Research report

High frequency electro-acupuncture enhances striatum DAT and D1 receptor expression, but decreases D2 receptor level in 6-OHDA lesioned rats

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HIGHLIGHTS
► 100 Hz EA halted DA neuron degeneration, improved behavior in 6-OHDA lesioned rats.
► 100 Hz EA raised the DA level in the ST although the difference was not significant.
► High frequency EA increases the DAT level in the ST and SN simultaneously.
► 100 Hz EA suppressed D2R up-regulation secondary to 6-OHDA lesioning.
► The D1R expression level was obviously elevated after high-frequency EA.

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ABSTRACT
The direct effects of electro-acupuncture (EA) on the dopaminergic neurotransmitter system in Parkinson’s disease (PD) patients remain elusive. In the present study, 0.2 or 100 Hz EA was applied to acupoints Sanyinjiao (SP6), Yanglingquan (GB34) and Zuanshi (ST36) in a rat model unilaterally lesioned by 6-hydroxydopamine. Rotational behavior tests were performed and the animals were then decapitated. Levels of striatal dopamine (DA), dopamine transporter, and D1- and D2-like DA receptors were subsequently evaluated. EA at 100 Hz was shown to significantly enhance survival of dopaminergic neurons in the substantia nigra (52.10 ± 11.41% of the level on the non-lesioned rats vs. 21.22 ± 5.52% in the non-EA group, P < 0.05) and reduce motor deficits (207.80 ± 31.14 vs. 476.11 ± 68.80 turns/30 min, P < 0.05), whereas it only slightly restored the 6-hydroxydopamine-induced loss of striatal DA (P > 0.05 vs. the non-EA group). There was a 253.78% increase in dopamine transporter protein expression in the striatum in the 100 Hz EA group (P < 0.05 vs. the non-EA group). Moreover, high frequency EA induced increases in striatal D1-like receptor mRNA and protein levels of 81.88% and 62.62%, respectively (P < 0.001 and P < 0.05 vs. the non-EA group). However, the D2-like DA receptor up-regulation observed in the non-EA group was suppressed in high frequency group (P > 0.05 vs. the sham operation group). These findings suggest that high-frequency EA might work by acting on presynaptic dopamine transporter and postsynaptic dopamine receptors simultaneously to achieve a therapeutic effect in PD patients and models. This might shed some light on the mechanism by which EA affects the DA neurotransmitter system.

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1. Introduction

Although evidence of the effectiveness of acupuncture for treating Parkinson’s disease (PD) remains unconvincing [1], the modality has been shown to produce some clinical benefits [2,3].

Applications of acupuncture in animal PD models have shown that the beneficial effect might originate from the collaboration of its anti-inflammatory and neurotrophic actions. In addition, to increase the levels of various neuroprotective agents, such as brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor and cyclophilin A [4–10], acupuncture therapy attenuates oxidative stress in substantia nigra dopaminergic neurons [11–13].

The depletion of dopamine (DA) and disruption of the DA neurotransmitter system homeostasis sets a cascade of alterations, which prime the brain for complicated PD motor symptoms [14,15]. Presynaptic dopamine transporter (DAT) and postsynaptic dopamine
receptors (D1- and D2-like DA receptors (D1R and D2R, respectively)) are influenced at the transcriptional and/or translational levels by the disease process; these variations change with time and medications [16,17]. It is therefore of considerable importance to examine whether these alterations are affected by acupuncture treatment. However, little is known about the direct effects of acupuncture on dopaminergic neurotransmitter system.

In this study, dopamine axon terminal lesions induced by injections of 6-hydroxydopamine (6-OHDA) into the striatum were adopted for observing the effects of electro-acupuncture (EA) on partial lesions of the nigrostriatal DA system. Two weeks after EA we performed a detailed examination of striatal dopaminergic neurotransmitter system alterations. Such a study would provide a more complete and accurate analysis of the protective effects of EA on the dopaminergic neurotransmitter system, perhaps explaining the mechanisms underlying the beneficial effect of EA in PD movement disorders.

2. Materials and methods

2.1. Animals

Sixty-three adult male Sprague-Dawley rats, weighing 220–250 g, were used in this study. They were housed at a room temperature (22 ± 3°C) under standard 12-h light/dark cycles (lights on at 07:00), with unlimited access to food and water. The experiments were performed according to the guidelines of the Institutional Animal Care Committee of the University. All efforts were made to minimize the number of animals used and their suffering. The drugs used in this study were desipramine hydrochloride, 6-OHDA hydrochloride and apomorphine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA). Desipramine and apomorphine were prepared in 0.9% saline and 6-OHDA was prepared in distilled water containing 0.02% ascorbic acid. Drugs were prepared on the day of the experiment.

2.2. Surgery and grouping

Unilateral 6-OHDA lesioning was carried out as previously described [18]. Briefly, animals were anesthetized with 4% chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic instrument. Rats received two unilateral stereotaxic injections of 6 μg of 6-OHDA (Sigma-Aldrich) into the left striatum (AP +0.5 and −0.5, ML −2.5 and −4.2, DV −5.0 and −5.6, all coordinates represent millimeter adjustments from the bregma). Injections were made at a rate of 0.5 μl/min using a 10 μl microsyringe. The sham operation group (n = 9) was injected with vehicle (l-ascorbic acid). After each injection, the micropipette was left in place for an additional 5 min and then slowly withdrawn.

After the operation, the rats injected with 6-OHDA were randomly assigned to one of four groups: a non-EA group (Group A, n = 9), an EA 0 Hz group (Group B, n = 15), an EA 2 Hz group (Group C, n = 15), and an EA 100 Hz group (Group D, n = 15). EA was performed at the acupoints Sanyinjiao (SP6), Yanglingquan (GB34) and Zusanli (ST36), known to relieve PD-like symptoms such as muscle and movement disorders in traditional oriental medicine [8]. The positions of these acupoints in rats correspond anatomically to their original locations in humans [10]. Rats receiving sham operations (Group E, n = 9) were used as negative controls.

2.3. Acupuncture treatment

Stimulation was administered from the second day following striatal lesions. Rats were restrained in cylindrical restrainers, which had small holes at the anterior end for ventilation. Rats were partially immobilized and the hind legs of the rats were extended out through two rear openings. Stainless-steel needles, 0.25 mm in diameter, were bent into an ‘L’ shape. The proximal ends were soldered to a wire connected to an electric stimulator (HANS-LH800, Peking University, China), which generated biphasic square waves. The distal ends were inserted to a depth of 3 mm at Sanyinjiao (SP6), Yanglingquan (GB34) and Zusanli (ST36) bilaterally. EA was given for a total of 30 min each day (1 mA stepwise to 3 mA; 0.2 ms duration, 0.2 or 100 Hz), 5 days per week. During the first 2 weeks of the sham operations group and non-EA group were also immobilized in a similar fashion for 30 min as described [8]. A rotational behavior test was administered fourteen days after lesioning (12 sessions of EA in total). Subsequently the animals were decapitated swiftly.

2.4. Observation of motor deficits

Changes in motor deficits were assessed by monitoring body rotations induced by apomorphine (0.5 mg/kg, sc) in automated rotometer chambers [10]. The net number of rotations (contralateral–ipsilateral) was recorded over a time span of 30 min. Experiencers were blinded to the groups during this behavioral test.

2.5. Immunohistochemistry

Rats (five from each of groups B, C and D, and three from each of groups A and E) were given an overdose of urethane and perfused with saline followed by 4% paraformaldehyde. The brains were removed, post-fixed, and cryoprotected. Every sixth section of the striatum (ST) and every fourth section of the substantia nigra (SN) were selected from free-floating cryomicrotome-cut sections (40 μm thick). Tyrosine hydroxylase (TH) immunohistochemistry was performed as described previously [6]. Histological pictures were taken using a bright-field microscope (Olympus, Japan) and a DP70 camera (Olympus). The percent survival 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 lesioned rats (Group E). The mean optical density (OD) value in the striatum was evaluated using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). Density was consequently expressed as a percentage relative to that in lesioned rats (this calculation has been discussed and utilized in previous studies [5,12]). An independent investigator evaluated all sections in a blinded manner.

2.6. Determination of DA by high-performance liquid chromatography

The concentrations of DA in the ST were quantified using high-performance liquid chromatography (HPLC) combined with electrochemical detection. Striatal tissues were collected immediately after the animals were killed and then frozen rapidly for storage at −80°C until analysis. On the day of the assay, the tissue was homogenized with buffer A (0.1 M perchloric acid, 0.1 mM ethylenediaminetetraacetic acid, with dihydroxybenzylamine added as an internal standard). To measure DA, homogenized samples were centrifuged at 12,000 g for 10 min, the supernatants were filtered (0.2μm; Millipore, Billerica, MA, USA), and protein concentrations were measured using Bradford reagent (BioRad, Hercules, CA, USA). A 5-μl aliquot of each supernatant was injected onto a Shiseido C18 column (4.6 mm × 150 mm column; Waters Corp, Milford, MA, USA). The mobile phase consisted of 0.07 M sodium phosphate monobasic 2H2O, 1 mM sodium octanesulphonic acid, 0.14 M EDTA, and 3% acetonitrile (pH 3.2 with perchloric acid). The electrochemical detector was set at 700 nm. The levels of the neurotransmitter are presented as μg per gram of striatum tissue.

2.7. Quantitative analysis of DAT, D1R and D2R: QRT-PCR

The SN and ST were dissected from rat brains and RNA was extracted using the acid phenol method. Then, CDNA was synthesized from mRNA using the ReverTraAceTM First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Quantitative PCR reactions were carried out using the BioEasy SYBR Green Real Time PCR Kit (Bioer, Hangzhou, China) in an ABI PRISM 7900 apparatus (ABI, USA). Relative quantification of DAT in the SN and ST, and of D1R and D2R in the ST, was done via the comparative CT method. Thus, 2−ΔΔCT determined the relative amounts of the targets, normalized to the endogenous control β-actin.

The primers used for the amplification of β-actin (NM031144.2) are as follows: (forward) 5′-gagacctgctggcctgct-3′ and (reverse) 5′-gctactgctactgctgct-3′. The primers used for the amplification of DAT (M80233.1) are as follows: (forward) 5′-ctgctgctgctgctgctg-3′ and (reverse) 5′-ctgctactactactactact-3′. The primers used for the amplification of D1R (NM15727.1) are as follows: (forward) 5′-gctactagagacggtcagg-3′ and (reverse) 5′-gctgataggctagctgctg-3′. The primers used for the amplification of D2R (X56065.1) are as follows: (forward) 5′-gtcttctgacgcttgctc-3′ and (reverse) 5′-atgagctgctgctgctg-3′. PCR cycle conditions were as follows: 15 s at 95°C, 15 s at 58°C, and 40 s at 72°C for 40 cycles. The assays were performed at least thrice for all specimens.

2.8. Western blotting

For western blotting, samples (10–20 μg protein) were loaded on 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gels. After separation, the proteins were transferred to nitrocellulose membranes. The membranes were shaken for 1 h at room temperature in Tris–buffered saline (TBS) containing 0.1% Tween-20, 5% skim milk, and 0.2% BSA. Membranes were incubated for 1 h at room temperature with primary antibodies (mouse monoclonal anti-β-actin, anti-D1R, anti-D2R, anti-DAT; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:5000, 1:1000, 1:1000, 1:1000, respectively) in TBS containing 0.1% Tween-20. The primary antibodies were detected using a horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse, anti-rabbit, anti-goat; Santa Cruz, CA, USA), and then visualized using enhanced chemiluminescence. The band intensity of the detected proteins was measured by densitometry (GeneTools, Syngene, Cambridge, UK).

2.9. Statistical analysis

All values are expressed as mean ± SEM. For comparisons among groups, one-way analysis of variance (ANOVA) and Student–Newman–Keuls post hoc test were performed with P < 0.05 as an indication of statistical significance.
3. Results

3.1. High-frequency EA significantly improves apomorphine-induced motor deficits

The effect of EA on apomorphine-induced motor deficits was then investigated and the results were shown in Fig. 1. Rats of the non-EA group (Group A) exhibited significantly greater rotational asymmetry in the direction contralateral to the lesion as compared to rats of Group E fourteen days after lesioning \((F_{4,58} = 72.92, P < 0.001;\) one-way ANOVA). EA stimulation at 100 Hz at the acupoints significantly suppressed the numbers of rotations in comparison to those in the non-EA group \((207.80 \pm 31.14\) vs. \(476.11 \pm 68.80\) turns/30 min; \(P < 0.05\)). However, 0 and 2 Hz EA did not produce significant improvement in motor disorder symptoms as compared to Group A \((361.72 \pm 33.41\) and \(302.67 \pm 29.10\) turns/30 min, respectively; \(P > 0.05\) vs. Group A).

3.2. EA benefits survival of dopaminergic neurons after 6-OHDA lesioning

The changes in the number of TH-positive neurons in the SN were observed 2 weeks after the injection of 6-OHDA. In the non-EA group, the number of TH-positive neurons on the lesioned side significantly decreased to \(21.22 \pm 5.52\)% of the number in non-lesioned rats \((F_{4,58} = 56.71, P < 0.001;\) one-way ANOVA; Fig. 2). By comparison, in hemiparkinsonian rats of Group D, the TH-positive neuron number significantly increased to \(52.10 \pm 11.41\)% after receiving 100 Hz EA stimulation \((P < 0.05\) vs. Group A). However, rats that received EA stimulation at 0 or 2 Hz showed no significant difference in the survival rate of TH-positive neurons when compared to Group A \((P > 0.05)\). Similarly, there was a marked reduction of TH-positive dendritic fiber network in the striatum of Group A when compared with the unlesioned rats \((F_{4,58} = 54.22, P < 0.001;\) one-way ANOVA; Fig. 2), while a significant increase of TH-positive dendritic fibers was observed in the ipsilateral ST of rats received 100 Hz EA treatment \((P < 0.05\) vs. Group A).

3.3. EA did not substantially elevate extracellular DA levels in the ST-lesioned side

We evaluated striatal DA level to assess the protective effect of EA in 6-OHDA-lesioned rat brains. There was a 58.04% decrease in the extracellular level of DA in the non-EA group when compared with the sham operation group \((F_{4,58} = 10.16, P < 0.001;\) one-way ANOVA; Fig. 1). EA at 100 Hz restored the 6-OHDA-induced decrease in the levels of striatal DA by 37.78%, although the increase was not significant \((P > 0.05\) vs. Group A). Similarly, no significant differences of the extracellular DA levels were observed between the 0, 2 Hz EA groups and Group A \((P > 0.05;\) Fig. 1).

3.4. High-frequency EA significantly increased DAT expression in the ST and SN

Our data revealed a significant decrease of ST and SN DAT protein levels in Group A two weeks after lesion when compared with rats in the sham operation group \((F_{4,58} = 34.33, P < 0.001\) for ST; \(F_{4,52} = 29.52, P < 0.001\) for SN; one-way ANOVA; Fig. 3). Compared with the non-EA group, the DAT protein levels in the ST and SN on the lesioned side were increased by 253.78% and 194.33% in the 100 Hz EA group \((P < 0.001\) and \(P < 0.05\), respectively; Fig. 3), indicating high frequency EA stimulation prevented the 6-OHDA lesion-induced decrease in DAT protein levels in the ST and SN. Slight increases in the DAT protein levels in the ipsilateral ST and SN were also observed in the 0 and 2 Hz groups \((P > 0.05\) vs. Group A). The mRNA levels for DAT in the ST and SN of striatal lesion rats were affected similarly to the levels of DAT protein \((F_{4,79} = 12.81, P < 0.001\) for ST; \(F_{4,79} = 11.47, P < 0.001\) for SN; one-way ANOVA; Fig. 3).

3.5. High-frequency EA restored D1R expression and prevented 6-OHDA-induced up-regulation of D2R in the ST

Comparing with the sham operation group, 6-OHDA lesioning significantly decreased the striatal D1R expression in non-EA rats \((F_{4,79} = 8.49, P < 0.001\) for protein; \(F_{4,79} = 12.05, P < 0.001\) for mRNA; one-way ANOVA; Fig. 4). However, hemiparkinsonian rats that underwent 100 Hz EA showed 81.88% and 62.62% increases in striatal D1R mRNA and protein levels, respectively, when compared with the non-EA group \((P < 0.001\) and \(P < 0.05\), respectively). Only slight increases of D1R mRNA and protein levels were observed in the 0 and 2 Hz EA groups, as compared to Group A \((P > 0.05;\) Fig. 4).

Fourteen days after the ST lesion, the D2R protein level was significantly up-regulated in Group A animals when compared with the level in the sham operation group \((F_{4,79} = 8.07, P < 0.001;\) one-way ANOVA; Fig. 4). However, only a slight D2R protein increase
was observed in animals in the high-frequency group as compared to Group E ($P > 0.05$ vs. Group E; $P < 0.05$ vs. Group A), indicating that EA stimulation at a high frequency (100 Hz) prevented the 6-OHDA-induced D2R receptor up-regulation in the ST. Though EA stimulations of 0 and 2 Hz also suppressed the D2R protein increase in the lesioned striatum, the differences were not significant as compared to the levels in Group A ($P > 0.05$; Fig. 4). The D2R mRNA levels were affected similarly to the levels of D2R protein ($F_{4,70} = 28.36$, $P < 0.001$; one-way ANOVA; Fig. 4).

### 4. Discussion

In the present study, immunohistochemical examination showed that unilateral injection of 6-OHDA into the striatum produced a significant reduction in the number of neurons in the SN, with loss of TH-positive terminals in the striatum ipsilateral to the lesion [18]. By contrast, as shown in the previous and present studies, 100 Hz EA halted DA neuron degeneration and improved apomorphine-induced rotation behavior in animal models for PD [5,9]. Considering the results of the histological and behavioral examinations undertaken, high-frequency EA, but not stimulation at 2 Hz, appears to protect dopaminergic neurons against cell death, similar to the findings in other reports [9]. The discrepancy between the 2 Hz and 100 Hz groups implies that stimulation at different frequencies has different therapeutic effects, as one would expect [19,20]. Moreover, although there was a slight increase in the number of TH-positive neurons in the 0 Hz EA group, there was no significant difference in number compared with the non-EA group. This observation indicates that the enhanced survival of TH-positive neurons seen in the high-frequency group is not related to the stress induced by needling [20]. In addition to that, 0 Hz EA did not produce obvious improvement in motor disorder symptoms, indicating that the suppressive effects require high-frequency EA stimulation at the acupoints SP6, GB34 and ST36 [9].

Changes in striatal extracellular dopamine levels occur only after almost complete dopaminergic neurons destruction [14];
however, Yuan et al. reported an obvious decrease in DA levels in rats with partial DA neuron loss [15], as observed in our study. There has been no consensus regarding the effect of EA on dopamine levels in the striatum. Although study of Sun et al. showed that high-frequency EA treatment resulted in no obvious changes in DA levels in the striatum [21], other studies have pointed out that EA stimulation does affect extracellular DA content in the brain [9,22]. It is hard to make comparisons between studies because different acupoints, treatment durations and EA frequencies were adopted in these studies. These are the key factors that likely influence the effect of EA. Our data show that 100 Hz EA raised the DA level in the ST, although the difference was not significant. The slightly up-regulated DA level was speculated to be caused by enhanced activity of TH, which is possibly stimulated by high-frequency EA [23].

The physiological role of DAT is the re-uptake of released DA into presynaptic DA-terminals. DAT density has been reported to be down-regulated in response to dopaminergic lesions, presumably to maintain synaptic dopamine levels [24]. A correlation between the degree of striatal DAT loss and levodopa therapeutic effectiveness has been suggested by several studies [25]. However, few studies have investigated the relationship between DAT expression changes and the effects of acupuncture treatment. Our study shows that high-frequency EA significantly increased the DAT expression level in the ST and SN, simultaneously. It might be speculated that the enhancement of DAT expression by 100 Hz EA points to the potential neuroprotective effects of acupuncture, reflecting an effect of EA on the regulation and function of proteins involved in dopaminergic neurotransmission [26].

Although decreased levels of presynaptic reuptake transporter have been shown in both PD patients and 6-OHDA-lesioned rats, there has been no consensus regarding the changes in postsynaptic D2R receptor levels [27–34]. Similar to D2R, investigations of postsynaptic D1R levels have also yielded controversial results [35–37]. Our data suggest that 100 Hz EA had a suppressive effect on D2R up-regulation secondary to 6-OHDA lesioning. However, the D1R expression level was obviously elevated after high-frequency EA. These changes in the levels of postsynaptic receptors were supported by western immunoblotting and PCR results, indicating that high-frequency EA treatment could enhance D1R expression and prevent D2R up-regulation in rats with experimental hemiparkinsonism. According to the well-accepted theory of PD pathogenesis, the D1R subtype of DA receptors predominates in the direct pathway and has a permissive effect on D1R2 function, and the D2R subtype is the main subtype in the indirect pathway [38]. 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 [39,40]. In light of this and our findings, we speculate that high-frequency EA prevents the up-regulation of D2R expression secondary to DAergic neuron loss, while causing basal ganglia functional adjustments through increasing the postsynaptic dopaminergic D1R level. This might help maintain the balance in the control of basal ganglia outflow to the thalamus, which may modify the disease severity and ameliorate the degrees of the main clinical symptoms of PD. However, we only studied the effects of EA in an early-stage PD model (within 2 weeks); the long-term effects must be studied to further elucidate its therapeutic effects.

To our knowledge, this is the first study to evaluate the direct influence of EA on the DAergic neurotransmitter system by combining quantitative parameters of presynaptic dopamine transporter and postsynaptic dopamine receptor expression levels.
in a 6-OHDA-lesioned model. According to this investigation, the ST D1R and DAT levels were enhanced by high-frequency EA, and the elevated D2R expression secondary to 6-OHDA lesion was adjusted at the same time. Combined with former outcomes, we speculated that, although there was a slightly ST DA level increase after high-frequency EA treatment, EA mainly works by acting on presynaptic dopamine transporters and postsynaptic dopamine receptors simultaneously to achieve a therapeutic effect in a PD model. These findings may shed some light on the mechanism by which acupuncture cooperates with medications as a complementary and alternative medicine. It is plausible to speculate that the therapeutic effects of high frequency EA might be associated, at least partially, by virtue of its anti-inflammatory and neurotrophic actions, but this requires further research.

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References


