NEURAL REGENERATION RESEARCH
Volume 8, Issue 14, May 2013

www.nrronline.org

doi:10.3969/j.issn.1673-5374.2013.14.007 [http://www.nrronline.org; http://www.sjzsyj.org]
Dong CZ, Zhao WQ, Li WL, Lv PY, Dong XF. Anti-epileptic effects of neuropeptide Y gene transfection into the rat brain. Neural Regen Res. 2013;8(14):1307-1315.

Anti-epileptic effects of neuropeptide Y gene transfection into the rat brain☆

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Abstract

Neuropeptide Y gene transfection into normal rat brain tissue can provide gene overexpression, which can attenuate the severity of kainic acid-induced seizures. In this study, a recombinant adeno-associated virus carrying the neuropeptide Y gene was transfected into brain tissue of rats with kainic acid-induced epilepsy through stereotactic methods. Following these transfections, we verified overexpression of the neuropeptide Y gene in the epileptic brain. Electroencephalograms showed that seizure severity was significantly inhibited and seizure latency was significantly prolonged up to 4 weeks after gene transfection. Moreover, quantitative fluorescent PCR and western blot assays revealed that the mRNA and protein expression of the N-methyl-D-aspartate receptor subunits NR1, NR2A, and NR2B was inhibited in the hippocampus of epileptic rats. These findings indicate that neuropeptide Y may inhibit seizures *via* down-regulation of the functional expression of N-methyl-D-aspartate receptors.

Key Words

neural regeneration; brain injury; gene therapy; adeno-associated virus; neuropeptide Y; epilepsy; N-methyl-D-aspartate receptor; kainic acid; seizures; neuroregeneration

Research Highlights

(1) In this study, we established a chronic epilepsy model in rats using repeated injections of kainic acid. The experimental rats were transfected with a recombinant adeno-associated virus carrying the neuropeptide Y gene, resulting in continuous neuropeptide Y expression in the epileptic brain.
(2) A recombinant adeno-associated virus carrying the neuropeptide Y gene can relieve seizure severity 4 weeks after gene transfection in rats.
(3) Neuropeptide Y inhibited epileptic seizures by down-regulating N-methyl-D-aspartate receptor expression and reducing neuronal excitability.

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Received: 2012-08-20
Accepted: 2013-04-24
(N20120615002)

INTRODUCTION

Epilepsy is a chronic neurological disorder characterized by recurrent seizures, which may present as involuntary changes in sensation, awareness, behavior, body movements, and other functions. The main clinical approaches to control epilepsy are medications and surgery. It is well-documented that long-term application of antiepileptic drugs has many adverse effects, including dizziness, asthenia, somnolence, skin rashes, and allergic reactions[1-4]. In addition, approximately 30% of epileptic patients are unresponsive or resistant to current antiepileptic drugs[5-6]. For the estimated 10–20% of patients

that are pharmacoresistant, a resective surgery to remove the epileptogenic tissue is the only therapeutic option. However, ablative surgeries are only effective in about 70% of patients, require general anesthesia, and may have risks for postoperative complications, such as infection, hemorrhagic infarctions, hemiparesis, subdural effusion, aphasia, mutism, and late-onset psychosis[7-8]. Thus, new therapeutic approaches are needed to treat refractory epilepsy. Results from studies using animal experimental models suggest that gene therapy may represent a promising alternative to resective surgeries by targeting a “therapeutic” gene to the epileptic focus without ablating the morbid tissue[9-11].

Neuropeptide Y (NPY) is an endogenous 36-amino acid polypeptide. There is compelling evidence supporting the idea that NPY has powerful anticonvulsant properties[12-14]. Indeed, many studies have demonstrated that overexpression of NPY using a recombinant adeno-associated viral (rAVV) vector delivered into the rat hippocampus potently inhibits acutely induced seizures, delays kindling epileptogenesis, and reduces seizure susceptibility[15]. Moreover, some studies have shown that local hippocampal application of rAAV-NPY in a rat model of chronic epilepsy strongly decreased spontaneous seizure frequency and suppressed the symptomatic progression of seizures[16]. One likely mechanism for the anti-epileptic action of NPY is that NPY inhibits excitatory synaptic transmission by reducing glutamate release from presynaptic nerve terminals by suppressing Ca^{2+} influx through voltage-dependent Ca^{2+} channels[17-19].

N-Methyl-D-aspartate (NMDA) receptors are excitatory ionotropic glutamate receptors, which play multiple important roles in excitatory synaptic transmission and plasticity in the mammalian central nervous system. Evidence has indicated that excessive NMDA receptor activation results in excitatory neurotoxicity, which may be the main mechanism underlying the pathogenesis of epilepsy[20-21]. To date, no studies have evaluated the effects of NPY gene transfer on NMDA receptors located in neuronal postsynaptic membranes.

In the present study, we used intraventricular injection of rAAV-NPY in a rat model of chronic epilepsy induced by kainic acid, resulting in long lasting NPY overexpression in neurons. We investigated changes in epileptic rat behavior and electroencephalogram parameters, and expression of NMDA receptor subunits (NR1, NR2A and NR2B) to determine possible mechanisms mediating the effects of NPY overexpression on epileptic seizures.

RESULTS

Quantitative analysis of experimental animals

A total of 144 rats were randomly and equally assigned to control, kainic acid, rAAV-empty, and rAAV-NPY groups. The kainic acid, rAAV-empty, and rAAV-NPY groups were subjected to kainic acid injections to induce chronic epilepsy. The rAAV-empty and rAAV-NPY groups were additionally injected with rAAV-empty and rAAV-NPY, respectively, into the right lateral ventricle. Eighteen rats from each group were used for behavioral observations and electroencephalogram recordings, and the other 18 rats were used for PCR and western blot analyses. In the final analysis, 137 rats were included because seven rats (three from the rAAV-empty group and four from the rAAV-NPY group) that died of intracranial infection were excluded.

Exogenous NPY increased NPY mRNA and protein expression in the hippocampus of rats with chronic epilepsy

To assess NPY expression after vector injection, we performed quantitative PCR analysis of NPY mRNA levels in each group at different time points. As shown in Figure 1A, NPY mRNA expression was significantly increased in the kainic acid, rAAV-empty, and rAAV-NPY groups compared with the control group during the 4 weeks after vector injection ($P < 0.05$). We also found significant increases in NPY mRNA expression in the rAAV-NPY group compared with the kainic acid and rAAV-empty groups at 4 weeks ($P < 0.05$).

Furthermore, we performed a western blot assay to examine NYP protein expression under these conditions. Similar to the results from quantitative PCR, the NYP protein expression was also increased by kainic acid treatment, and was further increased by injecting the rAAV-NPY vector. Our quantitative PCR and western blot results were consistent in that there was a significant increase in NPY expression 4 weeks after injection of AAV2/1 encoding NPY ($P < 0.05$; Figure 1B).

Exogenous NPY decreased the severity of seizures

To access the effects of NPY gene transfer on the kainic acid-induced seizures in individual rats, we first observed the severity of behavioral seizures by continuous video monitoring. No seizures were found in the control group at different time points. The severity of the seizures was similar in the kainic acid and rAAV-empty groups over

the 4-week post-injection period.

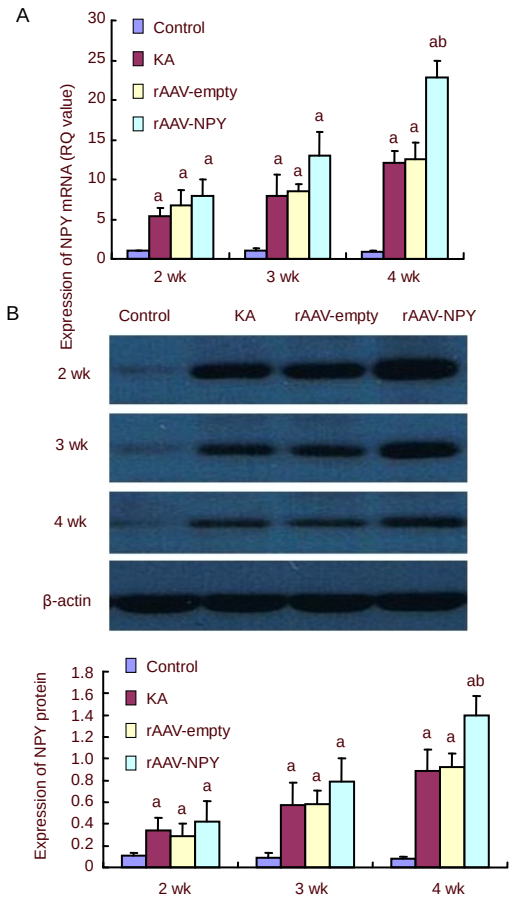

Figure 1 Influence of exogenous neuropeptide Y (NPY) on NPY mRNA and protein expression in the hippocampus of rats with chronic epilepsy.

The data are presented as mean ± SEM and were analyzed by the Student-Newman-Keuls test. [a]*P* < 0.05, *vs*. control group; [b]*P* < 0.05, *vs*. kainic acid (KA) and adeno-associated viral-empty (rAAV) groups. 2, 3, 4 wk: 2, 3, and 4 weeks after vector injection

(A) NPY mRNA expression in the rat hippocampus was detected by quantitative PCR. The threshold cycle (Ct) data of each target gene were normalized to the internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A comparative Ct method was applied to calculate relative quantification (RQ), which was used in the final statistical analyses.

(B) NPY protein expression in rat hippocampus was detected by western blot analysis. The expression of NPY is presented as the relative absorbance rate between NPY and β-actin.

Subsequently, we mainly analyzed the severity of the seizures over the 4 weeks after drug application among the kainic acid, rAAV-empty, and rAAV-NPY groups. At 2 and 3 weeks after injection, no significant differences were observed in seizure severity among these three groups (*P* > 0.05). In the rAAV-NPY group, the seizure severity at 4 weeks post-injection was almost 2-fold decreased compared with 2 and 3 weeks post-injection in the same rats (*P* < 0.05 by chi-square test; Table 1).

Table 1 Effects of exogenous NPY on the severity [*n*(%)] of seizures in rats

Group	*n*	2 weeks after vector injection	
		Mild	Severe
Kainic acid	18	5(28)	13(72)
rAAV-empty	15	2(13)	13(87)
rAAV-NPY	14	3(21)	11(79)
Group	***n***	**3 weeks after vector injection**	
		Mild	Severe
Kainic acid	18	6(33)	12(67)
rAAV-empty	15	2(13)	13(87)
rAAV-NPY	14	4(29)	10(71)
Group	***n***	**4 weeks after vector injection**	
		Mild	Severe
Kainic acid	18	5(28)	13(72)
rAAV-empty	15	3(20)	12(80)
rAAV-NPY	14	9(64)	5(36)[ab]

Differences in seizure severity across the groups were determined by the chi-square test. Mild: Grade I–III seizures; severe: grade IV–V seizures. [a]*P* < 0.05,*vs*. kainic acid and rAAV-empty groups; [b]*P* < 0.05, *vs*. 2 and 3 weeks groups. NPY: Neuropeptide Y; rAAV: recombinant adeno-associated viral.

Exogenous NPY decreased brain electrical activity in rats

After kainic acid induced seizures, rats presented different degrees of spike-wave discharge on electroencephalograms (Figure 2C). During the second and third weeks after vector application, no significant differences in mean spike frequencies were observed among the rAAV-NPY, kainic acid, and rAAV-empty groups (*P* > 0.05). However, in the fourth week after vector injection, the rAAV-NPY rats presented significantly lower spike frequency than the other two groups (*P* < 0.05). Rats in the rAAV-NPY group had approximately 60% lower electroencephalogram seizure frequencies compared with the other groups (Figure 2B).

As another indicator of electroencephalogram seizures, we calculated the mean seizure latency over 4 weeks of recording in these three groups. In the rAAV-NPY group, no statistical differences were found in the mean latency of individual electroencephalogram seizures in the second and third weeks of post-injection recording compared with the same recording period in the other groups. However, in the fourth week after vector injection, the rAAV-NPY rats had approximately 2.4-fold longer seizure latencies than the other groups (*P* < 0.05). No statistical differences were found between the kainic acid and rAAV-empty groups (Figure 2A).

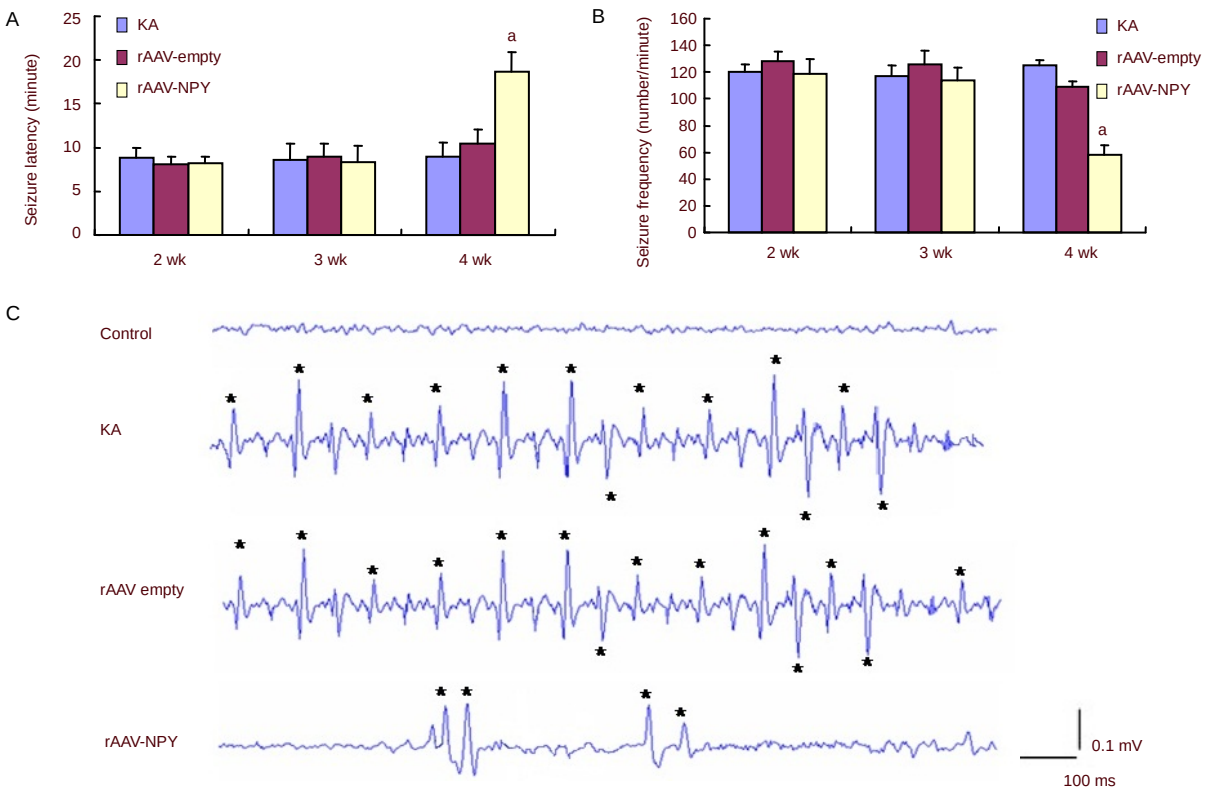

Figure 2 Effects of exogenous neuropeptide Y (NPY) on seizure frequencies and prolonged seizure latencies as seen in rat electroencephalogram activity.

Seizure latency is shown in A and seizure frenquency is shown in B. The data are presented as mean ± SEM and were analyzed by the Student-Newman-Keuls test. [a]$P < 0.05$, *vs*. kainic acid (KA) and recombinant adeno-associated viral (rAAV)-empty groups; spike-wave discharge (asterisk) on the electroencephalogram monitoring is shown in C. 2, 3, 4 wk: 2, 3, 4 weeks after vector injection.

Exogenous NPY decreased the expression of NMDA receptor subunits in the rat hippocampus

As shown in Figure 3, the expression of NR1, NR2A, and NR2B NMDA receptor subunit mRNA significantly increased in the rAAV-NPY, kainic acid, and rAAV-empty groups at 2, 3 and 4 weeks post-injection compared with the control group. Rats in the rAAV-empty group showed no significant difference in the expression of NMDA receptors compared with the kainic acid group at any time point ($P > 0.05$). Increased expression of NMDA receptors elicited by kainic acid-induced seizures was significantly lower ($P < 0.05$) in the rAAV-NPY group at 4 weeks post-injection.

Furthermore, western blotting was performed to examine protein expression of the NR1, NR2A, and NR2B NMDA receptor subunits under the same conditions. As shown in Figure 4, we found an increase in the protein expression of NMDA receptors in the kainic acid group compared with the control group. Similar to the results from quantitative PCR, the rAAV-empty group did not suppress the protein expression of NMDA receptors in the post-injection period. No significant differences were observed in the protein expression of NMDA receptors between the rAAV-empty and rAAV-NPY groups in the second and third weeks after vector application. Up to 4 weeks post-injection, the rAAV-NPY group showed a statistically significant decrease in the protein expression of NMDA receptors, but there were no statistical differences between the kainic acid and the rAAV-empty groups in the expression of NMDA receptors.

DISCUSSION

Our study presents the novel and important finding that rAAV vector-mediated NPY gene overexpression in the brain of chronic epileptic rats results in powerful inhibition of spontaneous recurrent seizures. From our data, it can be seen that rats in the rAAV-NPY group, compared with the rAAV-empty group, showed significantly alleviated seizure latencies and lower frequencies of abnormal spikes on electroencephalograms. These data suggest that transgenic NPY expression can inhibit seizure activity.

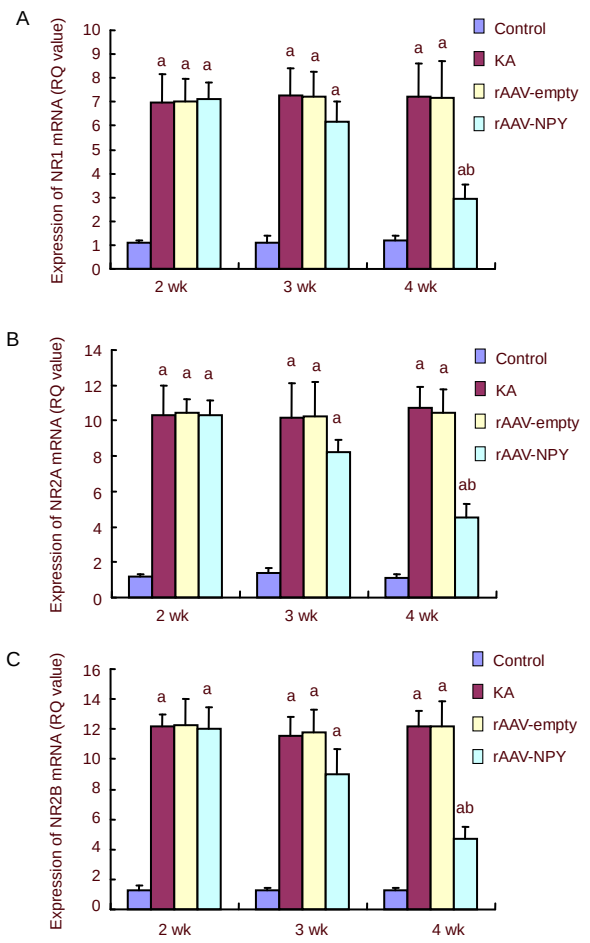

Figure 3 Effects of exogenous neuropeptide Y (NPY) on the mRNA expression of N-methyl-D-aspartate receptor subunits NR1 (A), NR2A (B), and NR2B(C) in the rat hippocampus.

The threshold cycle (Ct) data of each target gene were normalized to the internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A comparative Ct method was applied to calculate relative quantification (RQ), which was used in the final statistical analysis. The data are presented as mean ± SEM and were analyzed by the Student-Newman-Keuls test. $^{a}P < 0.05$, *vs*. control group; $^{b}P < 0.05$, *vs*. kainic acid and recombinant adeno-associated viral (rAAV)-empty groups. 2, 3, 4 wk: 2, 3, 4 weeks after vector injection.

NPY is considered to be a good therapeutic candidate for epilepsy because it acts as an endogenous anticonvulsant factor, as shown by genetic and pharmacological approaches[22]. Initial experimental attempts established that AAV-mediated NPY overexpression in the “normal rat brain” has strong anti-ictal effects on acutely induced seizures[23-24]. In experimental chronic epileptic models, however, the epileptic brain has abnormalities such as synaptic rearrangements and neuronal loss that could affect the efficacy of therapeutic gene transfection to a sufficient number of neurons. Here, we used a rat model of chronic limbic seizures induced by repeated kainic acid injections, which could imitate some neuropathological features of human temporal lobe epilepsy, including hippocampal sclerosis[25-26]. The results of quantitative PCR and western blotting analysis demonstrated that rAAV vector-mediated NPY gene expression increased significantly in this pathological state, even when neurons normally expressing NPY were partially lost.

The possible mechanisms underlying the anticonvulsant actions of NPY are mainly connected with the capacity of NPY, which potently suppresses glutamatergic excitatory synaptic transmission in areas CA1 and CA3[27-30], and significantly inhibits glutamate release *via* activating presynaptic Y2 receptors[31-33]. Up to now, no attempts have been made to investigate the effects of NPY on the expression of NMDA receptors. NMDA receptors are considered to play critical roles in modulating seizure activities[34-36]. Enhanced expression of NMDA receptor subtypes is required for the development of epilepsy[37-38]. Pratt *et al* [39] observed that NR2A and NR2B transcript levels were increased by 102% and 46%, respectively, compared with control values in fully kindled rats. Zhu *et al* [40] showed that the levels of NR1 subunit were significantly increased in the hippocampus of rats at different time points after pentylenetetrazol injection using western blotting analysis. In contrast, reduced expression of NMDA receptor subunits may have suppressed neuronal hyperexcitability induced in epileptic tissue by inhibiting Ca^{2+} influx[41]. Furthermore, our results show that the expression of NMDA receptor subunits mRNA and protein levels was significantly lower in the rAAV-NPY group than that in the rAAV-empty and kainic acid groups. Thus, our data support the hypothesis that NPY overexpression resulting in a reduction in epileptic seizures initiates a series of adaptive responses manifested as downregulation of the expression and functional activity of NMDA receptors.

In conclusion, we demonstrate that NPY protects against kainic acid-induced epileptic seizures possibly through downregulation of NMDA receptor expression. Our results suggest a possible strategy for the treatment of chronic epilepsy through elevation of NPY levels mediated by an AAV2/1 vector.

MATERIALS AND METHODS

Design

A randomized, controlled, animal study.

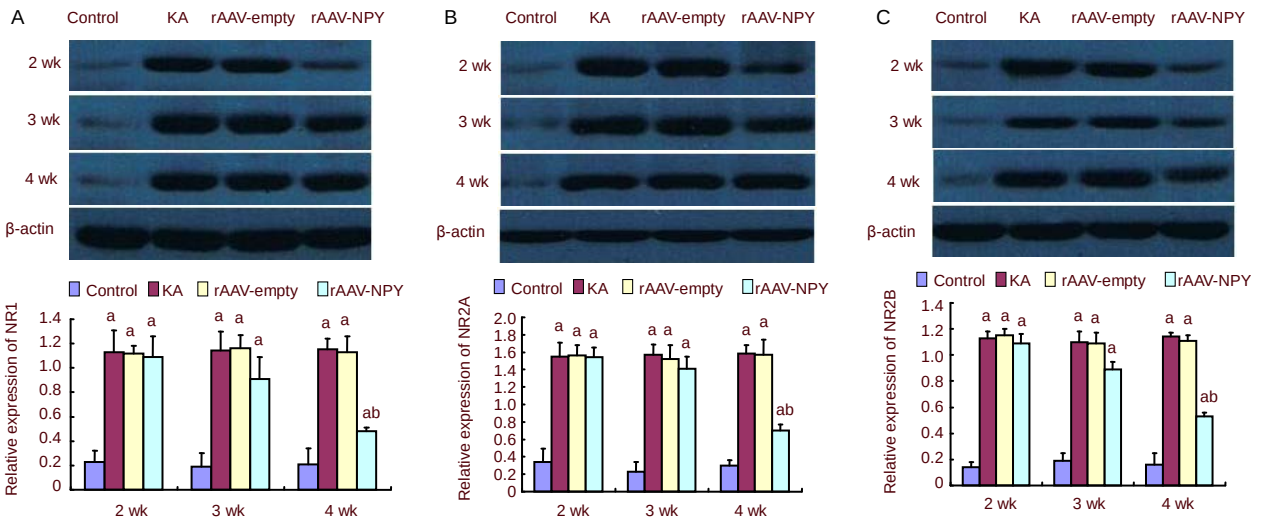

Figure 4 Effects of exogenous neuropeptide Y (NPY) on the expression of N-Methyl-D-aspartate receptor subunits NR1 (A), NR2A (B), and NR2B (C) in the rat hippocampus.

The expression of a target protein is presented as its absorbance relative to β-actin. The data are presented as mean ± SEM and were analyzed by the Student-Newman-Keuls test. [a]*P* < 0.05, *vs*. control group; [b]*P* < 0.05, *vs*. kainic acid (KA) and recombinant adeno-associated viral (rAAV)-empty groups. 2, 3, 4 wk: 2, 3, 4 weeks after vector injection.

Time and setting

This experiment was performed at the Clinical Research Central Laboratory of Hebei General Hospital, China from 2009 to 2011.

Materials

Male Wistar adult rats of 10–12 weeks of age, weighing 250–270 g, and of clean grade were purchased from the Laboratory Animal Center of Hebei Medical University, China (license No. SCXK (Ji) 2008-1-003). They were housed at a constant temperature (23°C) and relative humidity (60%) with a fixed 12-hour light/dark cycle. They were allowed free access to food and water. All experimental disposal of animals was conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[42].

Methods

rAAV-vector construction

The plasmid rAAV-NPY was constructed with standard molecular cloning procedures[43]. Briefly, the prepro-NPY cDNA (Beijing Vector Gene Technology Company, Beijing, China) was subcloned into an expression cassette including a cytomegalovirus promoter and a bovine growth hormone poly (A) signal. The same expression cassette lacking NPY (rAAV-empty; Beijing Vector Gene Technology Company) was used as a control vector. Expression cassettes were subcloned into the rAAV backbone, which was flanked by rAAV-inverted terminal repeats. To get high titers of chimerical rAAV vectors, a rAAV1 capsid helper plasmid was joined in a 1:1 ratio with the rAAV2 helpers during the packaging process.

Establishment of chronic epileptic model rats

Rats in the kainic acid, rAAV-empty and rAAV-NPY groups were deeply anesthetized by intraperitoneal injection with 4% chloral hydrate (3.5 mL/kg) and placed on a stereotaxic apparatus (Shenzhen Rui Ward Life Science and Technology Ltd., Shenzhen, Guangdong Province, China). The scalp was shaved and sterilized before a midline skin incision was cut to expose the calvarias. Burr holes were made using an electric drill (Shanghai Hefeng Medical Equipment Technology, Shanghai, China), and a microsyringe needle (Beijing Rapidbio Science, Beijing, China) was inserted into the right ventral hippocampus (coordinates: anterior = –5.3 mm, lateral = +4.0 mm, depth = –6.0 mm) according to a rat atlas provided by the stereotaxic apparatus. Kainic acid (1.5 μL, 0.4 μg/μL; Sigma, St. Louis, MO, USA) was slowly injected into the right ventral hippocampus over a 2-minute period with a 10 μL microsyringe. Kainic acid was re-injected once every 3 days, for five times in total. Control rats were injected with 1.5 μL of normal saline. Rats from all groups except the control group showed spontaneous recurrent seizures over 3 months after kainic acid injection. This experimental model of seizures was chosen because it is highly sensitive to NPY modulation of epileptic seizures[44] and produces minimal mortality.

Gene transfection

After the chronic kindled rat model of epilepsy was

successfully established, rats in the rAAV-empty and rAAV-NPY groups were administrated distinct vectors. Rats underwent surgeries as described above, and the rAAV-vector was injected unilaterally into the right lateral ventricle (coordinates: anterior = –1.5 mm, lateral = + 1.5 mm, depth = –3.8 mm). The total volume injected in the rats in the rAAV-NPY group was 10 μL of rAAV-NPY from a stock solution containing 5×10^{11} particles/mL. The same volume of rAAV-empty was injected into the same neuroanatomical target in the rAAV-empty group. A total of 10 μL of solution was infused over 5 minutes using a 10-μL microsyringe under stereotactic guidance. Rats in the control and kainic acid groups received no vector injection.

Behavior observations and electroencephalogram recordings in rats

At 2, 3, and 4 weeks after vector injection, the seizure severity was observed and graded according to a modified Racine six evaluation criteria[45]. We classified grade I–III seizures as mild and grade IV–V seizures as severe. Behavioral seizure manifestations were recorded and monitored using a video camera. Twenty-minute electroencephalogram recordings were made before kainic acid injection (serve as the electroencephalogram baseline), and electroencephalogram activity was monitored with the MS4000U-1 biological signal recording system (Guangzhou Dragon Feida Technology, Guangzhou, China). Electroencephalogram seizure activities were quantified by determining the frequency of seizures and the latency to the first seizure. In each rat, electroencephalogram seizures were characterized by the occurrence of discrete ictal episodes of high-frequency and high-voltage synchronous spike or wave activity in the injected hippocampus. The electroencephalogram seizures were visually analyzed and manually identified to find any activity different from baseline by two independent investigators blinded to the treatments.

Real time reverse transcriptase-quantitative PCR for detection of mRNA expression of NPY and NMDA receptor subunits in the rat hippocampus

At 2, 3, and 4 weeks after vector injection, animals were deeply anesthetized by intraperitoneal injection with 4% chloral hydrate (6 mL/kg). After quick decapitation, the hippocampus was rapidly dissected. Total RNAs were isolated from the hippocampal tissues using TRIZol reagents (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions, and were subsequently reverse transcribed to cDNA by Oligo. The resulting cDNA was used as the template for PCR. Specific primers for NPY, NR1, NR2A, NR2B, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) used in quantitative PCR are shown as follows.

Primer	Sequence (5′–3′)	Product size (bp)
GAPDH	Forward: TGA ACG GGA AGC TCA CTG G	120
	Reverse: GCT TCA CCA CCT TCT TGA TGT C	
NPY	Forward: TCG TGT GTT TGG GCA TTC TG	164
	Reverse: TCA GTG TCT CAG GGC TGG AT	
NR1	Forward: CAA CGA CCA CTT CAC TCC CAC	288
	Reverse: CAC CTT CTC TGC CTT GGA CTC	
NR2A	Forward: ATG GCT GAC AAG GAT CCG AC	165
	Reverse: CAC AAA GCT GTT GTC CAC TGT	
NR2B	Forward: GGA GAT GGA AGA ACT GGA AGC T	152
	Reverse: AGA TGA AGG TGA TGA GGC TGA G	

PCR amplification was performed using a Hot Start Fluorescent PCR Core Reagent Kits (Bio Basic Inc. Markham, Canada) and an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Briefly, 1 μL of cDNA template was added to 2 μL of 2 × SYBR green PCR master mix (Bio Basic Inc.), 2 μL of each primer and nuclease-free water 7 μL to a final volume of 20 μL. The reaction was initiated with thermal denaturation at 96°C for 4 minutes, in 20 μL. The reaction was initiated with thermal denaturation at 96°C for 4 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and product elongation and fluorescent signal acquisition at 72°C for 30 seconds. The threshold cycle (Ct) values were analyzed with ABI 7300 SDS software (Applied Biosystems) to detect fluorescence. The Ct data of each target gene were normalized to the internal control gene, GAPDH. A comparative Ct method was applied to calculate relative quantification, which was used in the final statistical analyses.

Western blot analysis of protein expression of NPY and NMDA receptor subunits in the rat hippocampus

At 2, 3, and 4 weeks after vector injection, the hippocampal tissues were homogenized in RIPA buffer containing NaCl (150 mmol/L), NP-40 (1.0%), sodium deoxycholate (0.5%), sodium dodecyl sulfate (0.1%) , Tris-HCl (50 mmol/L, pH 7.8), and ethylenediamine tetraacetic acid (0.1%). Extracted proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked at room temperature for 2 hours with 5% non-fat dried milk in Tris-buffered saline Tween-20 buffer containing Tris-HCl (10 mmol/L, pH 8.0), NaCl (150 mmol/L) and Tween-20 (0.05%), and subsequently incubated with rabbit anti-NPY, NR1, NR2A, NR2B, and β-actin polyclonal antibodies (1:200 dilution; Beijing

Biosynthesis Biotechnology, Beijing, China) overnight at 4°C. After three washes with PBS plus Tween-20 (0.1%), the blots were detected with goat anti-rabbit IgG (1:10 000; Beijing Biosynthesis Biotechnology) tagged with horseradish peroxidase at room temperature for 2 hours. Protein expression was visualized according to the manufacturer's instructions using an enhanced chemiluminescence system (Thermo Fisher Scientific, Beijing, China) and exposed to X-ray film. Finally, relative absorbance of each band was analyzed with the software Image J (V1.41, NIH, USA) after normalization to the amount of individual β-actin protein.

Statistical analysis

Data are presented as mean ± SEM. Differences in the percentage of seizure severity of rats in different groups were calculated by chi-square test. Differences in the electroencephalogram seizure latency and frequency of rats, results of quantitative PCR and western blotting in different groups were analyzed by one-way analysis of variance and Student-Newman-Keuls test. A value of $P < 0.05$ was considered statistically significant.

Acknowledgments: We thank Linhua Jiang from the University of Leeds for help with preparation of the manuscript.

Author contributions: Changzheng Dong wrote the manuscript and performed the study. Xiufang Dong collected and analyzed the data. Wenling Li designed the study. Wenqing Zhao and Peiyuan Lv directed the research. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: The study was approved by the Animal Ethical Committee of Hebei Medical University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

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(Reviewed by Murnane K, Stow A, Wang KL, Shi RF)
(Edited by Yu J, Su LL, Li CH, Song LP)