rats in the drug-resistant group received intraperitoneal injection of phenytoin (PHT, 75 mg/kg); 1 h later, the after-discharge threshold (ADT) was measured (initial current intensity was 20% of the ADT value in the normal saline group; subsequent intensities were added by 20% each time with 5 min once). Rats in the saline group received intraperitoneal injection of normal saline at the same volume with that of screening drugs. The detected ADT value after the injection of PHT was compared with that after the injection of normal saline. If the fluctuation range was within \pm 20%, the PHT screening was performed one week later, and rats passing PHT screening for three times were considered resistant to PHT. One week later, phenobarbital (PB, 25 mg/kg) screening was conducted, whose method was the same as the above. All the rats that passed both PHT and PB screenings were drug-resistant epilepsy rat models. Subsequently, all the rats were anesthetized by intraperitoneal injection of 4% chloral hydrate (1 mL/100 g), and the hippocampus and temporal lobe cortex were isolated and stored.

Immunohistochemistry

Paraffin tissue sections were taken and used for immunohistochemistry. After xylene dewaxing, ethanol at gradient concentrations was used for dehydration, and antigen retrieval was conducted with sodium citrate buffer using a microwave. Peroxidase was blocked by 3% H₂O₂ blocker, and after 10% donkey serum was used for blocking, sections were dropwise added with the primary antibody (Nurrl, Abcam, Cambridge, MA, USA; diluted at 1:500), placed in the wet box, and incubated overnight at 4°C. The next day, sections were washed with phosphate-buffered saline (PBS) for three times, the ready-to-use universal secondary antibody was used for incubation, and 3,3'-diaminobenzidine (DAB) color development was conducted, followed by photographing under a microscope. Brown and median brown nuclei or cytoplasm shown under the microscope were positive cells, and the number of positive cells was counted.

Detection of the Protein Levels of Interleukin-6 (IL-6) and Tumor Necrosis Factor-Alpha (TNF-α) in Tissues by Reverse Transcription Polymerase Chain Reaction (RT-PCR), Western Blotting and Immunohistochemistry

The total messenger ribonucleic acid (mR-NA) in tissues was extracted using the RNeasy Kit (Qiagen, Hilden, Germany). Reverse transcription kit [SuperScript® VILO complementary DNA (cDNA) Synthesis Kit and Master Mix, Thermo Fisher Scientific, Waltham, MA, USA] was applied to synthesize 1.0 µg total mRNA into cDNA. Expressions of B-cell lymphoma 2 (Bcl2) associated X protein (Bax) and Bcl-2 were detected by quantitative RT-PCT (qRT-PCR) (GeneCopoeia Inc., Guangzhou, Guangdong, China) and Real-time fluorescence qPCR apparatus (Thermo Fisher Scientific, Waltham, MA, USA). β -actin was taken as an internal control for correction, and the relative expression level of each indicator was calculated as $2^{-\Delta Ct} \left[\Delta Ct = Ct \text{ (target gene)} - Ct (\beta - actin) \right]$. All the primers were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. After homogenate process was conducted for the hippocampus and temporal lobe tissues, proteins were extracted. After the protein concentration (Beyotime Institute of Biotechnology, Shanghai, China) was detected using the bicinchonininc acid (BCA) protein concentration assay kit, samples were loaded at the equal quantity of 40 µg, followed by electrophoresis. The protein was transferred to a polyvinylidene difluoride (PVDF) membrane. After 5% skim milk was used for blocking at room temperature for 1 h, the Nurrl antibody (1:1000; Proteintech, Chicago, IL, USA) was applied for incubation; P-glycoprotein (P-gp), monocyte chemoattractant protein-1 (MCP-1), multi-drug resistance protein (MRP) and leukemia-associated phenotype (LAP) were all diluted at 1:1000 (Abcam, Cambridge, MA, USA) with β-actin as an endogenous reference (1:2000; Abcam, Cambridge, MA, USA). Horseradish peroxidase (HRP)-labeled secondary antibody was used for incubation (1:5000; Beyotime Institute of Biotechnology, Shanghai, China). The membrane was visualized with an enhanced chemiluminescence (ECL) detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and grayscale analysis was performed with a gel analyzer. The relative content of the target protein was the ratio of the gray value of the target protein to that of the corresponding internal reference band.

Statistical Analysis

Data analysis was conducted using statistical product and service solutions (SPSS) 19.0 software (Armonk, NY, USA). Measurement data were expressed as $(\bar{x} \pm s)$, and the *t*-test was used to compare the differences in two groups of indicators. Correlation analyses of Nurr1 in hippocampus and temporal lobe cortex tissues were conducted using Pearson correlation analysis. p < 0.05 represented that the difference was statistically significant.

Results

Detection of the mRNA Level of Nurr1 by RT-PCR

RNAs in hippocampus and temporal lobe cortex tissues of the two groups were extracted, respectively, and the mRNA level of Nurrl was detected. As shown in Figure 1, the mRNA level of Nurrl in hippocampus tissues of the drug-resistant group was significantly increased compared with that of the control group, and the difference was statistically significant (p < 0.05). In temporal lobe cortex tissues, there was no significant difference in the mRNA level of Nurrl between the control group and the drug-resistant group (p > 0.05).

Detection of the Protein Expression Level of Nurr1 by Western Blotting

Proteins in hippocampus and temporal lobe cortex tissues of the two groups of rats were extracted, respectively, and the expression level of



Figure 1. Detection of the mRNA level of Nurr1 in hippocampus and temporal lobe cortex tissues of the two groups of rats.*Note*: Compared with the control group, *p < 0.05.

Nurr1 was detected. Results also showed that the expression level of Nurr1 in hippocampus tissues was significantly increased (p < 0.05). In temporal lobe cortex tissues, there was no significant difference in the protein expression of Nurr1 between the control group and the drug-resistant group (Figure 2).

Detection of the Expression of Nurr1 Tissues of Two Groups of Rats by Immunohistochemistry

The hippocampus and temporal lobe cortex of epilepsy model rats were taken, respectively, and the expression level of Nurr1 was further detected by immunohistochemistry. Results revealed that the positive rate of Nurr1 in the drug-resistant group was significantly increased compared with that in the control group. However, in the temporal lobe cortex, the expressions of Nurr1 in the drug-resistant group and the control group were not significant (Figure 3).

Further Statistical Analysis According to the Results of Immunohistochemistry

The analysis of immunohistochemistry results showed that Nurrl in the control group was significantly increased compared with that in the experimental group (p < 0.01). However, in the temporal lobe cortex, there was no statistically significant difference in Nurrl expression, suggesting that Nurrl is mainly upregulated in hippocampus tissues, and it is positively associated with drug resistance of rats with epilepsy (Table I).

Staining by the Budding Tissue Marker Growth-Associated Protein-43 (GAP43)

It has been confirmed in animal models of epilepsy that the abnormal budding leads to changes in temporal lobe, especially in the structure of the hippocampal pathway and excitability. Hippocampus tissues were stained with the fluorescence-labeled GAP43 antibody, and the results revealed that the fluorescence intensity of GAP3 in hippocampus tissues of the drug-resistant group was significantly higher than that of the control group (Figure 4).

Detection of the mRNA and Protein Levels of Indicators Related to Drug Resistance in Hippocampus Tissues of the Two Groups of Rats

According to the previous experimental results, it was confirmed that Nurrl was up-regulated in hippocampus tissues, and there was no difference in temporal lobe cortex tissues. Therefore, drug-resistant epilepsy-related protein indicators (LAP, MRP, P-gp and MCP-1) in hippocampus tissues were further detected. The results manifested that the mRNA and protein levels of epilepsy-related indicators in hippocampus tissues of rats in the drug-resistant group were significantly increased (p < 0.05) (Figures 5 and 6).

Correlation Analyses of the Expression Level of Nurr1 with Drug-resistant Proteins in Hippocampus Tissues

Pearson correlation analyses (Table II) indicated that Nurr1 level in hippocampus tis-



Figure 2. Detection of the protein expression level of Nurrl in hippocampus and temporal lobe cortex tissues of the two groups of rats. *Note:* Compared with control group, **p < 0.01.



Figure 3. Detection of the expression level of Nurr1 by immunohistochemistry.

sues was positively correlated with LAP, MRP, P-GP and MCP-1 levels in the corresponding regions.

Discussion

Drug resistance is a major barrier to the treatment of many diseases, including cancer, infectious diseases and epilepsy⁹. Recently, the mechanism of AEDs has drawn increasing interests. Based on clinical data and experiments, researchers have proposed two main hypotheses to explain the resistance of epilepsy: target hypothesis and the multi-drug transport hypothesis¹⁰. The target hypothesis suggests that the intrinsic or acquired change in AED targets in the brain is the basis of resistant epilepsy, whereas the

Table I. Comparisons of Nurrl in hippocampus and temporal lobe cortex among each group ($\bar{x} \pm s$, n = 20).

| | Hippocampus tissues | | Temporal lobe cortex tissues | |
|---|---|--|---|--|
| Group | Grayscale ratio | The number of positive cells (n) | Grayscale ratio | The number of positive cells (n) |
| Control group Drug-resistant group F-value <i>p</i> -value | $\begin{array}{c} 1.282 \pm 0.012 \\ 15.523 \pm 0.063^{**} \\ 2.65 \\ < 0.01 \end{array}$ | $8.46 \pm 2.16 \\ 22.43 \pm 3.26 ** \\ 2.32 \\ < 0.01$ | $\begin{array}{c} 0.282 \pm 0.012 \\ 0.423 \pm 0.063 \\ 0.42 \\ > 0.05 \end{array}$ | 5.46 ± 2.16 6.43 ± 3.26 0.36 > 0.05 |

Note: Compared with control group, *: p < 0.05, **: p < 0.01.



Figure 4. Detection by the budding tissue marker GAP43.



Figure 5. Detection of the mRNA level of proteins related to drug resistance in hippocampus tissues. *Note:* Compared with the control group, **p < 0.01.



Figure 6. Detection of proteins related to drug resistance in hippocampus tissues. *Note:* Compared with the control group, **p < 0.01.

 Table II. Correlations of Nurr1 level with drug-resistant proteins in hippocampus tissues.

| | Nurr1 level in hippocampus tissues | | |
|-----------------------------|------------------------------------|--------------------------------------|--|
| Indicator | <i>r</i> -value | <i>p</i> -value | |
| MRP P-gp MCP-1 LAP | 1.883 2.738 1.704 2.836 | < 0.05 < 0.05 > 0.05 < 0.05 | |

multi-drug transport hypothesis states that drugs do not reach the brain since the intrinsic or acquired overexpression of a multi-drug transporter within the blood-brain barrier (BBB) limits the absorption of drugs by the brain's AED¹¹. Nurrl explored in this study, also known as NOT/TIN-UR/HZF-3, is a product of an early gene that is mainly expressed in the central nervous system and belongs to the class of steroid, retinoid and thyroid hormone nuclear receptor superfamilies¹². The nuclear receptor superfamily refers to transcription factors that regulate gene expression in response to various chemical signals. The nuclear receptor superfamily is divided into two groups: the ligand-activating group, including receptors for specific ligands such as retinoids, steroids and thyroid hormones¹³, and the so-called "orphan receptor" group whose gene activation mechanism and whether there are homologous ligands are unknown. Nurr1 belongs to the orphan receptor subfamily¹⁴. The amino acid sequence of mouse Nurr1 is a 66 kDa protein, a DNA binding region containing a zinc finger structure. Nurr1 expression has recently been found to be crucial for the differentiation of midbrain dopaminergic neurons in the developing brain. In adults, Nurr1 helps maintain dopamine's phenotype by regulating enough dopamines¹⁵. On the other hand, Nurr1 mRNA is present in brain regions unrelated to dopaminergic neurons, such as the cerebral cortex and hippocampus¹⁶. A recent study¹⁷ has revealed that after the induced epileptic seizure occurrs, the mRNA and protein levels of Nurr1 in the rat brain are increased, while Nurr1 expression level becomes different in the case of electrical spasms and ignition. These observations suggest that this orphan receptor may play a role in the synaptic plasticity of the nervous system by mediating activity-dependent gene regulation. A research¹⁸ has shown that high-level P-gp in the cerebrovascular endothelium contributes to the function of BBB, whereas the overexpression of P-gp leads to multi-drug resistance of some

cancers. Presumably, the overexpressions of P-gp and other transporters in the brain endothelium in the focal areas of epilepsy may also lead to the drug resistance of epilepsy. The increased expression of P-gp in epileptic foci in patients with drug-resistant epilepsy can induce epileptic seizures in animal models, and it is a common substrate for AEDs¹⁹. The above experimental evidence confirms this hypothesis. In this study, it was found that the tissue, protein and mRNA levels of multi-drug resistance-related proteins such as P-gp, MRP-1, LAP and MCP-1 were detected to be significantly increased in the hippocampus of rats with drug-resistant epilepsy, whereas there were no differences in their expressions in the temporal lobe cortex. In addition, studies have reported that the abnormal budding leads to changes in temporal lobe, especially in the structure of the hippocampal pathway and excitability, which has been demonstrated in animal models of epilepsy²⁰. Fluorescence was used to label GAP43 in rat hippocampal tissues, and the results showed that the fluorescence intensity of GAP43 in the drug-resistant group was significantly higher than that in the control group.

Conclusions

We showed that Nurr1 might regulate the drug resistance of drug-resistant epilepsy by up-regulating the expression of drug resistance-related proteins. As an upstream gene, Nurr1 activates DNA transcription and up-regulates the protein level by binding to drug resistance-related protein DNA. After the expression level of drug transport protein is elevated, namely, the drug transporter is increased, the concentration of anti-epileptic drugs entering BBB is decreased, thus showing resistance. However, the specific molecular mechanism of Nurr1 has not yet been explored in depth, but it is believe that with the research on neurobiology, molecular biology, molecular pharmacology and electrophysiology, the mystery of drug-resistant epilepsy will be gradually revealed, so as to provide new ideas and methods for the diagnosis and prevention of drug-resistant epilepsy and open up new areas for treatment strategies as well as research and development of new drugs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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