Inhibition of glutamate and acetylcholine release in behavioral improvement induced by electroacupuncture in parkinsonian rats

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HIGHLIGHTS

- Electroacupuncture (EA) stimulation improved motor impairment in parkinsonian rats.
- EA protected dopaminergic neurons but did not increase dopamine levels.
- Glutamate and acetylcholine levels decreased after EA stimulation.
- Glutamate and acetylcholine levels correlated with rotational behavior.

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ABSTRACT

Prior evidence shows that acupuncture improves symptoms in both Parkinson’s disease (PD) patients and animal models. We examined the effects of high-frequency (100 Hz) electroacupuncture (EA) on behavior in a rat PD model induced by medial forebrain bundle (MFB) transection. Neurotransmitters levels in the striatum were measured using in vivo microdialysis and high performance liquid chromatography (HPLC). High-frequency EA stimulation at Dazhui (GV14) and Baihui (GV20) acupoints decreased rotational behavior induced by apomorphine (APO) and improved motor coordination, protected axotomized dopaminergic neurons from degeneration in the substantia nigra (SN), it did not increase striatal dopamine (DA) levels. However, EA stimulation at acupoints significantly decreased the abnormally elevated glutamate (Glu) and acetylcholine (ACh) levels in the lesioned side of striatum. Moreover, the Glu levels correlated significantly with survival ratios of dopaminergic neurons in the SNc and rotational behavior. These data suggested that behavioral alleviation with EA stimulation may be associated with modulation of neurotransmitters release, such as Glu and ACh in the striatum, rather than with DA restoration.

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Parkinson’s disease (PD) is a common age-related neurological disorder, marked by the relatively selective and progressive loss of dopaminergic neurons in the substantia nigra (SN) and subsequent dopamine (DA) depletion in the striatum. The motor symptoms of PD such as resting tremor, rigidity, and bradykinesia appear, when striatal DA depletion exceeds 60–80% [3].

In mammals, the striatum is a large subcortical structure that mainly receives dopaminergic projections from the SN and glutamatergic projections from the cortex and thalamus. As such, it is rich in neurotransmitters including DA, glutamate (Glu), γ-aminobutyric acid (GABA), and acetylcholine (ACh). Increased Glu, ACh and GABA levels were reported in many studies in PD animals [1,17,18]. Using in vivo microdialysis in 6-hydroxydopamine (6-OHDA) lesioned rats, Meshul et al. [17] reported that Glu levels in the striatum significantly increased after 1 month and then decreased after 3 months compared with NS-lesioned rats, while DA levels progressively decreased. In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated monkeys, Boulet et al. [1] found a large increase in Glu and GABA levels in the striatum and a marked decrease in DA levels and metabolism. Increased ACh levels were also reported in PD animal models, while administration of Glu N-methyl-d-aspartic acid (NMDA) receptor antagonists or ACh muscarinic receptor agonists could alleviate PD symptoms [18]. Thus, these studies suggest that elevated neurotransmitters levels may be associated to the symptoms of PD.

Acupuncture is a popular treatment in traditional Chinese medicine. Studies showed that acupuncture alleviated the symptoms of PD patients and animal models [10,11,20]. We have previously demonstrated that 4 weeks of high-frequency electroacupuncture (EA) stimulation alleviated movement disorders following medial forebrain bundle (MFB) axotomy [9]. However, the mechanisms underlying the improvement remain unstudied. Thus, in the present study we set to examine intrastriatal release of the neurotransmitters DA, ACh, Glu, and GABA by microdialysis
in conscious rats following transection of the MFB, and to explore their correlation with the symptoms of PD. We hypothesized that improvement in motor behavior observed in high-frequency EA stimulation in PD model rats would associate with the reversal of neurotransmitter levels.

This study was conducted in accordance with the Animal Use and Care Guidelines issued by the National Institutes of Health and was approved by the Animal Use and Care Committee at Capital Medical University, and all efforts were made to minimize animal suffering.

Forty-eight adult male Wistar rats weighing 180–200 g were supplied by the laboratory animal center at Capital Medical University, and were housed in a standard 12 h light-dark cycle with ad libitum access to food and water.

Rats were anesthetized with 350 mg/kg chloral hydrate and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) with the tooth bar set at −3.3 mm. MFB lesions were performed using a retractable Scouten wire knife in the right side of rats. After axotomy, six rats in each group were used for microdialysis assay. A second hole was drilled at 1.0 mm rostral to bregma and 2.5 mm lateral to midline. Four additional holes were drilled to place four flathead screws for holding the entire cannula assembly in place. A stainless steel guide cannula (15 mm long, 21 gauge; CMA, Acton, MA, Sweden) was placed with the tip located 3.5 mm below the dura, and was attached to the skull using a thin layer of dental cement and allowed to dry.

Rats were randomly divided into four groups: the sham group, model group, acupoints group, and non-acupoints group. Rats received sham lesioned which means wire knife was not stretched out. In the acupoints or non-acupoints group, rats received an MFB lesion followed by a 100 Hz EA stimulation at the acupoints Dazhui (GV14, just below the spinous process of the vertebra prominens) and Baihui (GV20, at the midpoint of the line connecting the two ears) or on the hips as previous reported [11]. Rats in the sham or model group did not receive any stimulation. The EA stimulation was administered on day 2 following the MFB lesion. Two stainless steel needles (0.25 mm in diameter, 5 mm in length) were inserted obliquely at the acupoints or the hips. Bidirectional square wave electrical pulses (0.2 ms duration, 100 Hz), designated as EA, were given for a total of 30 min each day, 6 days per week. The duration of EA stimulation lasted for 4 weeks. Schedule was shown in Supplemental Fig. 1. 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 relaxed during stimulation, so anesthesia was not performed.

Behavioral tests were assessed at the second day after 4 weeks of EA stimulation. Rotational test was performed in automatic rotemeter bowls (Columbus Instruments, Columbus, OH, USA). Changes in rotational behavior were assessed by monitoring body rotations induced by apomorphine (APO) (0.5 mg/kg, s.c.). The net number of turns was determined by contralateral turns minus ipsilateral turns counted by an observer blinded to the treatment groups in 30 min as previously reported [9]. The motor coordination of rats was evaluated using rotarod treads (Stoelting Company, WoodDale, IL, USA). The accelerating speed of the rotarod was set to increase from 6 rpm/min to 40 rpm/min within 2 min. The rats were placed on the treadmill and the timers were set with acceleration programmed to automatically stop when the rat fell off. The time that the rats stay on the treadmill was calculated. All experiments were examined blind.

After behavioral tests, the rats implanted with guide cannulas were used for microdialysis. A 2 mm long, 0.5 mm diameter microdialysis probe (CMA, Solna, Sweden) catheter with a 20 KDa cut-off was inserted into the guide cannula and perfused with artificial cerebrospinal fluid (2 µl/min; 140 mM NaCl, 3.4 mM KCl, 1.5 mM CaCl2, 1.0 mM MgCl2, 1.4 mM NaH2PO4, 4.85 mM Na2HPO4, and 5.4 mM glucose, pH 7.4) until the baseline stabilized. Samples were collected every 15 min for the detection of neurotransmitters. After sampling, Nissl staining was performed to determine the location of the probe (Supplemental Fig. II). Briefly, the rats were deeply anesthetized and perfused with 100 ml saline followed by 200 ml of 4% paraformaldehyde in phosphate buffer. Brain slices were incubated with Nissl dye. All of the probes were placed in the striatum.

The samples were injected into a high performance liquid chromatography (HPLC) system (Model 5600A; CoulArray Detector System ESA, Brighton, MA, USA) for analysis. For DA assay, the mobile phase was 0.125 M sodium citrate buffer containing 20% methanol, 0.1 mM Na2EDTA, and 0.5 mM 1-octanesulfonic acid sodium salt (Acros Organics, Morris Plains, NJ, USA). For Glu and GABA assays, the samples were mixed with the same volume o-phthalic dicarboxaldehyde solution (Sigma, St. Louis, MO, USA) for precolumn derivation. The mobile phase for isotonic elution consisted of 100 mM NaH2PO4 (pH 3.5), 10% methanol, and 0.5 mM Na2EDTA. ACh was converted into hydrogen peroxide. The mobile phase contained 60 mM Na2HPO4 and 0.5% Kathon CG at pH 8.5.

To observe the protective effect of EA on dopaminergic neurons, immunohistochemical and immunoblotting analysis on tyrosine hydroxylase (TH) were performed. After microdialysis, rats processing and sliced for immunohistochemical as previous reported [9], TH-positive neuronal profiles with distinct nuclei were counted in ten sections throughout the entire rostrocaudal extent of the substantia nigra pars compacta (SNc). All sections were coded and examined blind. Protein extraction from the ventral midbrain and western blot analysis were performed as previously described [16]. An anti-TH mouse monoclonal antibody (Sigma, St. Louis, MO, USA) was used at 1:10,000 dilutions for analysis.

Data were reported as mean ± SEM. Statistical significance was assessed using a one-way ANOVA followed by Newman–Keuls post hoc test of difference between groups. A value of P < 0.05 was considered statistically significant. Using regressive analysis assessed if the changes of neurotransmitters (Glu, ACh) was correlation with rotational behavior, TH-positive neuron respectively, also P < 0.05 was considered statistically significant.

The Rotational and rotarod test were used to measure motor ability of rats. Rats in the model group exhibited obvious rotational asymmetry in the direction contralateral to the lesion (P < 0.001; Fig. 1A). In rats that received acupoints EA stimulation, the net number of rotations was significantly decreased (P < 0.01). Moreover, acupoints EA stimulation also increased the reduced treadmill occupancy time in parkinsonian rats (P < 0.05; Fig. 1B). By contrast, there was no change in rotational behavior or treadmill occupancy time in rats that received non-acupoints EA stimulation compared with the model group (P > 0.05). Overall, these data suggested that EA stimulation at the acupoints improved the abnormal behavior of the model rats.

TH is a key enzyme in dopamine metabolism. To measure the effects of EA on TH expression in MFB lesioned rats, protein levels were measured. A significant reduction of the TH-positive profile on the lesioned side was seen after MFB lesioned (P < 0.001, Fig. 2A and B). After EA stimulation at acupoints, the profile of TH-positive neurons increased significantly (P < 0.001). This neuroprotective effect of EA was also shown with Western blot analysis (Fig. 2C and D). Rats that received non-acupoints EA stimulation showed no significant increase in TH protein levels. These results indicated EA stimulation prevents the loss of dopaminergic neurons in parkinsonian rats.

Since dopaminergic neurons project to the striatum to exert its function, we next detected the striatal DA levels. The axotomy of MFB was confirmed by a decrease of DA content in the striatum (Supplemental Fig. III), while whether EA stimulation at acupoints or non-acupoints did not result in significant changes. These results suggested that the improvement in behavior was not
Fig. 1. Effects of EA on movement behavior at the end of 4 weeks after MFB axotomy. (A) Rotational behavior induced by apomorphine (APO, 5 mg/kg) in each group. (B) Motor coordination of rats was measured by the rotarod treadmill test. Values are expressed as mean ± SEM (n = 12). *P < 0.05, **P < 0.01 and ***P < 0.001 vs. sham lesioned group. *P < 0.05 and **P < 0.001 vs. MFB lesioned group.

fully dependent on the restoration of the striatal DA content after EA stimulation.

To provide further insight into the mechanism of behavioral improvement of EA, the levels of striatal neurotransmitters were examined with microdialysis. Similarly to the content in the tissue, EA stimulation did not increase DA levels. A large increase in ACh, Glu and GABA levels was observed in the lesioned side of the striatum after MFB axotomy (P < 0.05, 0.01 and 0.01, respectively, Fig. 3). After 4 weeks of EA treatment at acupoints, ACh and Glu levels decreased significantly (P < 0.05 and 0.01, respectively), while GABA

Fig. 2. Neuroprotective effects of EA stimulation on TH-positive neurons and TH protein expression at 4 weeks after MFB axotomy. (A) EA stimulation protected TH-positive neurons from injury in SNc after MFB axotomy with immunohistochemical staining. a, c, e, g showed the TH-positive neurons in the unlesioned side of sham, model, acupoints and non-acupoints group, respectively. b, d, f, h showed the TH-positive neurons in the lesioned side of each group. (B) Statistic histogram of survival ratios of TH-positive neurons in the lesioned side compared with unlesioned side. (C) Effects of EA on expression of TH protein in ventral midbrain by Western blot. Strip in order from left to right represents the unlesioned side and lesioned side of sham, model, acupoints and non-acupoints group. (D) Statistic histogram of TH protein levels. Scale bar = 200 μm. Data are presented as mean ± SEM (n = 6). **P < 0.01, ***P < 0.001 vs. sham lesioned group. ##P < 0.01, ###P < 0.001 vs. MFB lesioned group.
levels did not change significantly. Moreover, in model group, rats Glu level in striatum showed a significantly positive correlation with rotational behavior and a negative correlation with survival ratio of TH-positive neurons in the SNc \( (P < 0.05) \). ACh level was not significantly correlated with these two factors, though there was a associated tendency \( (P < 0.05) \). This finding suggested that Glu levels in the striatum was more sensitive than others to reflect the injury extent of SNc.

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 [23]. In the present study, high-frequency acupoints EA stimulation reduced abnormal rotation behavior and increased treadmill occupancy time in our MFB axotomy rat PD model. The improvement of abnormal behavior after EA stimulation was consistent with our previous reports showing that 100 Hz, but not 0 Hz or 2 Hz, EA stimulation reduced abnormal rotation behavior and increase the treadmill occupancy time after MFB axotomy \( (8,9) \). Furthermore, in a 6-OHDA-lesioned rat PD model, acupunctural treatment at acupoints GB34 and L13 significantly reduced rotation behavior \( (19) \); while in the MPTP model of PD, 100 Hz EA stimulation at ST36 and SP6 protected dopaminergic neurons from MPTP toxicity and produced antioxidative effects \( (24) \). Our results also showed acupoints EA stimulation prevented the degeneration of dopaminergic neurons observed after MFB axotomy. The mechanism of protection may involve neurotrophic and anti-inflammation effects, such as an increase in the expression of neurotrophic factors and the inhibition of the activation of microglia or the release of inflammatory cytokines \( (14,15) \). The relationship between neurotrophic effects and improvement of abnormal behavior need further study.

Other studies also suggested that striatal dopamine content was not necessarily associated with the improvement of motor behavior in animal models \( (5,23) \). We previously reported that high-frequency EA stimulation protected dopaminergic neurons in the SNc, enhanced synthesis and release of neurotrophic factors, and alleviated inflammation \( (14,15) \). Furthermore, high-frequency EA stimulation was shown to increase GABAergic inhibition in the output structure of the basal ganglia \( (8) \). Thus, the effects of EA stimulation may not be limited to dopaminergic neurons, it may involve other neurotransmitters in the basal ganglia as well.

MFB axotomy resulted in decrease in levels of DA but increase in levels of ACh, Glu, and GABA in the lesioned side of the striatum. These results are consistent with other reports in animal models and PD patients \( (1,7) \). Loss of striatal dopaminergic terminals can reduce this inhibitory control in PD, and the abnormal elevation of ACh promotes the occurrence of PD symptoms \( (22) \). Increased levels of Glu can also accelerate PD symptoms, therefore

\[ \text{Values at levels} \]

\[ \text{Fig. 3. Effects of EA on extracellular levels of DA (A), ACh (B), Glu (C) and GABA (D) by in vivo microdialysis in the lesioned side of the striatum at 4 weeks after MFB axotomy. Values are expressed as mean ± SEM (n = 6). } \]
inhibition of Glu is a strategy being examined for treatment of PD [4, 21]. Increased GABA concentrations have also been suggested to be a compensatory effect of the increased Glu release [1].

Central and peripheral electrical stimulation may induce release of neurotransmitters and neuropeptides in the brain [2, 6]. Lee et al. reported that acupuncture at the Yanglingquan (GB 34, anterior and inferior to the fibular headanterior and inferior to the fibular head) and Xuanzhong (GB 39, distal four-fifths points on the imaginary line connecting the lateral side of the knee and the lateral malleolus of the tibiofibula) acupoints reduced the increase in Glu levels observed during ischemia [13]. In the present study, EA stimulation significantly decreased Glu and ACh release, which was also correlated with an attenuation of PD symptoms. Overall, these data suggest that the improved behavior following EA stimulation may occur via down-regulation of levels of Glu and ACh, but not DA. We found that acupuncture EA stimulation improved abnormal behavior and decreased the abnormal elevation of Glu and ACh following MFB transection while non-acupoints EA stimulation had no such effects, suggesting a specific action of acupoints stimulation. These data are consistent with previous studies [11, 13]. For example, Kim et al. [11] reported that acupuncture at non-acupoints had no effect on degeneration of dopaminergic neurons in a 6-OHDA-induced PD rat model. Similar effects of acupoints EA stimulation have also been confirmed by imaging study [12].

In conclusion, our findings suggest that decreased Glu and ACh levels, not DA levels may play an important role in the improvement of abnormal rotation behavior and reduced treadmill capacity of high-frequency acupoints EA stimulation in MFB axotomy-lesioned rats. The generalizability of the findings to humans is unclear. Also, how EA stimulation downregulates neurotransmitters levels requires further studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2012.05.021

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