

Neuropeptide Y suppresses epileptiform discharges by regulating AMPA receptor GluR2 subunit in rat hippocampal neurons

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Abstract. The present study aimed to investigate the effects of neuropeptide Y (NPY) on the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor glutamate receptor 2 (GluR2) subunit in epileptiform discharge hippocampal neurons. Hippocampal neurons were harvested from neonatal Sprague-Dawley rats aged <24 h and primarily cultured *in vitro*. At day 12 following culture, hippocampal neurons were divided into the following groups: Control, Mg^{2+} -free, NPY+ Mg^{2+} -free and BIBP3226+NPY+ Mg^{2+} -free. The action potential of neurons was measured using the whole cell patch clamp technique in the control, Mg^{2+} -free and NPY+ Mg^{2+} -free groups. AMPA current (I_{AMPA}) was detected and peak current density was calculated in each group. Alterations in total protein and phosphorylation of the GluR2 subunit were detected by western blot analysis, and GluR2 mRNA expression levels were detected by reverse transcription-quantitative polymerase chain reaction, in each group. The whole cell patch clamp technique demonstrated an abnormal action potential in the Mg^{2+} -free group. The frequency and amplitude of the action potential were significantly greater in the Mg^{2+} -free group compared with the control group, and significantly reduced in the NPY+ Mg^{2+} -free group compared with the Mg^{2+} -free group ($P<0.05$). In the Mg^{2+} -free group, compared with the control group, peak current density was significantly reduced ($P<0.05$), GluR2 subunit protein content was slightly reduced ($P>0.05$), phosphorylation levels of GluR2 subunit were significantly greater ($P<0.05$) and GluR2 mRNA was significantly reduced ($P<0.05$). In the NPY+ Mg^{2+} -free group, compared with the Mg^{2+} -free group, peak current density was significantly greater ($P<0.05$), phosphorylation levels of GluR2 subunit were significantly reduced ($P<0.05$) and

GluR2 mRNA expression was significantly greater ($P<0.05$). In the BIBP3226+NPY+ Mg^{2+} -free group, compared with the NPY+ Mg^{2+} -free group, peak current density was significantly reduced ($P<0.05$), phosphorylation levels of GluR2 subunit were significantly greater ($P<0.05$) and GluR2 mRNA expression was significantly reduced ($P<0.05$). After 3 h of treatment with Mg^{2+} -free extracellular fluid, epileptiform discharge was detected in the cells. NPY inhibited the discharge and its underlying mechanism may be that epileptiform discharge suppressed the function of the AMPA receptor GluR2 subunit. NPY relieved the inhibition of the GluR2 subunit via the Y1 receptor. This may provide a novel direction for future studies on the pathogenesis and treatment of epilepsy.

Introduction

Epilepsy is a common clinical disease characterized by abnormal discharge from neurons in the brain. The pathogenesis of epilepsy remains to be fully elucidated and its causes are complex. The hippocampus is the site of high concentration of neurons and has an important role in the pathogenesis of epilepsy (1). Excitatory and inhibitory neurotransmitters present in the central nervous system maintain normal cerebral function, and glutamate is an important excitatory neurotransmitter in the brain. Glutamate receptor (GluR) dysfunction may be an important cause of epilepsy (2). Excessive activation of GluRs may cause neuronal damage, a variety of neurological damage, and chronic neurodegenerative diseases (3). The α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is an important subtype of ionotropic GluRs (4) and is composed of four subunits (GluR1, 2, 3 and 4). The primary features of AMPA receptors are determined by GluR2, with GluR2 protein downregulation considered to be a molecular switch. Blocking or reducing GluR2 expression forms a calcium-permeable AMPA receptor, which increases Ca^{2+} influx and enhances endogenous glutamate excitotoxicity (5). In the central nervous system, the GluR2 subunit is highly expressed in various neurons. Under normal conditions, GluR2 is abundant in synapses. Following hypoxia, the expression of GluR2 on the surface of the neuron membrane is significantly decreased, indicating that the mechanism of blocking Ca^{2+} influx has weakened. Without a GluR2 subunit, the Ca^{2+} permeability of

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AMPA receptors increases and a large Ca^{2+} influx is detected, which may activate a series of intracellular protein kinases or immediate early genes. The decreased GluR2 expression induced by epileptic seizure causes an increase in the Ca^{2+} permeability of AMPA receptors, resulting in Ca^{2+} overload, which is an important cause of delayed neuronal death in hippocampal neurons (6). Neuropeptide Y (NPY) contains 36 amino acids and was first extracted from the brain tissue of pigs by Tatemoto *et al.* (7) in 1982. It is widely distributed in the central and peripheral nervous systems (8). In the central nervous system, NPY concentrations are greatest in the hippocampus. NPY has a protective effect on cerebellum neuronal cells cultured *in vitro* and has been demonstrated to possess an anti-epileptic effect (9); however, its protective effects are weakened following blocking of NPY Y1 and Y2 receptors, suggesting that NPY exerts neuroprotective effects via these receptors (10). The present study aimed to investigate the functional alterations in the GluR2 subunit induced by epileptiform discharge in hippocampal neurons and to investigate whether NPY affects these functional alterations.

Materials and methods

Animals and reagents. A total of 64 clean male Sprague-Dawley rats, which were born within 24 h were provided by the Experimental Animal Center of Hebei Medical University (animal license no. SCXK (Ji) 2013-1-003; Shijiazhuang, China). The rats were maintained in a 12 h light/dark cycle, humidity of $60\pm5\%$, $22\pm3^\circ\text{C}$. All rats were allowed free access to food and water. All animal experimental procedures were performed in strict accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals of National Institutes of Health (U.S.) and the protocol was approved by the Institutional Animal Care Committee of Hebei Medical University. Neurobasal medium, B-27, L-glutamine, fetal bovine serum (FBS; special grade) and Dulbecco's modified Eagle's medium (DMEM)/F-12 medium were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA); poly-L-lysine and trypsin from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany); and AMPA, NPY and BIBP3226 from Enzo Life Sciences, Inc. (Farmingdale, NY, USA).

Rabbit anti-microtubule-associated protein 2 (MAP-2) (cat. no. 17490-1-AP) polyclonal antibody and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G (cat. no. SA00003-2) were obtained from ProteinTech Group, Inc. (Chicago, IL, USA); rabbit anti-phosphorylated (p)-GluR2 (Tyr876) (cat. no. 4027) antibody and rabbit anti-GluR2 (cat. no. 5306) antibody from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti- β -actin (cat. no. sc-130300) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (cat. nos. 7072 and 7071) from Cell Signaling Technology, Inc. (Danvers, MA, USA). The random primers were obtained from Promega Corporation (Madison, WI, USA).

Culture of hippocampal neurons. Hippocampal neurons were primarily cultured as described by Yang *et al.* (11). Following intraperitoneal injection of anesthetic (pentobarbital sodium, 3 $\mu\text{l/g}$), Neonatal Sprague-Dawley rats aged <24 h were

Table I. Specific primers for GluR2 and GAPDH used in reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')	Product size (bp)
GluR2	F: CAAGTTCGCATACCTCTA R: TTATCCCTTTCACAGTCC	207
GAPDH	F: TGAACGGGAAGCTCACTGG R: GCTTCACCACCTTCTTGATGTC	120

GluR2, glutamate receptor 2; F, forward; R, reverse.

surface sterilized with disinfectant (75% alcohol) and sacrificed by decapitation and the brains were extracted and placed in DMEM/F-12 medium at 0°C . Bilateral hippocampi were harvested under an anatomical microscope. Following removal of the meninges, the brain tissue was cut into pieces and immersed in 0.125% trypsin (5X volume of brain tissue). The samples were digested in a 5% CO_2 incubator at 37°C for 15 min. The digestion was terminated by adding DMEM/F-12 medium containing 10% serum. All samples were triturated and filtered with a 200-mesh screen. The resulting cell suspension was adjusted to $\sim 1 \times 10^5/\text{ml}$ with DMEM/F-12 medium containing 10% FBS, seeded into 6-well plates with polylysine-coated coverslips (3 ml/well) and placed in a 5% CO_2 incubator at 37°C for 24 h. The medium was subsequently replaced with neuronal medium (Neurobasal medium), supplemented with 2% B-27, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Half of the neuronal medium was replaced with fresh every 3 days, for 7-9 days, following which the purified neurons were harvested.

Identification of hippocampal neurons. MAP-2 is a neuron-specific protein and a marker of neuronal differentiation (12). At day 9 of *in vitro* culture, the hippocampal neurons on glass slides were fixed with 4% paraformaldehyde for 30 min, washed three times with PBS for 5 min each time, permeabilized with 0.3% Triton X-100 for 10 min and washed three times with PBS for 5 min each time. Cells were blocked with 3% goat serum (OriGene Technologies, Inc., Beijing, China) at room temperature for 30 min and incubated with MAP-2 antibody (1:100) at 4°C overnight. Following three washes with PBS for 5 min each time, cells were incubated with a FITC-conjugated secondary antibody (1:100) at 37°C for 1 h, followed by a further three washes with PBS for 5 min each time. Cells were counterstained with Hoechst 33258 for 5 min, washed three times with PBS for 5 min each time and observed under a fluorescence microscope.

Group assignment. Following 12 days of *in vitro* culture, hippocampal neurons were assigned to the following groups: Control, Mg^{2+} -free, NPY+ Mg^{2+} -free and BIBP3226+NPY+ Mg^{2+} -free. In the control group, neurons were treated with normal extracellular fluid for 3 h. In the Mg^{2+} -free group, neurons were treated with Mg^{2+} -free extracellular fluid for 3 h. In the NPY+ Mg^{2+} -free group, neurons were incubated with cell culture fluid containing NPY at a final concentration of

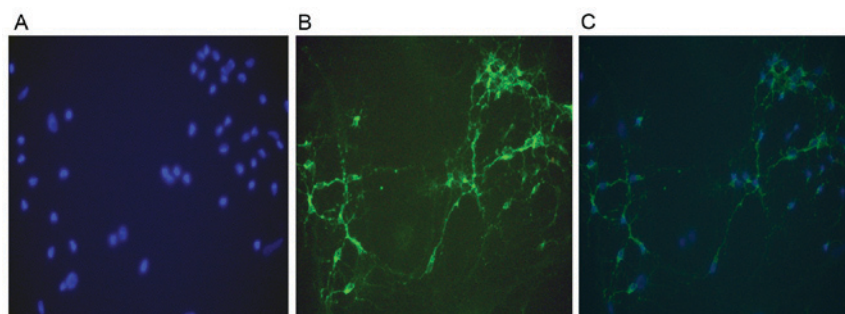


Figure 1. Primary cultured hippocampal neurons and cortical neurons demonstrated positive immunofluorescence staining with an MAP-2 antibody. (A) Blue fluorescent Hoechst 33258 nuclear staining, (B) green fluorescent MAP-2 staining and (C) merged image. Original magnification, x400. MAP-2, microtubule-associated protein 2.

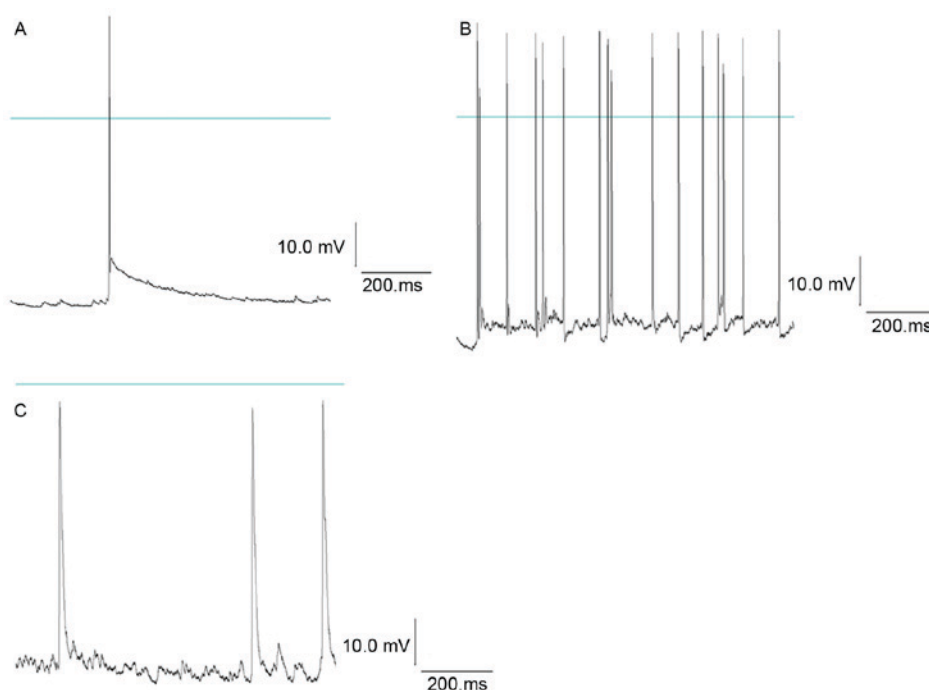


Figure 2. Action potential of rat hippocampal neurons treated with Mg^{2+} -free and NPY extracellular fluid. Action potential of (A) control group and hippocampal neurons treated with (B) Mg^{2+} -free and (C) $1 \mu M$ NPY-containing extracellular fluid. NPY, neuropeptide Y.

$1 \mu mol/l$ for 30 min and then with Mg^{2+} -free extracellular fluid for 3 h. In the BIBP3226+NPY+ Mg^{2+} -free group, neurons were incubated with cell culture fluid containing the NPY Y1 receptor blocker BIBP3226 at a final concentration of $1 \mu mol/l$ for 30 min, with NPY at a final concentration of $1 \mu mol/l$ for 30 min and finally with Mg^{2+} -free extracellular fluid for 3 h. Afterwards, all groups received normal extracellular fluid for 1 h. Cells were subsequently analyzed using the patch clamp technique, western blot analysis to measure GluR2 and phosphorylated GluR2 protein expression levels, and RT-qPCR to measure GluR2 mRNA expression levels.

Normal extracellular fluid comprised: NaCl, 147 mM; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM; glucose, 13 mM; KCl, 2 mM; $CaCl_2$, 2 mM; and $MgCl_2$, 2 mM; adjusted to pH 7.3 with 5 mM NaOH; osmotic pressure 280-320 mM. Mg^{2+} -free extracellular fluid comprised: NaCl, 147 mM; HEPES, 10 mM; glucose, 13 mM; KCl, 2 mM; and $CaCl_2$, 2 mM; adjusted to pH 7.3 with 5 mM NaOH; osmotic pressure 280-320 mM.

Using the patch clamp technique, the action potential of neurons was recorded in the control, Mg^{2+} -free and NPY+ Mg^{2+} -free groups in accordance with the method of DeLorenzo *et al* (13).

Detection of AMPA current (I_{AMPA}). I_{AMPA} in neurons was recorded using the patch clamp technique (14,15). Cell slides were placed in a 0.3 ml bath and perfused with extracellular fluid at 2 ml/min to ensure fluid exchange in 2 min. Cells were observed under an inverted microscope. Neurons with a distinct stereoscopic outline and smooth surface were used for the sealing experiment. With a three-dimensional manipulator, a glass microelectrode with impedance of 1-3 M Ω and pipette solution (Cs-gluconate, 110 mmol/l; CsCl, 30 mmol/l; HEPES, 10 mmol/l; EGTA, 0.2 mmol/l; NaCl, 8 mmol/l; Mg-ATP, 2 mmol/l; Na_3GTP , 0.3 mmol/l; phosphocreatine, 10 mmol/l; pH 7.2 adjusted with NaOH) was connected to the cell surface. Negative pressure was increased until the cells ruptured. Capacitive current and series resistance were compensated and

Table II. Comparison of frequency of action potential in three groups.

Group	Frequency (number/s)
Mg ²⁺ -free	13.86±2.19 ^a
NPY+Mg ²⁺ -free	1.89±0.69 ^b
Control	0.85±0.22

Data are expressed as the mean ± standard deviation (n=5). NPY, neuropeptide Y. ^aP<0.05 vs. control group; ^bP<0.05 vs. Mg²⁺-free. NPY, neuropeptide Y.

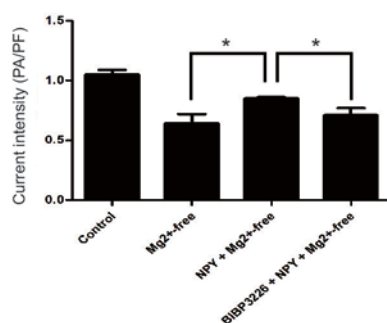


Figure 3. Current density of primary cultured rat hippocampal neurons induced by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid in control, Mg²⁺-free, NPY+Mg²⁺-free and BIBP3226+NPY+Mg²⁺-free groups. Data are expressed as the mean ± standard deviation (n=5). *P<0.05. NPY, neuropeptide Y.

a whole-cell recording created. Voltage was held at -70 mV for 5 min, following which the intracellular fluid and pipette solution were completely replaced, and 100 μ mol/l AMPA was administered to induce inward current. This current was I_{AMPA} . To further verify this, AMPA was measured following elution and the membrane current returned to normal. When 100 μ mol/l AMPA was administered, 10 μ mol/l 6-cyano-7-nitroquinoxaline-2,3-dione was used to block the AMPA receptor and the original inward current could not be detected. Thus, the recorded current was I_{AMPA} . To exclude error in different cells, peak current density was measured using pA/pF.

Western blot analysis of GluR2 protein expression and phosphorylation levels. Cells were harvested, washed with PBS, lysed with precooled radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) and centrifuged at 12,000 \times g at 4°C for 10 min. The supernatant, containing the total proteins from the hippocampal neurons, was collected. Protein concentrations were determined by the Lowry method. Total proteins (100 μ g) were separated by electrophoresis on 5% stacking and 10% separating gels at 100 V for 150 min. Separated proteins were transferred onto polyvinylidene difluoride membranes. Membranes were blocked by 5% non-fat milk in 0.01 mol/l PBS at room temperature for 2 h, incubated with primary antibody at 4°C overnight (1:1,000), washed three times, incubated with a horseradish peroxidase-conjugated secondary antibody (1:400) at 37°C for 1 h and washed three times. Protein bands

Table III. Comparison of amplitude of action potential in three groups.

Group	Amplitude (mV)
Mg ²⁺ -free	81.25±5.18 ^a
NPY+Mg ²⁺ -free	40.06±2.31 ^b
Control	35.56±1.23

Data are expressed as the mean ± standard deviation (n=5). ^aP<0.05 vs. control group; ^bP<0.05 vs. Mg²⁺-free. NPY, neuropeptide Y. NPY, neuropeptide Y.

were visualized with 3,3'-diaminobenzidine (Sangon Biotech Co., Ltd., Shanghai, China) and the gray values were measured and quantitatively analyzed with BandScan version 5.0 (Glyko; BioMarin Pharmaceutical, Inc., San Rafael, CA, USA). The experiments were performed in triplicate.

RT-qPCR analysis of GluR2 mRNA expression levels. Following the removal of cell medium, 1 ml TRIzol® was added to each well. The samples were triturated and placed in a ribozyme-free centrifuge tube for 5 min. Subsequently, 0.2 ml chloroform was added to each tube, which were vigorously agitated for 15 sec, rested for 5 min and centrifuged at 13,800 \times g at 4°C for 15 min. The supernatant was transferred to a new centrifuge tube, treated with an equal volume of isopropanol and centrifuged at 13,800 \times g at 4°C for 10 min. A feathery white precipitate was observed at the bottom of the tube, the supernatant was discarded and 1 ml 75% ethanol [prepared with diethyl pyrocarbonate (DEPC)-treated water] was added. The precipitate was washed and centrifuged at 5,400 \times g at 4°C for 5 min. Following removal of the supernatant, the sample was air-dried for 3-5 min. RNA was fully dissolved with 20-30 μ l of DEPC-treated water and its purity measured using an ultraviolet spectrophotometer. cDNA was synthesized using the EasyScript First-strand cDNA synthesis superMix kit (Beijing TransGen Biotech Co., Ltd., Beijing, China). Rat GluR2 primer sequences were synthesized by Promega Corporation (Table I). The reaction mixture was as follows: 2X UltraSYBR® Mixture (with ROX), 10 μ l; forward primer (10 μ mol/l), 1 μ l; reverse primer (10 μ mol/l), 1 μ l; cDNA, 8 μ l. The total reaction volume was 20 μ l. qPCR cycling conditions were as follows: An initial predenaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 20 sec and extension at 72°C for 27 sec. Following amplification, results were analyzed with an ABI 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative value (RQ value) of target gene expression to the internal reference gene GAPDH was detected and calculated (16).

Statistical analysis. The data were analyzed with SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA) using tests for normality and homogeneity of variance. Data that obeyed normality and homogeneity of variance were analyzed using analysis of variance for completely random design. Paired comparison was conducted with the least significant difference post hoc test.

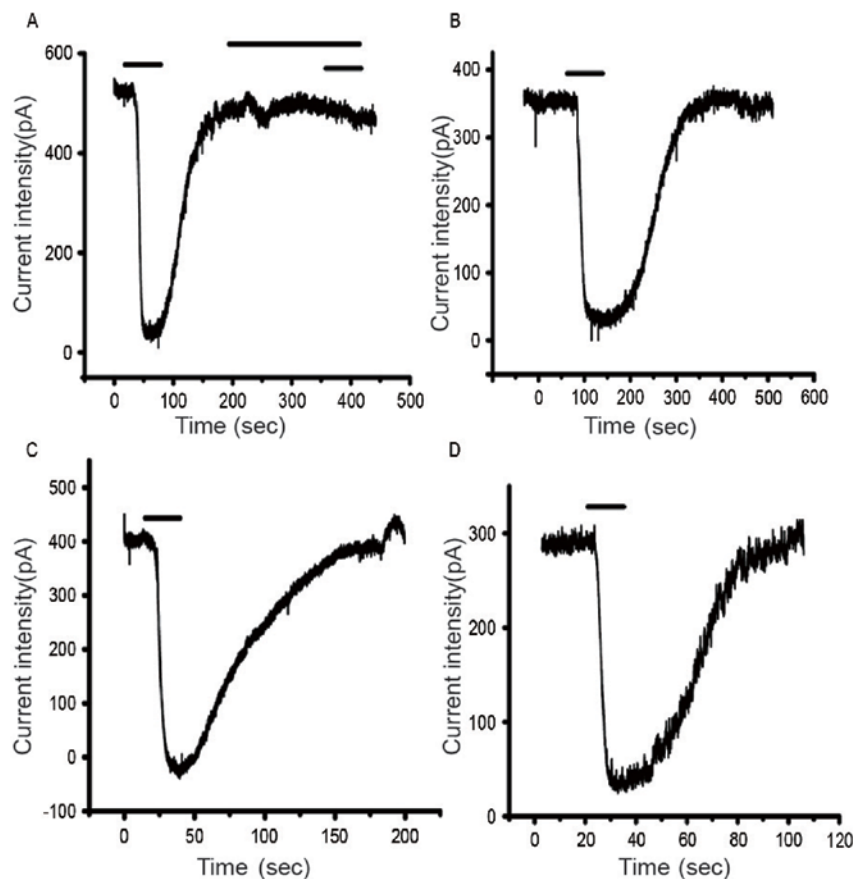


Figure 4. Current of primary cultured rat hippocampal neurons induced by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid. (A) Control, (B) Mg^{2+} -free, (C) NPY+ Mg^{2+} -free and (D) BIBP3226+NPY+ Mg^{2+} -free groups. NPY, neuropeptide Y.

Data were expressed as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

NPY suppresses epileptiform discharges in hippocampal neurons. Primary cultured hippocampal neurons had axons and dendrites. Immunostaining of MAP-2 revealed that neuronal purity was $>95\%$ (Fig. 1). The patch clamp technique records the normal action potential of hippocampal neurons. Following treatment with Mg^{2+} -free extracellular fluid for 3 h followed by normal extracellular fluid, a continuously stable action potential was detected in the neurons; the frequency and amplitude appeared greater compared with the control group, indicating spontaneous epileptiform discharges in the neurons. Following treatment with NPY $1 \mu\text{mol/l}$ for 30 min, Mg^{2+} -free extracellular fluid for 3 h and normal extracellular fluid, the frequency and amplitude of the action potential were significantly reduced in NPY+ Mg^{2+} -free group compared with the Mg^{2+} -free group ($P < 0.05$; Fig. 2; Tables II and III). This suggested that NPY significantly inhibited abnormal discharges in hippocampal neurons.

Epileptiform discharges in hippocampal neurons inhibit the function of AMPA receptor GluR2 subunit. I_{AMPA} detection results demonstrated that peak current density was significantly reduced in the Mg^{2+} -free group compared with the control group ($P < 0.05$; Figs. 3 and 4; Table IV). Protein

expression levels of GluR2 were slightly reduced ($P > 0.05$; Fig. 5; Table V) and those of p-GluR2 were significantly greater ($P < 0.05$; Fig. 6; Table VI) in the Mg^{2+} -free group compared with the control group. GluR2 mRNA expression levels were significantly reduced in the Mg^{2+} -free group compared with the control group ($P < 0.05$; Fig. 7; Table VII). These results suggested that epileptiform discharges in hippocampal neurons may induce the suppression of the function of the GluR2 subunit.

NPY relieves the inhibition of GluR2 subunit function induced by epileptiform discharges in hippocampal neurons. Peak current density was significantly greater in the NPY+ Mg^{2+} -free group compared with the Mg^{2+} -free group ($P < 0.05$; Figs. 3 and 4; Table IV). Protein expression levels of GluR2 were slightly increased ($P > 0.05$; Fig. 5; Table V) and those of p-GluR2 were significantly reduced ($P < 0.05$; Fig. 6; Table VI) in the NPY+ Mg^{2+} -free group compared with the Mg^{2+} -free group. GluR2 mRNA expression levels were significantly greater in the NPY+ Mg^{2+} -free group compared with the Mg^{2+} -free group ($P < 0.05$; Fig. 7; Table VII). These findings indicated that NPY weakened the inhibition of GluR2 function induced by epileptiform discharges in neurons.

NPY regulates GluR2 subunit function possibly via the Y1 receptor. Peak current density was significantly reduced in the BIBP3226+NPY+ Mg^{2+} -free group compared with the NPY+ Mg^{2+} -free group ($P < 0.05$; Figs. 3 and 4; Table IV). Protein

Table IV. Current density of primary cultured hippocampal neurons induced by α -amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid.

Group	Current density (pA/pF)
Control	1.05 \pm 0.04
Mg ²⁺ -free	0.64 \pm 0.08 ^a
NPY+Mg ²⁺ -free	0.85 \pm 0.01 ^b
BIBP3226+NPY+Mg ²⁺ -free	0.71 \pm 0.06 ^a

Data are expressed as the mean \pm standard deviation (n=5).

^aP<0.05 vs. control group; ^bP<0.05 vs. Mg²⁺-free and BIBP3226+NPY+Mg²⁺-free groups. NPY, neuropeptide Y.

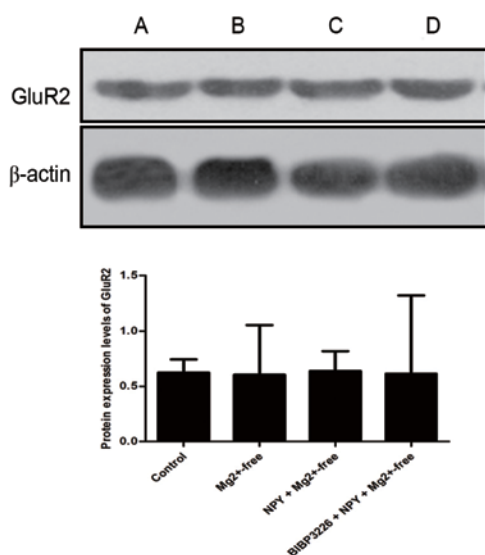


Figure 5. Protein expression of GluR2 as assessed by western blot analysis. (A) Control, (B) Mg²⁺-free, (C) BIBP3226+NPY+ Mg²⁺-free and (D) NPY+Mg²⁺-free groups. GluR2, glutamate receptor 2; NPY, neuropeptide Y. Protein expression levels of GluR2 as assessed by densitometry following western blot analysis. Data are expressed as the mean \pm standard deviation (n=5). GluR2, glutamate receptor 2; NPY, neuropeptide Y.

expression levels of GluR2 were slightly reduced ($P>0.05$; Fig. 5; Table V) and those of p-GluR2 were significantly greater ($P<0.05$; Fig. 6; Table VI) in the BIBP3226+NPY+Mg²⁺-free group compared with the NPY+Mg²⁺-free group. GluR2 mRNA expression levels were significantly reduced in the BIBP3226+NPY+Mg²⁺-free group compared with the NPY+Mg²⁺-free group ($P<0.05$; Fig. 7; Table VII).

Discussion

Epilepsy is a brain disorder characterized by the abnormal discharge of neurons in the brain. The pathogenesis of epilepsy remains unclear and it may be that a variety of factors contribute to its occurrence. Neuronal loss occurs, and is accompanied by a large number of abnormal discharge neurons in the lesion. Ion channel dysfunction in cells causes seizures; this is the 'epileptic neuron' theory (17,18). The hippocampus is the site of a high concentration of neurons and

Table V. Protein expression levels of GluR2, as assessed by western blot analysis.

Group	GluR2
Control	0.6241 \pm 0.15
Mg ²⁺ -free	0.6057 \pm 0.25
NPY+Mg ²⁺ -free	0.6397 \pm 0.18
BIBP3226+NPY+Mg ²⁺ -free	0.6146 \pm 0.21

Data are expressed as the mean \pm standard deviation (n=5). GluR2, glutamate receptor 2; NPY, neuropeptide Y.

Table VI. Protein expression levels of p-GluR2, as assessed by western blot analysis.

Group	p-GluR2
Control	0.3879 \pm 0.21
Mg ²⁺ -free	1.3173 \pm 0.17 ^a
NPY+Mg ²⁺ -free	0.8918 \pm 0.07 ^b
BIBP3226+NPY+Mg ²⁺ -free	1.0483 \pm 0.08 ^a

Data are expressed as the mean \pm standard deviation (n=5).

^aP<0.05 vs. control group; ^bP<0.05 vs. Mg²⁺-free and BIBP3226+NPY+Mg²⁺-free groups. GluR2, glutamate receptor 2; p, phosphorylated; NPY, neuropeptide Y.

possesses an important role in the pathogenesis of epilepsy: Hippocampal sclerosis is a common cause of temporal lobe epilepsy. The structure of the nervous system is complex and there are numerous factors restricting its study *in vivo*. Electrophysiological testing, including the patch clamp technique, is not easy to implement. Hippocampal slices and neuronal cultures have attracted increasing attention in the study of epilepsy (19).

Mg²⁺ serves an important role in maintaining normal electric activity in the central nervous system (20). DeLorenzo *et al* (13) reported that the removal of Mg²⁺ from the cell medium, or treatment with Mg²⁺-free extracellular fluid for 3 h, may successfully induce spontaneous recurrent epileptiform discharges in primary cultured hippocampal neurons. Such cultured neurons do not possess real anatomical connections or clinical manifestations, but the spontaneous recurrent action potential evoked by Mg²⁺-free conditions is similar to the electrophysiological activity during epileptic seizures and anti-epileptic drugs may prevent such action potentials (21-22). This technique has been used in studies of the biochemical, electrophysiological and molecular mechanisms of acquired epilepsy (23).

NPY is widely distributed in the central nervous system (cerebral cortex, hippocampus, thalamus, hypothalamus and brain stem) and the peripheral nervous system. In the central nervous system, NPY concentration is greatest in the hippocampus. NPY has an anti-epileptic effect (24,25) and a protective effect on cerebellum neuronal cells cultured *in vitro*. These protective effects are weakened following blocking of

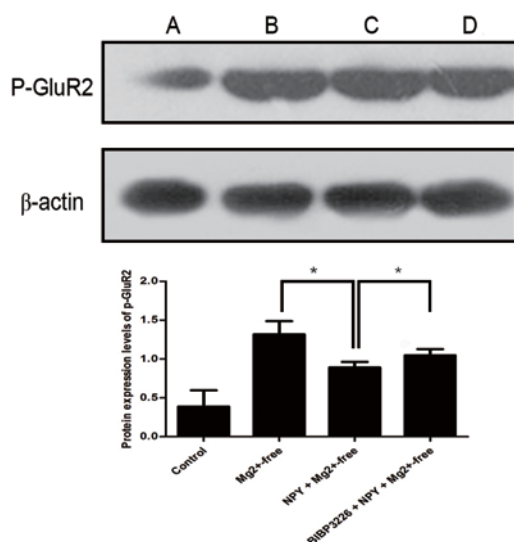


Figure 6. Protein expression of p-GluR2 as assessed by western blot analysis. (A) Control, (B) Mg^{2+} -free, (C) BIBP3226+NPY+ Mg^{2+} -free and (D) NPY+ Mg^{2+} -free groups. GluR2, glutamate receptor 2; p, phosphorylated; NPY, neuropeptide Y. Protein expression levels of p-GluR2 as assessed by densitometry following western blot analysis. Data are expressed as the mean \pm standard deviation (n=5). * $P<0.05$. GluR2, glutamate receptor 2; p, phosphorylated; NPY, neuropeptide Y.

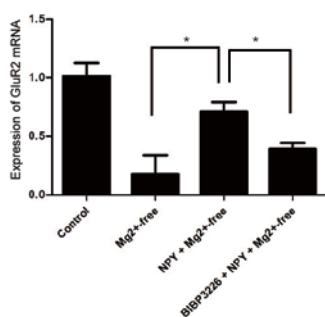


Figure 7. mRNA expression levels of GluR2, as assessed by reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean \pm standard deviation (n=5). * $P<0.05$ vs. control group. GluR2, glutamate receptor; NPY, neuropeptide Y.

the NPY Y1 and Y2 receptors, suggesting that NPY exerts neuroprotective effects via the Y1 and Y2 receptors (26-28). In the present study, epileptiform discharges were detected in hippocampal neurons of rats following treatment with Mg^{2+} -free extracellular fluid. Following treatment with NPY and Mg^{2+} -free extracellular fluid, epileptiform discharges were notably weakened, indicating that NPY may suppress epileptiform discharges in neurons.

Excitatory and inhibitory neurotransmitters present in the central nervous system are responsible for the balance of a complex network and maintain normal cerebral function. Following excitatory neurotransmitter increases, or inhibitory neurotransmitter decreases, the ratio may become unbalanced. GluR functional alteration is one of the important causes of epilepsy (29). Excessive activation of GluRs may cause neuronal damage, a variety of neurological damage, and chronic neurodegenerative diseases, including cerebral ischemia and hypoxia, epilepsy and brain trauma. Previous studies have confirmed

Table VII. mRNA expression levels of GluR2, as assessed by reverse transcription-quantitative polymerase chain reaction.

Group	GluR2
Control	1.016 \pm 0.11
Mg^{2+} -free	0.179 \pm 0.16 ^a
NPY+ Mg^{2+} -free	0.713 \pm 0.08 ^b
BIBP3226+NPY+ Mg^{2+} -free	0.394 \pm 0.05 ^a

Data are expressed as the mean \pm standard deviation (n=5).

^a $P<0.05$ vs. control group; ^b $P<0.05$ vs. Mg^{2+} -free and BIBP3226+NPY+ Mg^{2+} -free groups. GluR2, glutamate receptor 2; NPY, neuropeptide Y.

that following epilepsy, a massive release of glutamate and overactivation of its receptors causes a Ca^{2+} influx, followed by delayed neuronal death and secondary injury (30,31).

GluR may be divided into metabotropic types (coupled with G protein) and ionotropic types (containing ion channels). In accordance with pharmacological properties, molecular characteristics and electrophysiological properties, ionotropic GluR may be divided into three subtypes: AMPA receptor, N-methyl-D-aspartate receptor and kainate receptor (32).

The AMPA receptor, a type of ion channel protein, may be regulated by membrane potential, glutamate and AMPA. It is composed of four subunits (GluR1, 2, 3 and 4) encoded by different genes (33). Due to the low calcium ion permeability induced by mRNA Q/R site editing, the relative content of GluR2 subunit in the AMPA receptor determines the functional properties of AMPA receptors. An AMPA receptor containing the GluR2 subunit is permeable to monovalent cations (Na^+ , K^+), but not to divalent cations (Ca^{2+}); an AMPA receptor without a GluR2 subunit is highly permeable to Ca^{2+} (34,35). The primary features of AMPA receptors are therefore determined by GluR2 and GluR2 protein downregulation is considered to be a molecular switch (36). Blocking or reducing GluR2 expression forms a calcium-permeable AMPA receptor, increases Ca^{2+} influx and enhances endogenous glutamate excitotoxicity.

In the central nervous system, the GluR2 subunit is highly expressed in various neurons. Under normal conditions, GluR2 is abundant in synapses. Following hypoxia, the content of GluR2 on the membrane surface of the neuron is significantly decreased and the number of synapses containing GluR2 reduced, indicating that the mechanism of blocking Ca^{2+} influx has markedly weakened (37).

The results of the present study demonstrated that, following treatment with Mg^{2+} -free extracellular fluid, GluR2 subunit mRNA expression levels were decreased, but protein phosphorylation levels were increased, suggesting that the GluR2 subunit had been reduced or its activity decreased in neuronal membranes. Abnormal activation of neurons diminished AMPA receptor GluR2 subunit expression. It has previously been demonstrated that without a GluR2 subunit, the Ca^{2+} permeability of AMPA receptors increases and a large Ca^{2+} influx may be detected, able to activate a series of

intracellular protein kinases or immediate early genes (38) and resulting in a decrease in GluR2 subunit expression. Grooms *et al* (39) hypothesized that the decreased GluR2 expression induced by epileptic seizure caused an increase in the Ca^{2+} permeability of AMPA receptors, resulting in Ca^{2+} overload, an important cause of delayed neuronal death in hippocampal neurons. Sanchez *et al* (40) demonstrated that perinatal hypoxia-induced seizures increased the Ca^{2+} permeability of AMPA receptors.

The present study used a whole cell patch clamp technique to record I_{AMPA} in neurons cultured *in vitro*. AMPA is a selective agonist of the exogenous AMPA receptor, and is synthesized artificially. AMPA binding to AMPA receptor depolarizes the cell membrane and opens ion channels. Results from the present study demonstrated that I_{AMPA} was reduced in the Mg^{2+} -free group compared with the control group. The decreased degree of I_{AMPA} was markedly diminished in the NPY+ Mg^{2+} -free group compared with the Mg^{2+} -free group. This effect was inhibited by BIBP3226. These results indicated that epileptiform discharges in cells may induce a reduction in the functions of AMPA receptors.

The AMPA receptor is a membrane receptor, the function of which may be regulated by multiple internal and external cell processes; phosphorylation has the greatest influence on its function (41). PDZ domain-containing proteins may interact with the PDZ structural domain at the GluR2 C terminus following binding to activate protein kinase C- α , which induces ser880 phosphorylation at the GluR2 C terminus and endocytosis of the GluR2 complex, reducing the expression of AMPA receptor GluR2 subunit on the surface of neurons and thus serving an important role in AMPA receptor expression and transport (42,43).

I_{AMPA} is the most direct indicator reflecting electrophysiological alterations in AMPA receptors. A decrease in I_{AMPA} reflects a reduction in receptor function. During epileptiform discharges, I_{AMPA} falls, indicating that AMPA receptor activity is reduced. Based on the RT-qPCR and western blot analysis results, it is hypothesized that epileptiform discharges in hippocampal neurons led to the phosphorylation of the AMPA receptor GluR2 subunit on the membrane surface and suppressed GluR2 subunit mRNA. The AMPA receptor GluR2 subunit on the membrane surface was transported into cells, so AMPA receptor activity on the membrane surface diminished. Therefore, neuron total protein detection did not alter significantly between groups. The lack of the normal AMPA receptor GluR2 subunit pathway on the membrane surface causes a high Ca^{2+} permeability and may induce a rapid Ca^{2+} influx, Ca^{2+} concentration increase and a series of pathological reactions.

I_{AMPA} and GluR2 mRNA expression levels were significantly greater, but GluR2 protein phosphorylation levels were significantly reduced in the NPY+ Mg^{2+} -free group compared with the Mg^{2+} -free group. It is hypothesized that NPY may inhibit the alterations in AMPA receptor function induced by epileptiform discharges, suggesting that NPY may have affected AMPA receptor functions and avoided excessive reduction of I_{AMPA} by regulating the GluR2 subunit. The inhibitory effect of NPY was suppressed by treatment with the Y1 receptor blocker BIBP3226. NPY exerts different effects by binding to different receptors; NPY receptors belong to

the G protein-coupled receptor family (44) and contain Y1, Y2, Y3, Y4 and Y5 subtypes in humans. Different NPY receptors are distributed in different regions of the body; the Y1 receptor is primarily expressed in the cerebral cortex, amygdala and hippocampus (45). Previous studies on the anti-epileptic effect of NPY have primarily focused on Y2 and Y5 receptors (46,47). It is hypothesized that NPY exerted an anti-epileptic effect in the present study via the Y1 receptor expressed on the membrane surface of hippocampal neurons. This may be a pharmacological mechanism underlying the anti-epileptic effect of NPY.

In conclusion, the present study investigated the functional alterations in the AMPA receptor GluR2 subunit and the effect of NPY on these alterations during epileptiform discharges in hippocampal neurons, to provide a theoretical basis for specifically blocking pathological process and for the design of therapeutic agents with low toxicity for the treatment of epilepsy.

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