Electro-acupuncture stimulation improves motor disorders in Parkinsonian rats

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Abstract

Electro-acupuncture (EA) is believed to be effective for alleviating motor symptoms in patients with Parkinson’s disease. In a rat hemiparkinsonian model induced by unilateral transection of the medial forebrain bundle (MFB), the effects of EA stimulation were investigated. EA stimulation at a high frequency (100 Hz) significantly reduced apomorphine-induced rotational behavior. Tyrosine hydroxylase immunohistochemical staining revealed that EA at 100 Hz protected axotomized dopaminergic neurons from degeneration in the substantia nigra (SN). Moreover, high frequency EA reversed the axotomy-induced decrease in substance P content and increase in glutamate decarboxylase-67 (GAD 67) mRNA level in the midbrain; however, it did not affect the axotomy-induced increase in enkephalin content in the globus pallidus. These results suggest that the effects of high frequency EA on motor symptoms of Parkinsonian rats may involve restoration of the homeostasis of dopaminergic transmission in the basal ganglia circuit.

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1. Introduction

Acupuncture has long been known to have therapeutic effects on chronic and acute pain. With the increasing use of alternative therapies for treatment of patients with Parkinson’s disease (PD), acupuncture has become popular in clinical practice [1]. In a clinical survey, many PD patients who received acupuncture reported improvement of motor disorder symptoms [2].

In a behavioral study using an rat model of hemiparkinsonism induced by MFB transection, we have demonstrated that high frequency EA stimulation improves motor deficits [3] and elevates mRNA levels of glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) in the SN [4]. However, the underlying mechanisms of EA stimulation in the improvement of motor disorders in PD models are poorly understood.

PD is a progressive neurodegenerative disorder in which the ability to control voluntary movement is lost as a consequence of profound changes in the functional organization of basal ganglia nuclei [5]. According to the “classic” model of the basal ganglia circuit, in the direct pathway, striatal GABAergic neurons [containing dynorphin (DYN) and substance P (SP) as co-transmitters] project mono-synaptically to the basal ganglia output nuclei, substantia nigra pars reticulata (SNr) and internal segment of the globus pallidus (GPI). In the indirect pathway, striatal GABAergic neurons [containing enkephalin (ENK)] project to the external segment of the globus pallidus (GPe), which sends GABAergic projections to the subthalamic nucleus (STN). The balance of activity in the two pathways projecting from the striatum is important for normal function of the basal ganglia in the control and initiation of movement [6–8]. However, in PD, dopamine depletion enhances the activity of striatopallidal neurons (indirect pathway) and reduces the activity of striatonigral neurons (direct pathway), leading to an imbalance in the control of basal ganglia outflow to the thalamus and an inability to move effectively in response to higher motor system commands [6].

We have hypothesized that EA stimulation improves Parkinson’s motor disorders by modulating neuronal activity in the basal ganglia circuit. The present study was conducted to evaluate the effects of different frequencies of EA stimulation (0, 2, or 100 Hz) on motor behavior and dopaminergic neuron survival in MFB-transected rats. Changes in ENK and SP content as well as GAD 67 gene expression were detected to assess the effects of EA stimulation on the neurochemical response to dopamine depletion in the basal ganglia.

2. Materials and methods

2.1. Animal care and MFB axotomy

Adult male Wistar rats weighing 200–230 g were obtained from the laboratory animal center, Capital Medical University, and housed in a standard 12-h on/off light

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cycle with ad libitum access to food and water. The rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and then positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) with the mouthbar set at −3.3 mm. MFB lesions were performed using a retractable wire knife (Scouten knife, USA), a method previously described by Tseng et al. [9]. The experimental procedures were approved by the Committee on Animal Care and Usage, Capital Medical University, and all efforts were made to minimize animal suffering.

2.2. EA stimulation

Rats were randomly divided into five groups: the sham group, MFB-lesioned group, and MFB-lesioned groups with EA stimulation at 0, 2, or 0.1 Hz. Animals of the sham group underwent the operation without knife blade extension or surgical transection. EA stimulation was administered from day 2 following the MFB lesion, a method previously described by Liang et al. [3,4]. Two stainless steel needles 0.25 mm in diameter and 5 mm in length were inserted obliquely at the acupuncture point DAZHUI (GV 14, directly below the spinous process of the vertebra prominens) and horizontally at BAIHUI (GV 21, at the midpoint of the line connecting the two ears). For the 0 Hz group, needles were placed into these acupoints without electrical stimulation. Bidirectional square wave electrical pulses (0.2 ms duration, 2 or 100 Hz), designated as EA, were administered for a total of 30 min each day, 6 days per week. The duration of EA treatment was limited to 4 weeks. 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. The animals remained in the cage during EA administration in an awake, unrestrained condition.

2.3. Behavioral testing

Rotational testing was performed in automatic rotometer bowls (Columbus Instruments, USA) prior to MFB lesion and 7, 14, 21, and 28 days following the lesion, a method previously reported by Liang et al. [3]. Changes in rotational behavior were assessed by monitoring body rotations induced by apomorphine (0.5 mg/kg, s.c.). The net number of rotations (contralateral–ipsilateral) was recorded over a time span of 45 min and the number of turns per minute was calculated, a method previously described by Park et al. and Kim et al. [10,11]. This behavioral test was performed blindly.

2.4. Immunohistochemical analysis

Rats were deeply anesthetized with 450 mg/kg chloral hydrate, and then transcardially perfused with 100 ml saline followed by 200 ml 4% paraformaldehyde in phosphate buffer. Brains were dissected and post-fixed in the same fixative and cryoprotected in 30% sucrose solution for 3–5 days. Frozen sections were cut into 30 μm-thick sections on a cryostat and processed for TH-immunohistochemistry. Every sixth section of the SN (bregma, −4.8 to −6.3 mm) was selected from each brain. After several washes, brain slices were incubated with TH antibody (diluted 1:2000; Chemicon, USA) for 24 h at 4 °C. The antibody was detected using an ABC Elite kit (Vector laboratories, USA) with 3,3′-diaminobenzidine (DAB) and nickel enhancement. The number of TH-immunoreactive neurons in the SN was counted in each section using a bright-field microscope (Olympic, Japan) and analyzed using an advanced image-analysis system (Metamorph). The survival percentage of TH-positive cells in the SN was calculated as the number of TH-positive cells in the lesioned side divided by the number of TH-positive cells in the unlesioned side (this calculation has been discussed and utilized in previous studies [4,10,11]). An independent investigator evaluated all sections in a blinded manner.

2.5. Radioimmunoassay (RIA) test

After decapitation, the brain was removed quickly and boiled for 5 min in 0.9% saline solution. The ventral midbrain and global pallidus were dissected out, rapidly frozen on dry ice, and stored at −80 °C. On the day of assay, tissue samples were weighed and then homogenized in 1 ml of 1N acetic acid and centrifuged at 4000 × g for 15 min at 4 °C. The supernatant was lyophilized and stored at −20 °C until analysis. ENK and SP kits (Neurobiology Laboratories of the Second Military Medical University, Shanghai, China) were used to analyze the samples.

2.6. Real-time quantitative RT-PCR analysis

Total RNA was extracted from the ventral midbrain using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). RNA was treated with RNase-free DNase I, and 5 μg of total RNA were used for cDNA synthesis with reverse transcription by the SuperScript™ III RT kit (Invitrogen Corporation). Real-time PCR was performed with the Applied Biosystems technique (Foster City, CA). The primer pairs for GAD 67 were 5′-AGA ACG GGG AGG ACC AAA CTT T-3′ and 5′-AAA CTT TTC TAC CCT GCC GTC TTC TCT TG-3′. The primer pairs for GADPH were 5′-GGT AGG CTT AGG GCT GCC GTC TCT TCT TG-3′ and 5′-CCT TGA CTC TGC CTT TGA ACT TG-3′. The SYBR Green PCR core reagents kit (Applied Biosystems) was used, with GAPDH as the endogenous control. PCR was performed in Micro-Amp Optic 96-well reaction plates (Applied Biosystems) using a Stratagene MX3000P Sequence Detection system (Stratagene, USA); the PCR protocol consisted of 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 1 min at 58 °C, and 30 s at 72 °C. The levels of target gene expression were quantified relative to the level of GAPDH using the standard curve method. The specificities of RT-PCR products were confirmed by the presence of both a single dissociation curve for the product and a single band with corresponding molecular weight revealed by agarose gel electrophoresis.

2.7. Statistical analysis

Data are expressed as means ± SEM. Statistical significance was assessed using one-way ANOVA followed by Newman–Keuls post hoc test of differences between groups. P < 0.05 was considered statistically significant.

3. Results

3.1. Effect of EA stimulation on rotational behavior

Results of the assessment of apomorphine-induced changes in rotational behavior are shown in Fig. 1. Rats of the MFB-lesioned group exhibited greater rotational asymmetry in the direction contralateral to the lesion (6.46 ± 0.64 turns/min) as compared to rats of the sham group (1.53 ± 0.29 turns/min, P < 0.01) at 4 weeks after MFB lesion. However, a significant decrease in the net number of rotations was observed in the 100 Hz EA stimulation group compared to the MFB-lesioned group from the 2nd to 4th week after MFB lesion. EA stimulation at 0 or 2 Hz did not produce an obvious improvement of motor disorder symptoms. Thus, high frequency EA stimulation alleviated the rotation behavior of MFB-transected rats.

3.2. Effect of EA stimulation on TH-immunoreactivity in the SN

Immunohistochemical staining for TH was performed 4 weeks after MFB lesion, and the number of TH-IR neurons in the SN was calculated. A significant reduction in the number of TH-IR neurons was seen in the MFB group, as compared to the sham group (Fig. 2A and B). In the MFB group, the number of TH-positive neurons on the lesioned side was approximately 38.2 ± 3.63% of that on the unlesioned side. In hemiparkinsonian rats that received 100 Hz EA stimulation, the number of TH-positive neurons significantly increased to 74.9 ± 5.68% (P < 0.01). However, animals that received EA stimulation at 0 or 2 Hz showed no significant increase in the survival rate of TH-positive neurons when compared to the MFB.
Fig. 2. Effect of EA stimulation on TH immunohistochemical staining in the SN. (a) Photomicrographs showing changes in the intensity of TH staining on the lesioned side (A′, B′, C′, D′, and E′) compared with the unlesioned side (A, B, C, D, and E). Representative photomicrographs were obtained from different groups of animals receiving the sham treatment (A and A′), the MFB lesion (B and B′), 0 Hz EA (C and C′), 2 Hz EA (D and D′), and 100 Hz EA (E and E′). Asterisks demarcate dendrites of the SNc located in the SNr of the lesioned side in the sham group and 100 Hz group. Scale bar: 200 μm. (b) Histogram shows the percentage of TH-positive SNc neurons on the right (lesioned) side versus left (unlesioned) side. Data are presented as means ± SEM (n = 5 per group). *P < 0.05 and **P < 0.01 versus sham rats (A). *P < 0.05 versus MFB rats (B).

Fig. 3. Effect of EA stimulation on ENK content in the globus pallidus (A) and substance P content in the ventral midbrain (B) of MFB-transected rats. The contents of ENK and SP were detected by RIA. The histograms represent the mean content on the unlesioned and lesioned sides of the MFB transection. The values are expressed as percentage ± SEM of the sham values (n = 5 per group). *P < 0.05 and **P < 0.01 versus sham rats (A). *P < 0.05 versus MFB rats (B).

In addition, there was a marked reduction of TH-IR fibers in the ipsilateral SNr in the MFB group (Fig. 2A). The reduced TH-IR in the dendrites indicates the decreased TH activity [12]. However, the high frequency EA stimulation seems to prevent the great reduction of TH-positive dendritic fibers in the ipsilateral SNr (Fig. 2A).

3.3. Effect of EA stimulation on the contents of ENK and SP

To investigate functional changes of direct and indirect pathways due to MFB axotomy, as well as the effect of EA treatment, we examined the levels of ENK in the GP and SP in the ventral midbrain area using a radioimmunoassay. Lesioning of the nigrostriatal pathway by MFB transection produced an increase in the ENK level in the GP (Fig. 3A) and a decrease in the SP level in the SN ipsilateral to the lesioned side (Fig. 3B). These changes are likely due to striatal dopamine denervation, and reflect increased neuronal activity in the striatopallidal pathway as well as decreased neuronal activity in the striatonigral pathway. No significant difference in ENK content was detected between rats with the MFB lesion and hemiparkinsonian rats at any frequency of EA stimulation. However, high frequency EA stimulation reversed the MFB lesion-induced reduction of substance P content in the SN region. This effect was not seen in hemiparkinsonian rats subjected to 0 or 2 Hz EA stimulation.

3.4. Effect of EA stimulation on GAD 67 mRNA in the midbrain

To determine functional changes in GABAergic neurons after EA treatment, we examined the level of GAD 67 (GABA synthesizing enzyme) mRNA in the ventral midbrain using RT-PCR. There was
a significant increase in the level of GAD 67 mRNA in the ventral midbrain of MFB-lesioned rats (Fig. 4). However, the upregulation of GAD67 mRNA was significantly reduced by 100 Hz (but not 0 or 2 Hz) EA stimulation of the midbrain (Fig. 4).

4. Discussion

Surgical transection of the nigrostriatal dopamine pathway at the MFB results in the progressive degeneration of dopaminergic neurons in the SNc and has been used as an animal model of PD [13,14]. This technique is an effective model of PD because it leads to axonal degeneration of dopaminergic neurons in the early course and subsequently results in reduced dopamine content in the striatum.

The striatum is the primary target for dopaminergic neurons in the SN. Thus, the restoration of dopamine levels in the striatum is critical for treatment of PD. On the other hand, intracerebral injection of GDNF has resulted in significant improvements in Parkinsonian behavioral symptoms but did not affect dopamine levels in the caudate nucleus or putamen of MPTP monkeys [15]. Continuous release of low levels of GDNF near the SN was also reported to protect nigral dopaminergic neurons from MFB axotomy-induced lesion and significantly improve apomorphine-induced rotational behavior; however, it did not prevent the loss of dopamine in the striatum [9]. Similar to these reports, our previous [3] and present studies also showed that 100 Hz (but not 0 or 2 Hz) EA stimulation significantly reduced abnormal rotational behavior in MFB-transected rats. High frequency EA treatment resulted in the enhanced survival of dopaminergic neurons in the SN, but had no obvious effect on dopamine levels in the striatum [3]. Therefore, the role of SN dopaminergic neurons in the improvement of motor disorders should be given greater attention.

Accumulating evidence indicates that dopaminergic neurons in the SNc uniquely synthesize, store, and release dopamine; not only from the terminal fields that innervate the striatum, but also from the dendrite network that arborizes in the SNr [16,17]. Previous work demonstrated that high frequency EA could enhance the survival of degenerating dopaminergic neurons following MFB axotomy, where this might be attributable to enhanced synthesis and release of neurotrophic factors [3,4] and alleviation of inflammatory reactions in the SN region [18] by high frequency EA. In the current study, we found that high frequency EA stimulation prevented the degeneration of not only dopaminergic cell bodies in the SNc, but also of dopaminergic neurons that arborize in the SNr. In animals that have undergone MFB axotomy, dopamine stores in the SNc are released at dendritic sites (as opposed to the axonal sites in the striatum) after administration of amphetamine [9]. This increase of dopamine release in the SNr may cause significant alternations in the output of SNr neurons. Hatzipetros and Yamamoto found that somatodendritically released dopamine in the SN regulates glutamate release from subthalamic axonal terminals by differentially activating dopamine D2 and D1 receptors [19]. Activation of D2 heteroreceptors, located on subthalamic axonal terminals, involves D2 receptor-mediated inhibition of glutamatergic input to the SNr from the STN [19].

Peripheral electrical stimulation is known to induce the release of neurotransmitters and neuropeptides in the brain [20]. Additionally, it has been shown that EA causes neuronal activity changes in the striatum and subsequent changes along the basal ganglia circuit [21,22]. In the current study, we confirmed that EA stimulation results in neuropeptide normalization in the basal ganglia circuit.

Dopamine depletion enhances the activity of striatopallidal neurons and reduces the activity of striatonigral neurons. In a unilaterally 6-OHDA-lesioned rat model of PD, synthesis of ENK was remarkably increased [23]. In the same model, decreases in striatal and nigral substance P content and preproenkephalin mRNA were reported [23]. In the present study, we found that the ENK level was increased in the GP of MFB-lesioned rats, and that the SP content was decreased in the midbrain. Notably, we observed that high frequency EA stimulation reversed the lesion-mediated decrease of SP in the ventral midbrain of the model but did not affect the lesion-induced increase of ENK in the GP.

A close interaction between DAergic and SPergic function has been demonstrated, and SP was thought to interact agonistically with DA neurons as a transmitter in the striatongrial projection [24]. Previous reports with 6-OHDA lesioned rats have shown that treatments with SP either pre- or post-lesion were able to promote functional recoveries, especially in cases of partial nigrostrial dopamine lesions [25,26]. The effect that SP treatments ameliorated the lesion-induced asymmetric turning behaviors may be related to the enhanced ventral striatal dopamine activity. Apart from its interactions with the DAergic system, SP may exert other neurotrophic and neuroprotective functions, including to inhibit the level of extracellular striatal acetylcholine and to modulate lesion-induced reactive gliosis. Such functions were linked to nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF), all of which were demonstrated to prevent deficits or promote recovery in meso-striatal DA lesions [26]. Further evidence with the unilateral dopamine lesion model implied that the promotive effects of SP treatment involved a residual dopamine mechanism and mediated by its C-terminus [27]. Intranigral injection of SP caused an immediate and long-lasting increase of DA release in the ipsilateral striatum [28]. Consequently, SP intranigral stimulation elicited contralateral rotational behavior, a response associated with enhanced dopaminergic transmission and modulation of the firing rate of nigrostriatal DA neurons. Therefore, EA-derived improvement of motor abnormalities might be ascribed to the modulation of synaptic transmission or the activities of neurons in the basal ganglia circuit.

In PD, dopaminergic neuronal degeneration leads to the tonic discharge of GABAergic output nuclei of the basal ganglia [29]. Increased output from the basal ganglia to the thalamus is thought to lead to Parkinsonian motor signs. In the present study, high frequency EA stimulation was found to normalize MFB lesion-induced changes in GAD 67 mRNA expression in the ventral midbrain. Previous studies [30,31] have shown that high frequency stimulation of the subthalamic nucleus (STN HFS) and subthalamotomy could reverse the upregulation of GAD 67 mRNA induced by depletion of dopamine in SNr neurons. This suggests that EA stimulation may reduce overactivity in the output structures of the basal ganglia.
Therefore, EA treatment may affect GABA transmission in the targets of the basal ganglia, specifically in SNr neurons. Therefore, we speculate that high frequency EA stimulation improves motor symptoms in MFB-transected rats through restoration of the homeostasis of the basal ganglia circuit.

Acknowledgments

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