

Neuroprotective effects of subthalamic nucleus high-frequency stimulation on substantia nigra neurons in a Parkinson's disease rat model*

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Abstract

BACKGROUND: Deep-brain stimulation has proven to be beneficial in the treatment of Parkinson's disease (PD) patients.

OBJECTIVE: To investigate the effects of high-frequency stimulation (HFS) to the subthalamic nucleus (STN) on neuronal apoptosis and apoptosis-related gene expression in the substantia nigra pars compacta, and to analyze the neuroprotective effect of HFS-STN.

DESIGN, TIME AND SETTING: Neuronal morphology experiments were performed in the Beijing Neurosurgical Institute from May to December in 2005.

MATERIALS: Forty healthy, adult, Sprague Dawley rats were used to establish a PD model with a unilateral microinjection of 6-hydroxydopamine into two target areas of the right medial forebrain bundle. 6-hydroxydopamine was purchased from Sigma (USA); high-frequency electrical stimulator was produced by World Precision Instruments (USA); Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit was a product of Nanjing Jiancheng Technology Co., Ltd. (China); and Bcl-2 and Bax protein assay kit were purchased from Wuhan Boster Bioengineering Co., Ltd. (China).

METHODS: Forty rats were randomly divided into three groups. The stimulation group ($n = 15$) received HFS-STN on the day of PD modeling. The PD model group ($n = 15$) was used to establish the PD model. The control group ($n = 10$) was injected with normal saline containing 0.2 g/L ascorbic acid into two areas of the right medial forebrain bundle.

MAIN OUTCOME MEASURES: Survival of dopaminergic neurons in the substantia nigra pars compacta was determined using Nissl staining. Apoptosis of dopaminergic neurons was detected using TUNEL techniques. Expression of anti-apoptotic protein, Bcl-2, and pro-apoptotic protein, Bax, were assayed by immunohistochemistry.

RESULTS: Following 6-hydroxydopamine injection, the number of substantia nigra pars compacta neurons was reduced in the stimulation and PD model groups, compared to the control group. At 2 and 4 weeks post-surgery, the grey value of Nissl stained images was significantly less in the PD model and stimulation groups ($P < 0.05$), and the stimulation group exhibited greater grey values compared to the model group ($P < 0.05$). At 2 and 4 weeks post-surgery, the number of apoptotic neurons was significantly less in the stimulation group compared to the model group ($P < 0.05$). In addition, Bcl-2 and Bax expression, as well as the Bcl-2/Bax ratio, was much higher in the stimulation group compared to the model group ($P < 0.05$).

CONCLUSION: HFS-STN has a neuroprotective effect on dopaminergic neurons in the substantia nigra pars compacta of PD rats by promoting Bcl-2 expression, inhibiting Bax expression, and reducing the number of apoptotic dopaminergic neurons.

Key Words: apoptosis; deep-brain stimulation; high-frequency stimulation; Parkinson's disease

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Supported by: Postdoctoral Science Foundation of China, No. 20070420399*

Ma Y, Wang ZC, Zhang JG, Liu HG, Zhang Y. Neuroprotective effects of subthalamic nucleus high-frequency stimulation on substantia nigra neurons in a Parkinson's disease rat model. Neural Regen Res 2008;3(9):1014-7

www.sjzsyj.com/Journal/0809/08-09-1014.html

INTRODUCTION

Many studies have demonstrated that cellular apoptosis plays an important role in the occurrence and development of Parkinson's disease (PD)^[1-4]. The process of establishing PD models using neurotoxins, such as 6-hydroxydopamine (OHDA) and 1-methyl-4-phenyl-tetrahydropyridine (MPTP),

can induce apoptosis of cerebral neurons in rats. Autopsy results also confirmed the appearance of apoptotic neurons in the substantia nigra of the midbrain in PD patients^[5-6].

Therefore, it is assumed that the disease process of PD might be delayed or inhibited by interfering with the process of cellular apoptosis.

Several studies^[7-8] have revealed that abnormal neuronal activity in the subthalamic nucleus (STN) of PD patients is a

Received: 2008-07-31; Accepted: 2008-08-20 (08-S-1-0022/H)

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leading cause of neuron loss in the substantia nigra pars compacta (SNc) of the midbrain. Inhibition of abnormal activity in the STN may result in neuroprotective effects. High-frequency stimulation (HFS) and posteroventral pallidotomy are two ways to treat PD; however, HFS is optimal [9-12]. In some studies [13-14], electrostimulation to the bilateral STN reduced neuronal loss in the rat SNc and increased tyrosine hydroxylase expression, thereby protecting the neurons. Neuronal apoptosis in the SNc of the midbrain has been seldom reported in PD. This study aimed to investigate the effects of HFS-STN on neuronal apoptosis in the SNc of PD rats.

MATERIALS AND METHODS

Design

Experiments of neuronal morphology.

Time and setting

Experiments were performed in the Beijing Neurosurgical Institute (Beijing Key Laboratory, Grade B) from May to December in 2003.

Materials

Fifty healthy, adult, male, Sprague Dawley rats of SPF grade were offered by Vital River Lab Animal Technology Co., Ltd. (No. SCXX2007-0001, China), and weighed (250 ± 30) g. The experimental procedures were performed in accordance with the animal care guidelines of the National Institute of Health.

Reagents and instrument	Sources
Brain solid positioner and automatic injection machine	Stoelting (USA)
6-OHDA and apomorphine	Sigma (USA)
Electrostimulator	World Precision Instruments (USA)
TUNEL kit	Nanjing Jiancheng Bioengineering Co., Ltd. (China)
Bcl-2 and Bax protein assay kits	Wuhan Boster Bioengineering Co., Ltd.(China)

Experimental procedure

Grouping intervention

Forty rats were divided into three groups: normal control ($n = 10$), PD model ($n = 15$), and stimulation ($n = 15$).

Stimulation group: rats were i.p. anesthetized and fixed to a brain solid positioner. According to the Rat Stereotaxic Atlas [15], two coordinates in the right medial forebrain bundle were defined: one was 3.5 mm posterior to the bregma, 0.5 mm right of the midline, 9 mm inferior to the dura; the other was 4.4 mm posterior to the bregma, 1.5 mm right of the midline, 7.8 mm inferior to the dura. Two burr holes were made with a dental drill. PD models were prepared by injecting 6-OHDA (2.5 μ L and 3 μ L, respectively) into the two holes with a 10- μ L microinjector. On the day of modeling, a stimulating electrode was implanted into the right STN at the coordinate of 4.0 mm posterior to the bregma, 2.6 mm right of the midline, and 8.0 mm inferior to the dura. The implanted electrode was fixed using dental base acrylic resin. HFS was

performed to the STN for 60 minutes every 24 hours at an intensity of 2 V, a width of 0.12 ms, a frequency of 130 Hz. Model group: 6-OHDA (2.5 μ L and 3.0 μ L, respectively) was injected into the two sides of the right medial forebrain bundle to establish PD models.

Control group: both sides of the right medial forebrain bundle were injected with normal saline containing 0.2 g/L ascorbic acid.

Pathological observation

Rats were sacrificed at 2 and 4 weeks after model establishment. The substantia nigra was paraffin-embedded, following routine perfusion and fixation, and was sectioned into continuous coronal slices at a thickness of 6–8 μ m. Nissl staining was utilized to observe the remaining neurons in the substantia nigra. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit was used to detect apoptotic cells. Bcl-2 and Bax protein immunohistochemistry methods were utilized to determine expression of the apoptosis-related proteins Bcl-2 and Bax. Image analysis of Nissl-stained slices was performed using the CIAS1000 color pathological analysis system (LEICA Company, Japan). Each slice was observed under high-power microscopy through three visual fields of the SNc, and the average grey value was measured.

Behavioral observations

All rats were injected with a subcutaneous injection of apomorphine (0.5mg/kg, 0.5 g/L) at 2 or 4 weeks post-surgery to induce rotational behavior. Ten minutes later, apomorphine-induced rotation frequency was recorded for a total of 30 minutes. PD models were considered successful if the frequency was more than 7 cycles per minute.

Main outcome measures

Survival of dopaminergic neurons in the SNc; dopaminergic neuronal apoptosis; Bcl-2 and Bax expression.

Design, enforcement and evaluation

Jianguo Zhang designed the study, and Yu Ma, Huanguang Liu, and Ying Zhang evaluated it. All authors received formal training.

Statistical analysis

Data management was completed by Yu Ma using SPSS 11.0 software. All data were expressed as Mean \pm SD by *t*-test and one-way analysis of variance. A level of $P < 0.05$ was considered statistically significant.

RESULTS

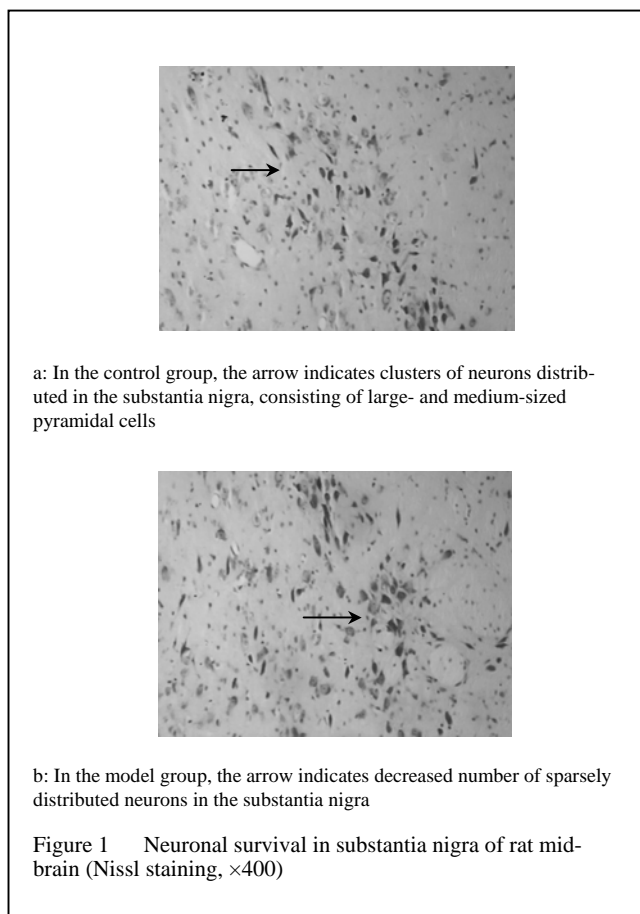
Quantitative analysis of experimental animals

Forty rats were included in the present study. Successful PD models were not established in three animals from the model group and two animals from the stimulation group. In summary, 35 rats were included in the final analysis, comprising 10 in the normal control group, 12 in the model group, and 13 in the stimulation group.

Survival and apoptosis of dopaminergic neurons, expression of Bcl-2 and Bax in the SNc rat midbrain

Compared with the control group, the number of neurons in the SNc was reduced in the model and stimulation groups following 6-OHDA injection. The Nissl bodies were fuzzy,

and particle density was decreased (Figure 1).



The Nissl-stained images of SNc at 2 and 4 weeks post-surgery exhibited significantly lower grey values in the model and stimulation groups compared to the control group ($P < 0.05$), and the stimulation group value was greater than the model group ($P < 0.05$) (Table 1).

Table 1 Percentage of Bcl-2-, Bax-, and TUNEL-positive cells in substantia nigra of rat midbrain ($\bar{x} \pm s$)

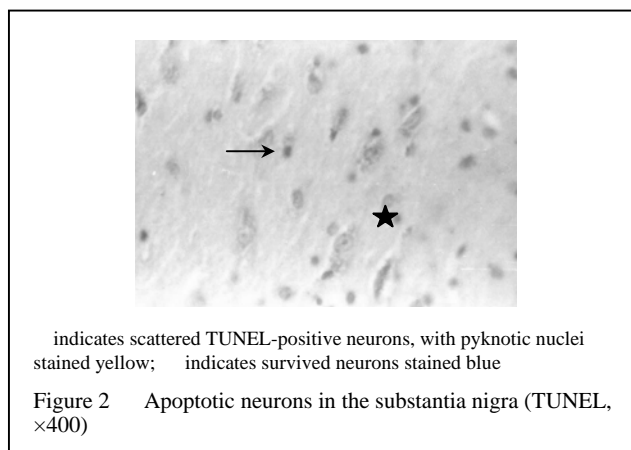
Index	Control group	Model group	
		2 wk	4 wk
Nissl (grey value)	168.16 \pm 11.59	117.29 \pm 10.62 ^a	119.13 \pm 11.78 ^a
TUNEL (%)	4.21 \pm 0.49 ^b	61.74 \pm 7.82	67.12 \pm 10.23
Bcl-2 (%)	32.14 \pm 1.12 ^b	18.57 \pm 0.92	15.12 \pm 2.11
Bax (%)	25.14 \pm 1.03 ^b	49.15 \pm 6.18	51.22 \pm 8.35
Bcl-2/Bax	1.33 \pm 1.11 ^b	0.39 \pm 0.15	0.30 \pm 0.58

Index	Stimulation group	
	2 wk	4 wk
Nissl (grey value)	129.97 \pm 14.33 ^{ab}	129.44 \pm 16.21 ^{ab}
TUNEL (%)	39.98 \pm 12.11 ^b	41.12 \pm 9.23 ^b
Bcl-2 (%)	25.63 \pm 3.76 ^b	26.24 \pm 4.12 ^b
Bax (%)	30.35 \pm 3.71 ^b	29.78 \pm 5.66 ^b
Bcl-2/Bax	0.84 \pm 1.01 ^b	0.88 \pm 0.81 ^b

^a $P < 0.05$, vs. control group; ^b $P < 0.05$, vs. model group

At 2 and 4 weeks post-surgery, neurons in the rat SNc

midbrain were TUNEL-positive in the model and stimulation groups (Figure 2).



At two and four weeks post-surgery, the percentage of positive apoptotic neurons in the stimulation group was significantly less than the model group ($P < 0.05$). Expression of Bcl-2 and Bax, as well as the Bcl-2/Bax ratio, was greater than the model group ($P < 0.05$).

Behavioral changes

The PD model group was the first to exert induced rotational behavior with the fastest frequency of rotation. At one week post-surgery, there were no significant differences in rotation frequency between the stimulation group and the model group ($P > 0.05$). However, the frequency was remarkably less in the stimulation group compared to the model group at 2 and 4 weeks ($P < 0.05$) (Table 2). The rats undergoing HFS-STN exhibited ameliorated abnormal activities, such as irritability, tail stiffness, and delayed walking.

Table 2 Comparison of rotation frequency and appearance ($\bar{x} \pm s$)

Group	Appearance time of postoperation rotation (d)	Rotation frequency (r/min)		
		1 wk postop-eration	2 wk postop-eration	4 wk postop-eration
Control group	-	1.75 \pm 0.87	2.11 \pm 1.56 ^a	1.23 \pm 1.11 ^a
Model group	6.4 \pm 0.84	9.85 \pm 2.59	13.45 \pm 2.07	12.13 \pm 4.54
Stimulation group	7.67 \pm 1.37	7.83 \pm 1.47	10.50 \pm 0.58 ^a	9.11 \pm 1.92 ^a

^a $P < 0.05$, vs. model group

DISCUSSION

Nissl staining does not only specifically stain for neurons, but can be utilized to detect pyknotic neurons. Mochizuki *et al* [5-6] have demonstrated that increased apoptosis of dopaminergic neurons in PD patients. Excessive apoptosis of dopaminergic neurons in the SNc is a dominant factor for neurological impairment. Ziv *et al* [2] hypothesized that regulation of cell apoptosis is crucial for preventing degradation of substantia nigra corpus striatum. In the present study, dopaminergic neurons in the substantia nigra evoked retrograde degeneration as a result of damaged nerve fibers in the substantia nigra corpus striatum pathway.

With this method, it was possible to study the apoptotic effects of 6-OHDA injections to the medial forebrain bundle^[16-17]. The number of positive apoptotic neurons in the SNc of rats undergoing HFS-STN was remarkably fewer than in the PD model group, which suggests that the SNc neurons were neuroprotected, and further verifies that abnormal STN excitability can delay neuronal loss in the SNc^[7, 18-19]. Cellular apoptosis is associated with anti-apoptotic proteins (Bcl-2 and Bcl-XL) and pro-apoptotic proteins (Bax, Bcl-XS, and Bad). The balance between cell death and survival is regulated by Bcl-2 and Bax expression, as well as the ratio of Bcl-2 to Bax^[20-22]. The Bcl-2/Bax ratio can be used as an indicator of apoptosis. Results from the present study showed high levels of Bcl-2 expression and a large Bcl-2/Bax ratio. These results suggest that HFS can induce *bcl-2* gene expression. According to the study by Temel *et al*^[13], bilateral HFS-STN may increase neuronal numbers in the SNc and tyrosine hydroxylase expression in PD rats. In addition, HFS-STN can increase the survival of dopaminergic neurons or delay cell death of dopaminergic neurons. Moreover, abnormal behavior in PD rats was delayed following HFS-STN treatment, and manifestations, such as irritability, tail stiffness, and delayed walking, were obviously improved. Similarly, motor symptoms of PD patients have been shown to slowly improve at 4 years post-surgery subsequent to clinical treatment of deep-brain stimulation to the STN^[9]. In the present study, 60 minutes of HFS exerted neuroprotective effects within 2 weeks. However, the long-term effects of HFS remain unclear, as well as the mechanisms involved in HFS-induced decreased apoptosis of dopaminergic neurons in the rat midbrain SNc, and the correlation between HFS and complex loop function of the basal nuclei.

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Background: The experiments have been sponsored by the China Post-doctoral Science Fund (No. 20070420399), which chiefly focuses on the application, underlying mechanism and effect on deep-brain stimulation.

Contributor: Professor Zhongcheng Wang, Doctoral supervisor, is an academician of Chinese Academy of Engineering. He is a specialist on the research of basic theory and clinical application of the neurosurgery in China, as well as neuroimaging. Professor Wang has performed over ten thousand neurosurgical operations and he is one of only some physicians who underwent cerebral aneurysm operation for over 1 000 cases. The present study was performed under his direction.

Bias or limitations: Deep-brain stimulation is a long-term treatment, so this study is failed to detect the protective effect of continuous deep-brain stimulation on substantia nigra pars compacta in rat model of Parkinson's disease.

(Edited by Chen B/Yang Y/Wang L)