Research report

Long-term high-frequency electro-acupuncture stimulation prevents neuronal degeneration and up-regulates BDNF mRNA in the substantia nigra and ventral tegmental area following medial forebrain bundle axotomy

Xi-Bin Liang, Xian-Yu Liu, Feng-Qiao Li, Yong Luo, Jun Lu, Wang-MingZhang, Xiao-Min Wang*, Ji-Sheng Han

Neuroscience Research Institute, Peking University, 38 Xueyuan Road, Beijing 100083, P.R. China

Abstract

Electroacupuncture (EA) has been used in China for many years to treat Parkinson's disease (PD) with reportedly effective results. However, the physiological and biological mechanism behind its effectiveness is still unknown. In the present study, different frequencies of chronic EA stimulation (0, 2, 100 Hz) were tested in a partially lesioned rat model of PD which was induced by transection of the medial forebrain bundle (MFB). After 24 sessions of EA stimulation (28 days after MFB transection), dopaminergic neurons in the ventral midbrain were examined by immunohistochemical staining, and brain-derived neurotrophic factor (BDNF) mRNA levels in ventral midbrain were measured by in situ hybridization. The results show a marked decrease of dopaminergic neurons on the lesioned side of the substantia nigra (SN) comparing with the unlesioned side. Zero Hz and 2 Hz EA stimulation had no effect on the disappearance of dopaminergic neurons. However, after 100 Hz EA, about 60% of the tyrosine hydroxylase (TH)-positive neurons remained on the lesioned side of the SN. In addition, levels of BDNF mRNA in the SN and ventral tegmental area (VTA) of the lesioned side were significantly increased in the 100 Hz EA group, but unchanged in the 0 and 2 Hz groups. Our results suggest that long-term high-frequency EA is effective in halting the degeneration of dopaminergic neurons in the SN and up-regulating the levels of BDNF mRNA in the subfields of the ventral midbrain. Activation of endogenous neurotrophins by EA may be involved in the regeneration of the injured dopaminergic neurons, which may underlie the effectiveness of EA in the treatment of PD.
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1. Introduction

The main pathological change of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Previous studies have shown that such neuronal degeneration may be the result of a reduction in the supply of neurotrophic factors that are required for the survival of the affected neurons. If this were the case, then neurotrophic factors could potentially be useful in treating

PD by slowing the ongoing loss of dopaminergic neurons in the SNpc, increasing the activity of the remaining dopaminergic neurons, or inducing sprouting of collateral axons from residual dopaminergic neurons [38]. BDNF is one such potential neurotrophic factor [5,18]. It has been shown to support the survival and differentiation of fetal mesencephalic dopaminergic neurons in vitro [39] and protect them from selective lesion induced by 1-methyl-4-phenylpyridinium (MPP$^+$) or 6-hydroxydopamine (6-OHDA). Administration of BDNF can also protect mesencephalic dopaminergic neurons from 6-OHDA lesions in vivo [13,37]. However, it has been reported that

*Corresponding author. Fax: +86-10-6209-1150.
E-mail address:* xmwang@bjmu.edu.cn (X.-M. Wang).

BDNF, if injected intermittently, was unable to prevent the loss of axotomized nigral neurons [19,20], questioning whether BDNF can be used as an effective tool in the treatment of PD. Continuous infusion of BDNF at a high dose can completely prevent the loss of axotomized nigral neurons in vivo [14]. Intrastriatal grafts of fibroblasts genetically engineered to produce BDNF can partially prevent the loss of nerve terminals and completely prevent the loss of cell bodies of the nigrostriatal dopaminergic pathway induced by intrastriatal injection of 6-OHDA [21,26]. These findings suggest that BDNF can support the neurons in vivo if diffusion limitation can be overcome.

BDNF is widely distributed throughout the brain including the striatum, SNpc and VTA [16]. Moreover, the finding that BDNF co-localizes with its functional receptor Trk B in the dopaminergic neurons suggests that BDNF may support the dopaminergic neurons via an autocrine or paracrine mechanism [40]. A reduced autocrine/paracrine effect of BDNF might contribute to the degeneration of the dopaminergic neurons owing to lack of trophic support. Therefore, approaches that can enhance the level of BDNF expression within dopaminergic neurons may be an alternative approach for treatment and improvement of PD.

In China, acupuncture has been used to treat PD for many years, and many clinical reports have illustrated the promising result of EA in alleviating the sufferings of PD patients [7,8,25,30,42,43]. Similar results have also been obtained by using EA to treat other central nervous system (CNS) disorders such as optic nerve atrophy, cerebral vascular disorder, and spinal cord injury. However, the physiological mechanism underlying this phenomenon is still unknown. We hypothesize that it might be related to the fact that EA could activate the endogenous neurotrophic factors. The present study was conducted to evaluate the effect of EA stimulation on both the dopaminergic neurons and BDNF mRNA in the SN and VTA in these regions in rats.

2. Materials and methods

2.1. Medial forebrain bundle axotomy

Adult female Wistar rats weighing 180–200 g were obtained from the Laboratory Animal Center, Peking University, and housed in a standard 12:12-h light/dark cycle with food and water ad libitum. Rats were anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and positioned in a stereotaxic apparatus (Kopf Instruments, Tuyunga, CA) with the mouthbar set at −3.3 mm. Lesions were performed using a retractable wire knife (Scouten knife, Kopf Instruments) as described in [6,41]. Briefly, the knife was lowered through a drill-hole 3.8 mm posterior and 2.4 mm lateral to bregma to a ventral position of 8.0 mm below bregma. The blade was extended by 2.0 mm and the knife slowly moved upward by 2.5 mm, and

subsequently down again by 2.5 mm. The blade was then retracted and the knife withdrawn. Then the skin was closed by stitches.

2.2. EA stimulation

Rats were divided into five groups: the normal group ($n=10$), the MFB lesioned control group ($n=10$), the MFB lesioned group followed by 0 Hz ($n=9$), 2 Hz ($n=8$) or 100 Hz ($n=8$) of EA. For the EA groups, stimulation was administered from the second day following MFB lesions. Two stainless-steel needles of 0.25 mm diameter and 5 mm length were inserted obliquely at the acupuncture point DAZHUI (Du 14, just below the spinous process of the vertebra prominens) and horizontally at BAIHUI (Du 21, at the midpoint of the line connecting the two ears). Bi-directional square-wave electrical pulses (0.2 ms duration, 2 and 100 Hz), designated as EA, were given for a total of 30 min each day, 6 days per week. The intensity of the stimulation was increased stepwise from 1 to 2 mA and then to 3 mA, with each step lasting for 10 min. Twenty-eight days after the lesion and a total of 24 sessions of EA, animals were decapitated.

2.3. Tissue collection and processing

Three rats from each group were randomly selected, anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and killed by transcardial perfusion of 100 ml saline followed by 200 ml 4% paraformaldehyde (PFA) in phosphate buffer (PB). Brains were dissected and post-fixed in the same fixative and cryoprotected with 30% sucrose for 3–5 days. The brains were frozen on powdered dry ice and then blocked for frontal sectioning according to the rat brain atlas of Paxinos and Watson [32]. Frozen sections (15 μm in thickness) were cut on the cryostat at −20 °C and mounted onto 3-aminopropyltriethoxysilane (APES)-coated glass slides. Alternate sections were used for immunohistochemistry and in situ hybridization.

2.4. Immunohistochemistry

All sections spanning the SN were collected and used to determine the number of TH-positive cells. The primary antibody used to visualize TH was purchased from Sigma–Aldrich (1:10 000). Sections were washed twice in PBS and then incubated for 48 h at 4 °C with primary antibody in PBS containing 2% bovine serum albumin, 0.2% Triton X-100. To block non-specific binding, 1% goat serum was present in the incubation medium for the primary antibody. Subsequent incubations at room temperature were performed with 1% anti-mouse immunoglobulin followed by a preformed conjugate of avidin and biotinylated horseradish peroxidase. The peroxidase was visualized with a standard diaminobenzidine-hydrogen peroxide chromogen reaction.

TH-Positive cell bodies were counted in eight equally spaced sections throughout the entire rostrocaudal extent of the SN. Dopaminergic neurons were counted in the SN (A9+A8) and the VTA (A10). Only cell bodies with a recognizable nucleus and at least one neuritic process were counted.

2.5. In situ hybridization with BDNF oligodeoxynucleotide probe

A non-radioactive in situ hybridization approach was used to localize BDNF mRNA expression in the SN and VTA. A BDNF oligo probe complementary to 746–795 of the pig BDNF cDNA [12,34] was synthesized by Sangon Co., Shanghai, China. This oligonucleotide has 90% identity with the rodent sequence and its specificity has previously been verified [12]. It was labeled with a digoxigenin oligonucleotide 3′-end labeling kit according to the protocol (Boehringer Mannheim). Sections were first warmed up to room temperature and then dried in an oven at 50 °C for 1 h. After rinses in PB and pretreatment with proteinase K (1 μg/ml), the sections were transferred into PFA/PBS and then acetylated. After pre-hybridization with hybridization mix (20% deioned formamide, 10% dextran sulphate, 500 μg/ml salmon sperm DNA, 2× SSC and yeast tRNA), the sections were covered with 25 μl hybridization mix containing 0.5 μg/ml DIG-labeled oligo probe, and hybridized for 12–16 h at 37 °C. Post-hybridization washes were undertaken at 37 °C in 2× SSC, 1× SSC and 0.25× SSC for 45 min with gentle shaking. For immunodetection and color staining, the procedures were performed as recommended by the detection kit from Boehringer Mannheim.

Methodological controls for ISH included pretreating the sections with RNase (20 μg/ml) or excluding probe from the hybridization buffer.

2.6. Quantitative analysis

For each slice, substantia nigra compacta (SNc), substantia nigra reticulate (SNr), and VTA on both the lesioned and non-lesioned side were analyzed using an advanced image-analysis system (MCID Model M2, Imaging Research Inc., St. Catherines, ON, Canada). Acquisition of the integrated optic density (IOD) was accomplished by using a 40×objective under normal bright-field illumination (Nikon, Japan). Data were resolved relative to a 255-level gray scale and the value was converted to IOD units. Outlines of SNc, SNr and VTA were identified according to the atlas of Paxinos and Watson [32]. IOD data were obtained by measuring four different regions in the same structure. Background values derived from areas where hybridization signal was absent were subtracted from the values recorded for the hybridized cells. At least five slices from each animal were recorded. Results are expressed as mean±S.E.M. Statistical significance was assessed using one-way analysis of variance followed by Newman–Keuls' post hoc test. Significance was set at $P<0.05$.

3. Results

3.1. Lesion identification

Alternate tissue sections through the lesion site were collected for Nissl staining to inspect the path of the knife and for TH immunohistochemical staining. If the path was too far from the MFB site, the rat was excluded from this study. MFB lesion reduced the number of TH-positive cell bodies to 40–50% of that on the control side in both SN and VTA.

3.2. Effect of EA on the survival of dopaminergic neurons after MFB axotomy

As shown in Figs. 1 and 2, the number of TH-positive neurons on the lesioned side decreased sharply compared with the control side 4 weeks after the lesion. In the MFB lesioned group, the number of TH-positive neurons on the lesioned side was 42.3±5.7% of the non-lesioned side. In animals that received 100 Hz EA (Fig. 1E), the number was 60.7±5.1%. However, in animals that received 0 Hz (Fig. 1C) and 2 Hz EA (Fig. 1D), only 40.4±4.9% and 52.7±4.7% remained compared to the unlesioned side (Fig. 2).

3.3. Effect of EA on the gene expression of BDNF in VTA

High levels of expression of BDNF mRNA can be found in VTA, SNc and SNr (Fig. 3). No signal was detected in the slices used for methodological controls (Fig. 3A), confirming the specificity of the BDNF oligodeoxynucleotide probe used.

In VTA, normal rat has a high level of BDNF mRNA expression (Fig. 3B). Twenty-eight days after the MFB lesion (Fig. 3C), the gene expression of BDNF did not show visible down-regulation compared with that of the normal controls. EA stimulations of 0 and 2 Hz produced no significant changes to the BDNF mRNA on either the lesioned side or the non-lesioned side (Fig. 3D,E). However, 100 Hz EA (Fig. 3F) significantly up-regulated BDNF mRNA expression on the lesioned side of the VTA compared with that of the lesioned control group (Fig. 4).

3.4. EA at 100 Hz enhances BDNF mRNA at the SNc but not SNr at the MFB lesioned side

As seen in Figs. 3 and 4, high levels of BDNF mRNA expression were also found in the SNc and SNr regions. The expression of BDNF mRNA in the SNc region was
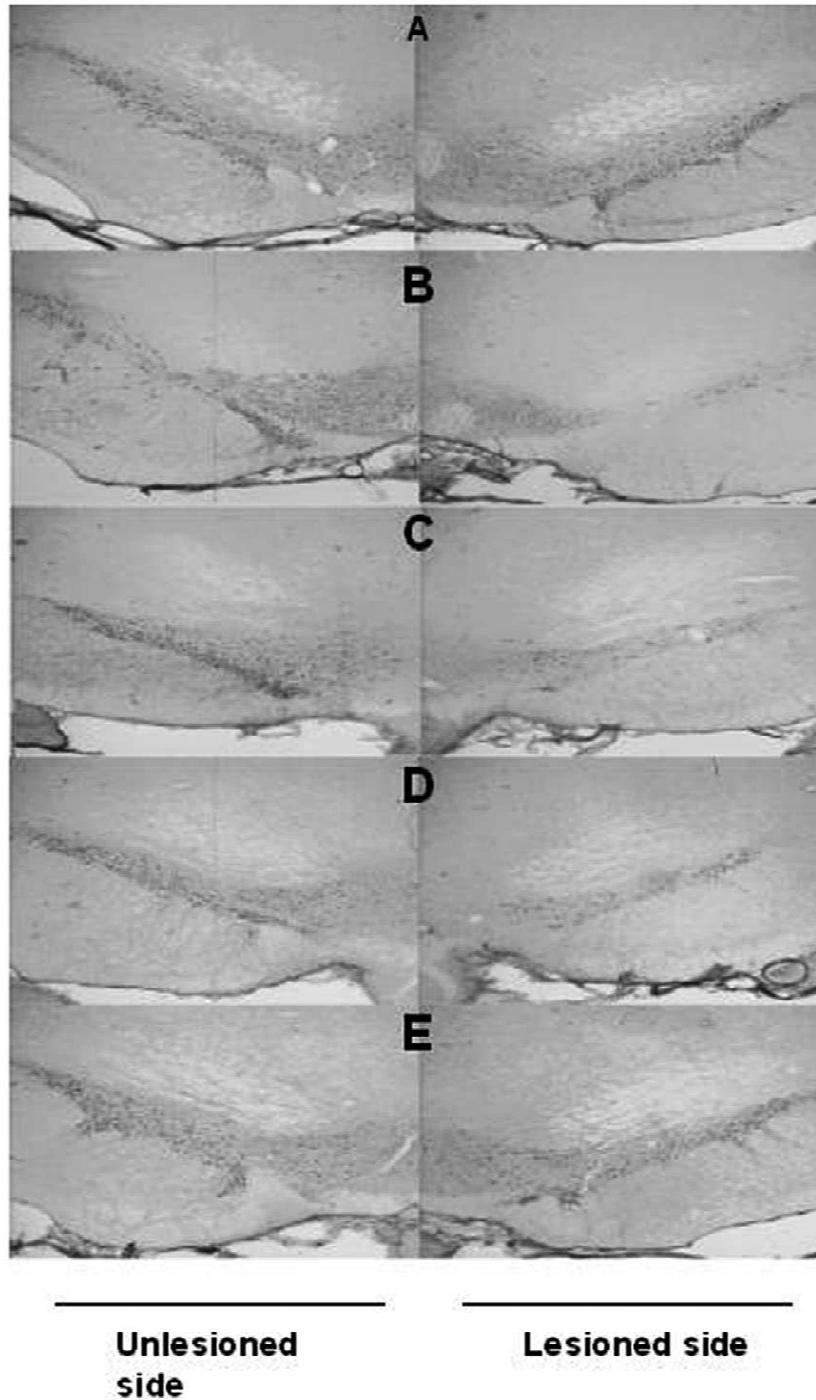
Fig. 1. Tyrosine hydroxylase (TH) immunohistochemical staining of dopaminergic neurons in the SN and VTA. Shown are the normal control group (A), the MFB lesioned group (B), and the MFB lesioned group followed by 0 (C), 2 (D) and 100 Hz (E) EA stimulation. Twenty-eight days after the MFB lesion, a pronounced loss of TH-positive cells is seen on the lesioned side in the MFB lesioned control group. Considerable loss of TH-positive neurons is also seen in the MFB lesioned groups followed by 0 and 2 Hz EA stimulation. But in animals that had been given 100 Hz EA, there remain relatively more neurons. Bar=200 μm.

more abundant than that in the SNr due its localization within the dopaminergic neurons. In the SNr region, no significant change could be found on either the lesioned or the non-lesioned side of the above groups. In the SNc, BDNF gene expression on the non-lesioned side of the

above groups remained stable; a slight, though non-significant decrease change was detected on the lesioned side of the MFB lesioned control group. No visible change in BDNF mRNA occurred in the 0 and 2 Hz EA treated groups. A higher level of BDNF mRNA expression can be
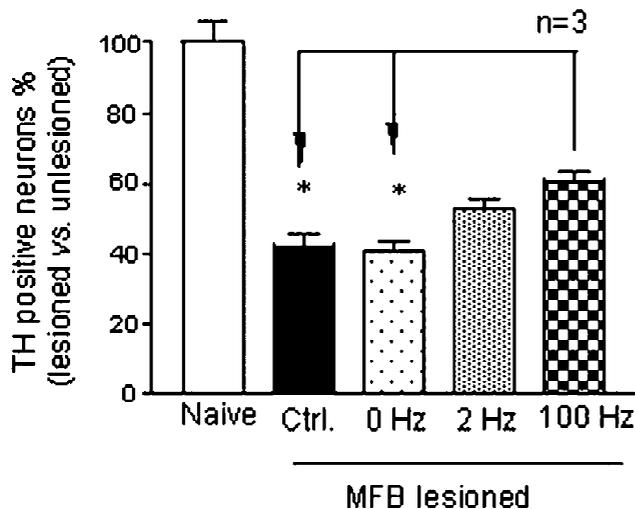
Fig. 2. Percentage of TH-positive neurons in the SNc and VTA (the lesioned side versus the unlesioned side). Dopaminergic neurons were counted in the SN (A9+A8) and VTA (A10). *$P<0.05$ (total of eight equally spaced sections throughout the entire rostrocaudal extent of the SN were counted).

seen in the 100 Hz EA group (Fig. 3F and Fig. 4). By comparing the IOD value of the lesioned side, a significant difference ($P<0.05$) was observed between the 100 Hz EA group and the MFB lesioned control group (Fig. 4).

4. Discussion

Employing EA in treating PD is relatively common in China, and clinical reports suggest effectiveness in this therapy [7,8,25,30,42,43]. Animal studies have shown that EA can attenuate the rotational behavior of PD rats and elevate the dopamine content in the brain of PD mice or rats [15,27,45]. The present study demonstrated for the first time that long-term high-frequency EA stimulation halts the degeneration of dopaminergic neurons following MFB axotomy and enhances BDNF mRNA levels in the SN and VTA region. Similar studies have also provided evidence that peripheral stimulation could affect the gene expression and activity of TH. Electric stimulation of preganglionic nerves for 30 min increases the TH activity 33% in the stimulated side over the control side, suggesting that the brief stimulation of a cell group or pathway may trans-synaptically activate gene expression [35]. We have found that electrical stimulation of acupoint ST-36 can rapidly induce expression of *c-fos*-encoded protein in the dopaminergic neurons of the VTA [28]. Therefore, we sought to examine the nature of peripheral stimulation on the nervous system.

After MFB transection, DA neurons in the rat showed a modest degree of cell degeneration, providing a good model for studying the regenerative growth capacity of injured SN dopaminergic neurons [6]. In the present study

we demonstrated that after long term 100 Hz stimulation, over 60% TH-positive neurons on the lesioned side of the SN still remained. This was significant ($P<0.05$) when compared with the lesioned control or 0 Hz EA group (but not the 2 Hz EA group), indicating that EA at high frequency, but not lower frequency, could halt the progressive degeneration of the dopaminergic neurons in the SN after MFB lesion.

Neurotrophic factors are considered potential therapeutic candidates for treating PD. BDNF is one of the most widely distributed such factor within the brain. Evidence demonstrates that BDNF has profound influences on the survival and development of dopaminergic neurons. However, the localization of BDNF in the caudate–putamen or the accumbens, the principal targets of the dopaminergic cells of the SNpc and the VTA, has not been reported. The trophic factor may be produced locally in the immediate vicinity of the responsive neuron. Accordingly the neurons could derive neurotrophic support via autocrine and paracrine mechanisms [36]. The finding that BDNF mRNA together with its receptor *trkB* mRNA was co-localized within the DA neurons [36,40] indicated that dopaminergic neurons might depend on the local synthesis of BDNF for neurotrophic support. Further studies have also demonstrated that BDNF and its specific *trkB* receptor immunoreactivity are widely distributed in the SN and VTA [2,10,44], providing good evidence that BDNF and *trkB* mRNA are translated to BDNF and *trkB* protein in the CNS. Neurotrophins produced by the dopaminergic neurons may act on the same cell through an autocrine mechanism or upon neighboring cells via a paracrine mechanism. Although there is still no evidence to support the hypothesis that PD results from the decrement in levels of neurotrophic factors or their receptors, two recent studies revealed that there are very substantial losses of BDNF mRNA and protein in the SN of PD patients [29,31]. The finding that antisense oligonucleotides to BDNF disrupt endogenous BDNF synthesis in mature sensory neurons, which results in their death by apoptosis [1] provides a further evidence that neurotrophic autocrine loops may exist and be essential for the maintenance of mature dopaminergic neurons. Thus it is conceivable that decreased levels of BDNF or a related neurotrophin in the SN could be a contributing factor in PD [23,24], and up-regulation of the levels of BDNF or other neurotrophins might be helpful for the maintenance of dopaminergic neurons.

By using in situ hybridization with a DIG-labeled oligodeoxynucleotide probe, we demonstrated that high-frequency EA can greatly enhance the levels of BDNF mRNA in the SNc and VTA on the lesioned side of MFB transected rats. This increase is probably an indication of the increased translation of BDNF, which may exert its trophic effect to the injured DA neurons and prevent the loss of their immunoreactivity.

Apart from BDNF, GDNF [33], neurturin [17], NT-3
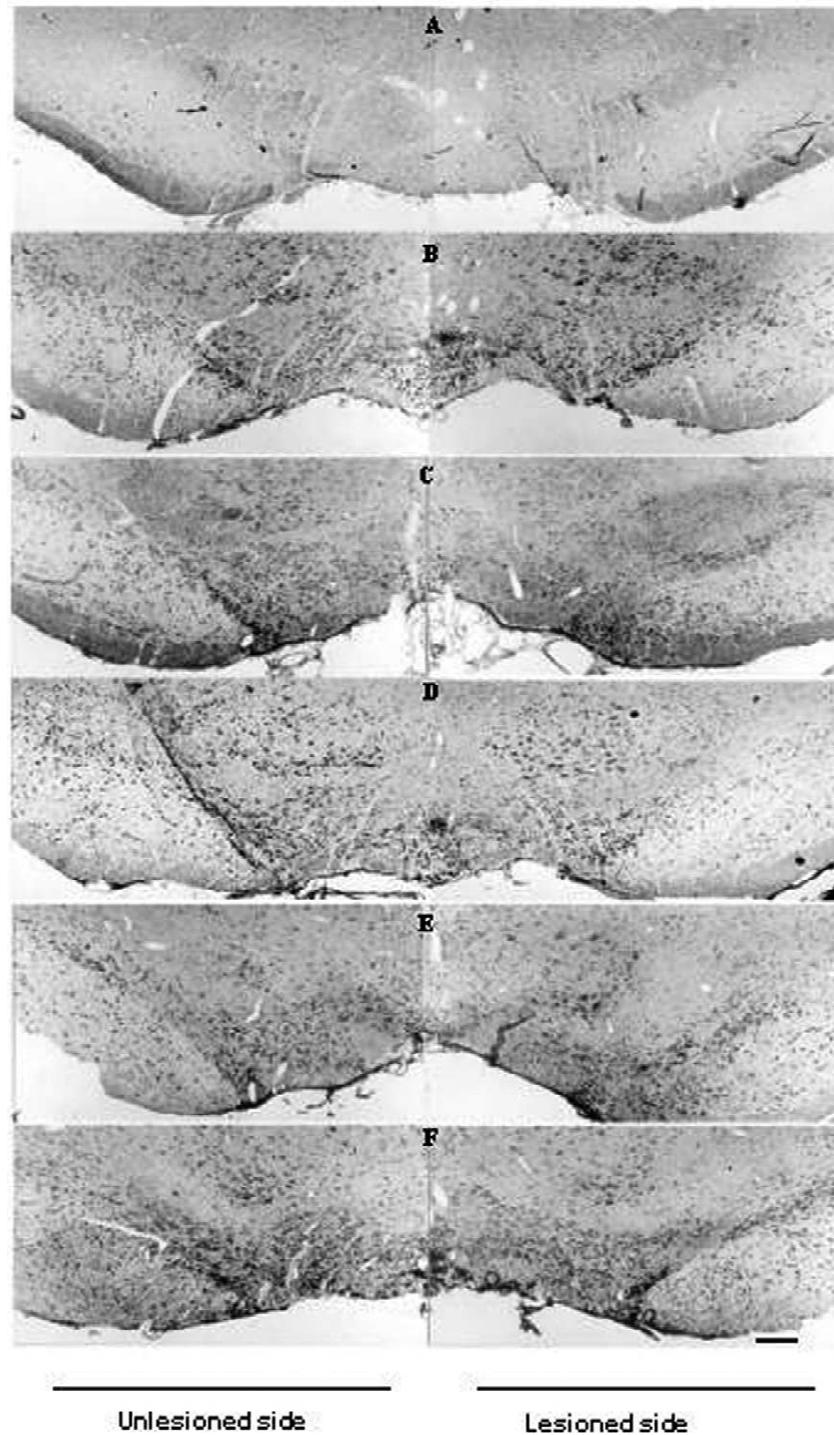
Fig. 3. Expression of BDNF mRNA in the ventral midbrain. No signal was detected in the slices treated for methodological controls (A). In the normal group, a high level of BDNF mRNA can be found in both the SNc and VTA (B). Twenty-eight days after the MFB lesion, the gene expression of BDNF did not show visible down-regulation compared with that of the normal controls (C). EA stimulations of 0 and 2 Hz produced no significant changes in BDNF mRNA either at the lesioned side or the non-lesioned side (D,E). However, 100 Hz EA stimulation (F) significantly up-regulated BDNF mRNA on the lesioned side of the VTA compared with that in the lesioned control group. Bar=200 μm.

[36], bFGF [9] and aFGF [3] were also found to co-localize with TH in the dopaminergic neurons of the SN and VTA. These factors have neurotrophic effects on the dopaminergic neurons in vitro [4,11,17,22]. However, we cannot exclude the possibility of the existence of more unknown neurotrophic factors that exert an influence over the dopaminergic neurons in the SN or VTA region. Therefore, multiple neurotrophic factors may be involved
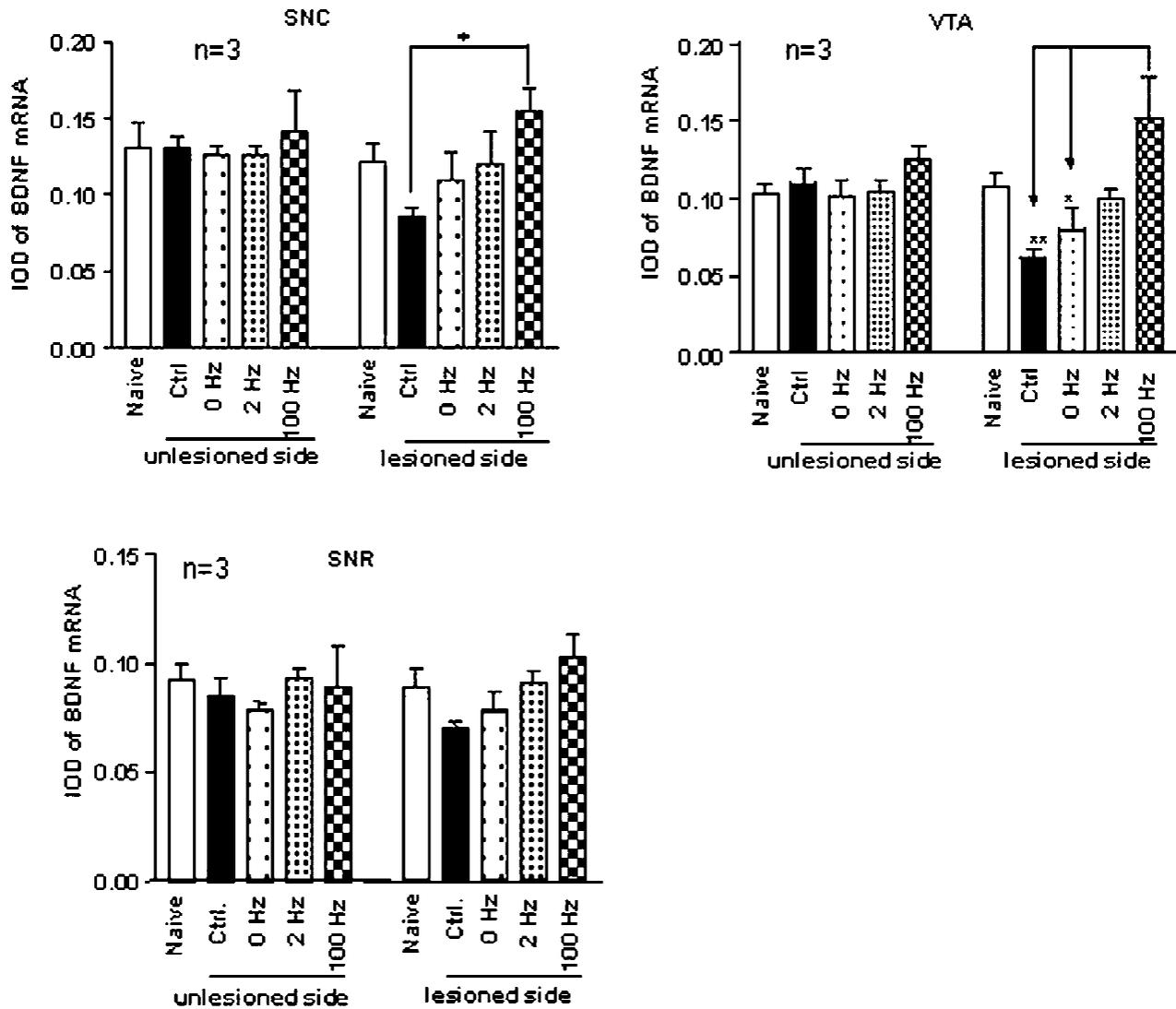
Fig. 4. Integrated optic density (IOD) of BDNF mRNA in the VTA, SNc and SNr. SNc, SNr and VTA were outlined according to the atlas of Paxinos and Watson [32]. IOD data were obtained by measuring four different regions in the same structure and subtracting a background value from areas where hybridized cells were absent. At least five slices from each animal were recorded. *$P<0.05$; **$P<0.01$.

in the maintenance of DA neurons. Their action in the process of EA still requires further investigation.

5. Conclusion

In conclusion, we have shown that long-term high-frequency, but not lower frequency, EA is effective in halting the degeneration of dopaminergic neurons in the SN and up-regulating the levels of BDNF mRNA in the lesioned side of the SNc and the VTA. BDNF may act locally on the dopaminergic neurons via an autocrine/ paracrine way or may be anterogradely transported to the site of the transected axons for repair of the resultant damage. High-frequency EA may increase the autocrine activity of BDNF. The activation of other endogenous neurotrophins by EA may also be involved in the regenera-

tion of the injured dopaminergic neurons, which may, together, underlie the potential effectiveness of EA in treating PD.

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