Electro-acupuncture improves behavior and upregulates GDNF mRNA in MFB transected rats

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Low and high frequency electro-acupuncture (EA) stimulation was used in rats that had been lesioned by medial forebrain bundle transection. Behavioral tests showed that both low and high frequency EA stimulation significantly reduced the amphetamine-induced rotation 2 weeks after the lesion but only high frequency EA improved the rotational behavior at 4 weeks. Analysis of the dopamine content in the striatum did not show any significant change after EA. In situ hybridization showed that high frequency EA stimulation up-regulated the glial cell line-derived neurotrophic factor (GDNF) mRNA in both sides of the globus pallidus, while low frequency EA only affected the unlesioned side. It suggests that the retrograde nourishment of GDNF to the dopaminergic neurons and the balanced activity of different nuclei in the basal ganglia circuit after EA may contribute to the behavioral improvement in these rats, which might be the factors that underlie the effectiveness of EA in the treatment of Parkinson's disease. NeuroReport 14:1177–1181 © 2003 Lippincott Williams & Wilkins.

Key words: Electro-acupuncture; Glial cell-line-derived neurotrophic factor; Parkinson's disease; Rat; Regeneration

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease characterized by the progressive loss of nigral dopaminergic (DAergic) neurons. Previous studies have shown that such neuronal loss may be the result of a reduction in the supply of neurotrophic factors that are required for the survival of the affected neurons [1,2]. Several neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor, epidermal growth factor, neurotrophin-4/5 and glial cell line-derived neurotrophic factor (GDNF) have been shown to promote the survival and physiological properties of mesencephalic DAergic neurons in vitro and to prevent neuronal degeneration after injury-induced cell death in animal PD models (for review see [3]). Among these factors, GDNF has been shown to be the most potent protective for DAergic nigral neurons [4]. Single or continuous injection of recombinant GDNF protein has been shown to rescue injured or axotomized DAergic neurons when administered before or shortly after insult and to preserve injured atrophic DAergic neurons during chronic neurodegeneration [5]. In addition, GDNF stimulates regenerative growth or axonal sprouting after partial lesions of the dopamine (DA) system and stimulates metabolism and function of lesioned DAergic neurons [6]. However, a major concern with all of these reports is that the large amounts (tens to hundreds of micrograms) of GDNF being injected into the brain may lead to deleterious side-effects (for review see [7]). Therefore, the ability to rescue neurons without the injection of massive amounts of factor or through activation of endogenous neurotrophic factors would be advantageous for clinical application.

Previous studies have shown that GDNF mRNA is widely expressed in the nigrostriatal system and the majority of DAergic neurons express GDNF [8,9]. This pattern of GDNF mRNA expression suggests a role for this factor as a local and target-derived survival factor for DAergic neurons. In addition, responses to GDNF are mediated through a receptor complex consisting of the ligand binding glycosyl-phosphatidyl inositol-linked protein, GFR-1 and a transmembrane tyrosine kinase termed Ret. Comparison of cellular expression of GFR-1 and Ret demonstrated the coexistence of both transcripts in DAergic cells of the ventral tegmental area (VTA) and substantia nigra (SN) [9]. The co-localization of GDNF with its functional receptors in the DAergic neurons suggests that GDNF may support DAergic neurons via an autocrine mechanism. Therefore, a reduced autocrine/paracrine effect of GDNF might contribute to the degeneration of the DAergic neurons owing to a lack of trophic support, and approaches that can enhance the level

of GDNF expression within DAergic neurons or within its target areas may be potential therapies for the treatment of PD.

Acupuncture has been reported effective in alleviating the symptoms and sufferings of PD patients [10–12]. However, the underlying mechanism is still unknown. A hypothesis is that EA stimulation might elevate the endogenous neurotrophic factors in certain brain areas. The present study was conducted to evaluate the effect of EA stimulation on GDNF mRNA in the midbrain and the striatum.

MATERIALS AND METHODS

Adult female Wistar rats (180–200 g) 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. Medial forebrain bundle (MFB) lesions were performed using a retractable wire knife (Scouten knife, Kopf Instruments) as described previously [5,14]. The experimental procedures were approved by the Committee on Animal Care and Usage of Peking University Health Science Center, and all efforts were made to minimize animal sufferings. After lesion, rats were divided randomly into three groups: MFB lesioned control, MFB lesion plus EA treatment at 2 Hz and MFB lesion plus EA treatment at 100 Hz. For the EA groups, stimulation was administered beginning from the day 2 following MFB lesion. Two stainless-steel needles 0.25 mm in diameter and 5 mm long were inserted obliquely at the acupuncture point DAZHUI (Du 14, just below the spinaous process of the vertebra prominens) and horizontally at BAIHUI (Du 21, at the mid point of the line connecting the two ears). Animals were, without fixation, put in a specially-made cage. 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/week. The intensity of the stimulation was increased stepwise from 1 mA to 2 mA and then to 3 mA, with each step lasting for 10 min. To enable the stimulation going, a researcher must stand beyond the cage to watch the whole process. At most of the circumstances, the animals were quietly stayed during EA administration. The rotation behavior was measured at 14 days (after 12 EA sessions) and 28 days (after 24 EA sessions) after lesion. Rats were first placed into bowls of 30 cm in diameter attached to rotometers (Animal Rotation Meter, Columbus Instruments, USA) and were allowed to rest for 5 min to adapt to the testing environment. Then they were injected i.p. with 5 mg/kg amphetamine sulfate (Sigma, USA. AMPH). Measurement of rotational activity began 5 min after injection. The animals were tested for 45 min in a quiet and dark environment. The rotometer recorded the number of clockwise turns (ipsilateral to the lesion) and counterclockwise turns (contralateral to the lesion). The net number of turns was that of clockwise turns minus counterclockwise turns and divided by 45 to calculate the change in the number of turns per minute. Animals were decapitated 24 h after the second rotation test (28 days after MFB lesion). Striatum was dissected and frozen under −80°C. Concentrations of DA were quantitated by a modified method of high performance liquid chromatography (HPLC) combined with electrochemical detection as described elsewhere [15]. Four to five rats from each

group were randomly selected and quickly decapitated. Brains were post-fixed, cryoprotected and then frozen sectioned. Coronal sections were cut at 15 m on a cryostat at −20°C and mounted onto 3-aminopropyltriethoxysilane (APES)-coated glass slides. Slices from the midbrain (4.8–5.8 mm posterior to bregma according to the rat brain atlas of Paxinos and Watson [16]) or the striatum (0.3–1.3 mm posterior to bregma) were selected for *in situ* hybridization. Alternate sections were used for Nissl staining to identify the MFB lesion. A non-radioactive *in situ* hybridization method was used to localize GDNF mRNA expression in the midbrain and the striatum. Two GDNF oligonucleotide probes complementary to nucleotides 340–398 and 256–305 of rat GDNF cDNA were synthesized and then labeled with a digoxigenin oligonucleotide 3′-end labeling kit (Roche, USA). *In situ* hybridization was performed according to the application manual of Roche Diagnostics Corporation. Controls included pretreating the sections with RNase or excluding probe from the hybridization buffer. For semi-quantitative analysis, each section was examined at × 100 magnification with a LEICA Q500MC image-analysis system (Leica, Germany). Images were digitized and stored on computer. Acquisition of the number of positive cells of GDNF mRNA was accomplished by imposing a frame of 283550.3 μm^2 area on regions to be measured. Outlines of substantia nigra pars compacta (SNc), substantia nigra pars reticulata (SNr) and VTA, dorsal caudate-putamen (dCPu), lateral caudate-putamen (lCPu), and globus pallidus (GP) were identified according to the atlas of Paxinos and Watson [16]. Two to three slices from each animal were recorded. Numbers of positive cells per region per rat were averaged to yield a mean value. Statistical significance was assessed using one-way ANOVA followed by Newman–Keuls *post-hoc* test. Significance was set at $p < 0.05$.

RESULTS

Figure 1 shows AMPH-induced rotational behavior in naive, MFB lesioned control rats, MFB lesioned rats stimulated with 2 Hz or 100 Hz EA for two weeks after MFB lesion (12 EA sessions). Slightly contralateral and ipsilateral rotation could be seen in naive rat (Fig. 1a,b). AMPH induced remarkable ipsilateral rotation in the MFB lesioned animals (Fig. 1b). The net turn for the animals in the MFB lesioned control group was 5.87 ± 1.34 turns/min. Both high and low frequency EA significantly reduced the rotation induced by AMPH ($p < 0.01$; $p < 0.05$ respectively; Fig. 1c). In the 100 Hz EA group, rotation numbers per minute (1.79 ± 0.68) decreased by 69% in comparison with that of the control group, while the 2 Hz EA group decreased to 2.60 ± 0.99 turns/min. this shows that at the early stage (2 weeks) after MFB transection, low and high frequency EA are both effective in improving the rotational behavior of the MFB transected rats. Figure 2 shows the result of the second rotational behavior test 28 days after MFB transection (24 EA sessions). No significant contralateral rotation was found in the MFB lesioned animals after AMPH injection (Fig. 2a), but different degrees of ipsilateral rotation could be seen (Fig. 2b). The average net number of turns/min in the MFB control group was 5.30 ± 0.85. EA stimulation at 100 Hz significantly reduced the rotational behavior (2.92 ± 1.09 net turns/min, $p < 0.05$), while 2 Hz
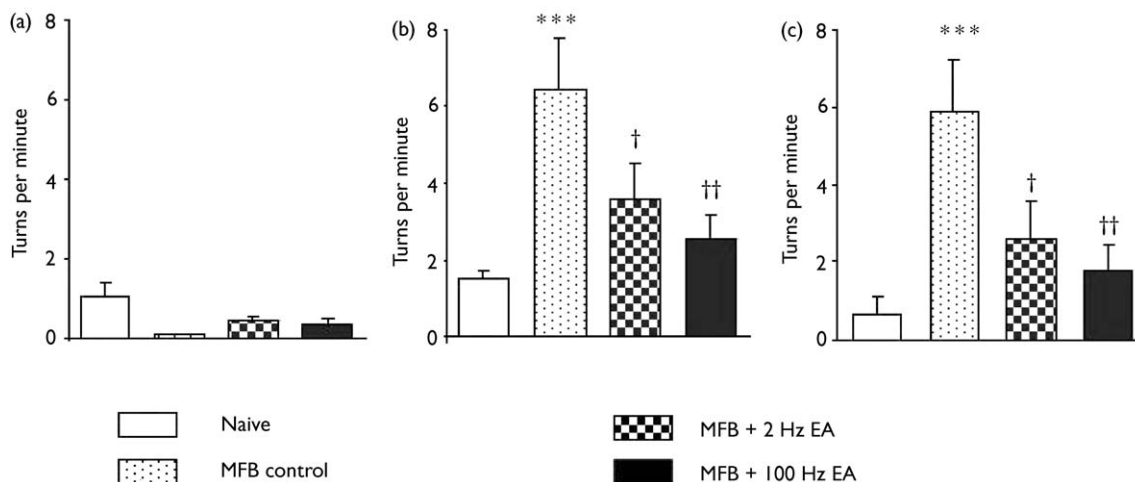
Fig. 1. Amphetamine-induced rotational behavior in naive, MFB (medial forebrain bundle) lesioned control rats and MFB lesioned rats stimulated with 2 Hz and 100 Hz EA on the 14th day of MFB lesion following 12 sessions of EA. (**a**) Contralateral rotation number, (**b**) Ipsilateral rotation number, (**c**) Net rotation number. The net number of turns/min was counted as ipsilateral (clockwise) turns minus countralateral (counterclockwise) turns. ***$p < 0.001$ vs naive group. [†]$p < 0.05$, [††]$p < 0.01$ vs MFB lesioned control group; $n = 11-15$.
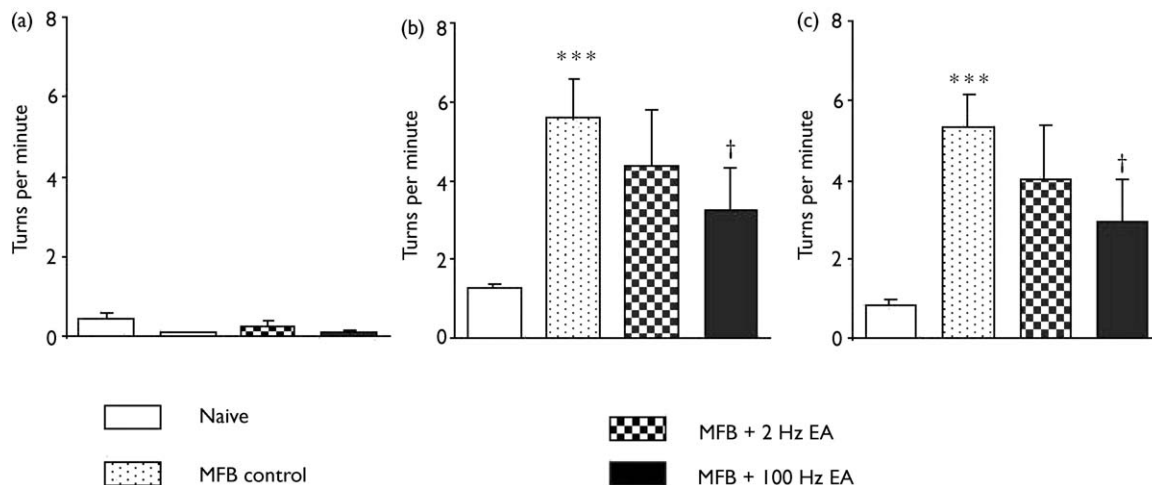

Fig. 2. Amphetamine-induced rotational behavior in naive, MFB (medial forebrain bundle) lesioned control rats and MFB lesioned rats stimulated with 2 Hz and 100 Hz EA on the 28th day of MFB lesion following 24 sessions of EA. (**a**) Contralateral rotation number, (**b**) Ipsilateral rotation number, (**c**) Net rotation number. The net number of turns/min was counted as ipsilateral (clockwise) turns minus countralateral (counterclockwise) turns. ***$p < 0.001$ vs naive group. [†]$p < 0.05$ vs MFB lesioned control group; $n = 11-15$.

EA did not produce a significant effect (4.00 ± 1.37 turns/min, Fig. 1c). Taken together, these results for both 14-day and 28-day post-MFB lesion indicate that EA stimulation can effectively alleviate the rotation behavior induced by AMPH in MFB transected rats. Low frequency EA stimulation takes effect in the early stage of lesion, while high frequency EA stimulation produces a longer-lasting effect to 4 weeks after the lesion.

Loss of DAergic innervation after MFB transection can be seen in Fig. 3, where the percentage of striatal DA content of the lesioned side versus the unlesioned side are much lower compared with the naive group ($p < 0.001$). But no difference was detected between the MFB lesioned control group and those treated by EA.

In situ hybridization showed that GDNF mRNA was highly expressed in VTA and SNc, and moderately expressed in SNr. MFB lesion did not affect GDNF mRNA expression in these areas. Neither 100 Hz nor 2 Hz EA stimulations induced GDNF mRNA changes in either side of the SNc and VTA. However, 100 Hz EA significantly increased the GDNF mRNA positive cell numbers in the SNr area of the unlesioned side ($p < 0.05$, data not shown). Widespread expression of GDNF mRNA could also be found in dCPu, lCPu and GP. Neither 2 Hz nor 100 Hz EA stimulations induced GDNF mRNA changes in either side of dCPu and lCPu. Low frequency (2 Hz) EA stimulation increased GDNF mRNA expression in the GP of the unlesioned side (Fig. 3 and 5c, $p < 0.01$ compared with the controls; Fig. 5a,b), but not in the GP of the lesioned side (Fig. 4 and 5g). High frequency EA stimulation increased GDNF mRNA level in both the lesioned and unlesioned side of GP ($p < 0.01$, Fig. 4 and 5d,h).
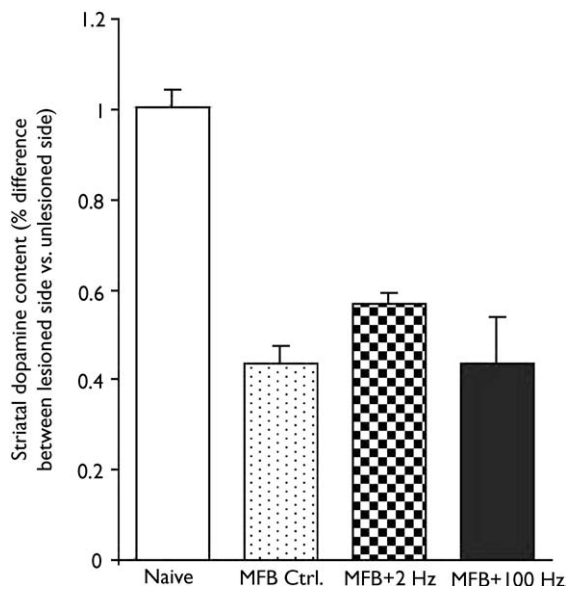
Fig. 3. Striatal dopamine content (percent difference between the lesioned side versus the unlesioned side, right side *vs* left side for the naive group), as determined by HPLC analysis. The striatal dopamine content of the MFB lesioned rats is much lower than that of the naive rats ($p < 0.001$, Student's *t*-test). No significant difference among the MFB lesioned control group and the group treated by 2 Hz or 100 Hz EA. Values are mean $\pm$ s.e.m.; $n = 6$–10.
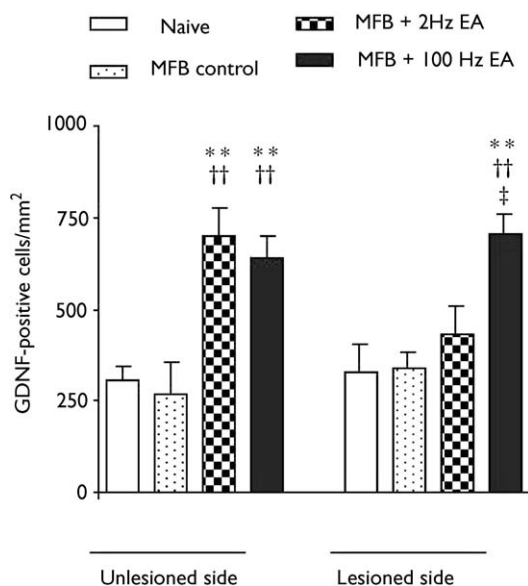
Fig. 4. Numbers of cells containing GDNF mRNA/mm^2 in the lesioned and unlesioned side of globus pallidus by *in situ* hybridization. **$p < 0.01$ *vs* naive group, $^{\dagger\dagger}p < 0.01$ *vs* MFB lesioned control group, $^{\ddagger}p < 0.05$ *vs* 2 Hz EA group; $n = 4$–5.

DISCUSSION

Acupuncture has been increasingly used as an alternative treatment for PD patients [10–12,17,18]. Since there is a lack of suitable animal models for the basic study to explore the potential mechanism, this therapy in the treatment of PD

remains controversial [19]. Mechanical transection of the MFB induces a relatively chronic degeneration of DAergic neurons and thus provides a potential model for studying the regenerative growth capacity of injured SN DAergic neurons [14]. By electrically needling this model, we have previously demonstrated that long-term, high frequency EA stimulation halted the degeneration of DAergic neurons following MFB axotomy and enhanced BDNF mRNA levels in the SN and VTA region [20]. In the current study, we showed that EA stimulation attenuated the rotational behavior induced by AMPH in MFB transected rats. Low frequency was effective for 2 weeks, and high frequency had an effect for ≥ 4 weeks. Since the functional recovery depends not only on the preservation of the nigral cell bodies, but also more critically on the reinnervation of the denervated striatum, we, therefore, attempted to find whether this rotational behavior improvement was related to the change of DA level in the striatum. The HPLC-ECD analysis data showed that neither low nor high EA stimulation could enhance the DA level in the striatum. It seems that the striatum is not a mediator for the reduction of drug-induced rotational asymmetry.

In this study, 2 Hz EA significantly increased GDNF gene expression in the non-lesioned side of GP, while 100 Hz EA increased GDNF gene expression in both sides of the GP and in the non-lesioned side of SNr. This indicates that different frequency EA stimulations may affect different brain areas. On the other hand, EA stimulation did not cause the GDNF mRNA changes in VTA, SNc, dCPu, lCPu and the lesioned side of SNr, suggesting that the endogenous GDNF in these areas do not participate in the process of the treatment of PD by EA.

As we have shown previously, EA stimulation can halt the degeneration of DAergic neurons in VTA and SNc [20]. This effect may be a result of trophic influences on DA neurons via multiple receptor complex that include several neurotrophic factor systems in these areas. Indeed, many of the factors and their receptors have been localized to the nigrostriatal system [3]. Several factors expressed in the striatum, such as GDNF and aFGF, show retrograde transport back to the substantia nigra [3], suggesting that DAergic neurons are trophically supported by striatal neurotrophic factors. Therefore endogenous neurotrophic factors are essentially important for the regeneration of DAergic neurons. In this study, we showed that high frequency EA stimulation increased GDNF mRNA level in the lesioned and unlesioned GP. GP receives a DAergic innervation from collateral fibers originating from the nigrostriatal pathway [13,21]. Accordingly, increased synthesis of GDNF in GP induced by EA stimulation may transport to the substantia nigra to nourish the DA neurons or rescue the injured DAergic neurons.

The GP is critical in processing afferent and efferent information within basal ganglia circuits. It has reciprocal connections with the striatum (the main input structure of the basal ganglia), and with the subthalamic nucleus, which directly innervates the basal ganglia output nuclei (the substantia nigra pars reticulata and the entopeduncular nucleus) [22]. Therefore, it is also possible that the effect of EA in improving the rotational behavior could have been due to that EA balances the activities of different nuclei in the basal ganglia circuit.
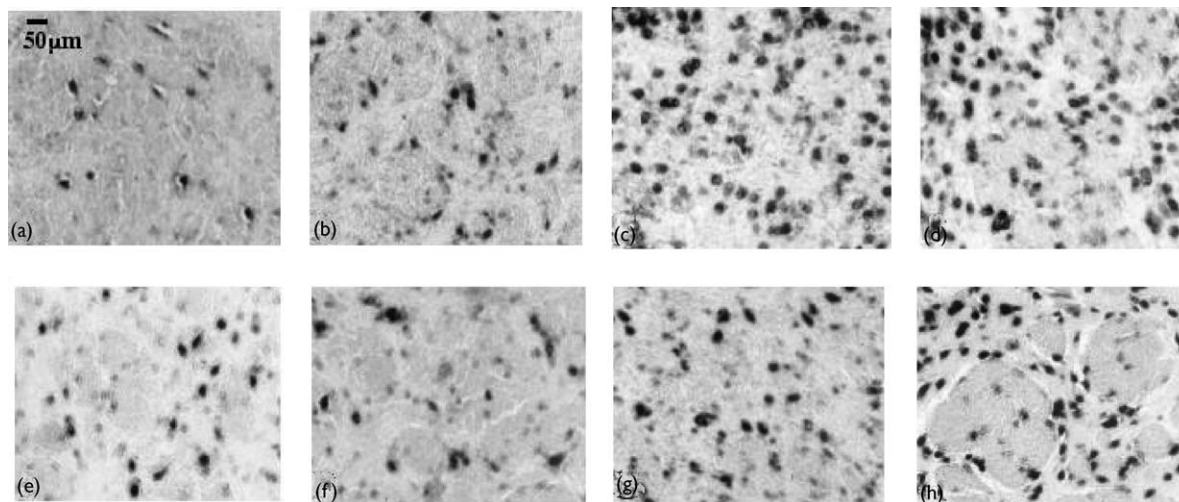
Fig. 5. Representative magnified histogram showing GDNF gene expression in the lesioned and unlesioned side of rat GP. (**a**) Naive; (**b**) unlesioned side of lesioned control group; (**c**) unlesioned side of 2 Hz EA group; (**d**) unlesioned side of 100 Hz EA group. (**e**) naive; (**f**) lesioned side of lesioned control group; (**g**) lesioned side of 2 Hz EA group; (**h**) lesioned side of 100 Hz EA group.

CONCLUSION

We have shown that EA is effective in attenuating the rotational behavior induced by AMPH in MFB transected rats. Low frequency EA may have an effect for 2 weeks after MFB lesion, while high frequency EA is effective for ≥ 4 weeks after MFB lesion. This effect is not related with the change of DA level in the striatum, but may be related with the GDNF expression changes in GP after EA stimulation. Low frequency EA stimulation significantly enhances GDNF mRNA level in the unlesioned side of GP while high frequency EA stimulation upregulates GDNF mRNA in both side of GP. The retrograde transportation of GDNF from GP to the substantia nigra and the balanced activities of different nuclei in the basal ganglia circuit after EA stimulation may contribute to the behavioral improvement induced by EA.

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