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Short Title: vibration training & PD

## Protection of vibration training on dopamine neurons and up-regulation of brain-derived neurotrophic factor in a MPTP Mouse Model of Parkinson's disease

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## **Summary**

It is unknown whether the longer duration of vibration training (VT) has a beneficial effect on Parkinson's disease (PD). And also, the mechanisms underlying the reported sensorimotor-improvement in PD induced by short-duration of VT has not been determined. Here, we investigated the effects of longer duration (4 weeks) of low amplitude vibration (LAV) training on the numbers of dopaminergic neurons in the substantia nigra by immunostaining and the levels of dopamine (DA) and brain-derived neurotrophic factor (BDNF) in the striatum by HPLC and ELISA in the chronic MPTP lesion mouse. We demonstrated for the first time that the longer duration of VT could significantly increase the numbers of nigrostriatal DA neurons and the contents of striatal DA and BDNF in the MPTP mice. Our findings implied that longer duration of VT could protect dopaminergic neurons from the MPTP-induced damage probably by upregulating BDNF and also provided evidence for the beneficial effect of longer duration of VT on PD at the cellular and molecular level.

## **Key words**

Vibration training (VT); 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); Parkinson disease (PD); substantia nigra; striatum; dopamine (DA); tyrosine hydroxylase (TH); brain-derived neurotrophic factor (BDNF)

## Introduction

During the past decade, whole-body vibration (WBV) therapy for the rehabilitation of various conditions has gained popularity ([Lau et al. 2011](#); [del Pozo-Cruz et al. 2012](#)). In WBV, the vibration signals are delivered via a vibratory platform or chair to expose a larger part of the body to the stimulation. Recent studies have demonstrated that this modality of training was useful for improving 'Timed up and Go' (TUG) performance, endurance capacity, hormonal production, arterial function, bone mass, balance, proprioception, and health related quality of life (HRQoL) in healthy subjects ([Di Loreto et al. 2004](#); [Cardinale et al. 2006](#); [Rehn et al. 2007](#); [Bogaerts et al. 2011](#); [Figueroa et al. 2012](#)).

Parkinson disease (PD) is a movement disorder that affects millions of people especially in the growing aging population ([Pothakos et al. 2009](#)) and has typically varying degrees of muscular weakness, mobility deficits, postural instability, and other motor impairments including rigidity and tremors ([Miyai et al. 2000](#); [Bergen et al. 2002](#); [Hirsch et al. 2003](#)). This disorder is mainly caused by degeneration of the nigrostriatal dopamine (DA) neurons in the substantia nigra pars compacta and the resultant deficiency in the neurotransmitter DA at the nerve terminals in the striatum ([Nagatsu and Sawada 2005](#); [Khan et al. 2012](#)). The decreased levels of neurotrophins such as brain-derived neurotrophic factor (BDNF) are found in the nigrostriatal region of postmortem brains and/or in the ventricular or lumbar cerebrospinal fluid (CSF) of the patients with PD, and also in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) animal models of PD ([Nagatsu and Sawada 2005](#)).

In view of the many neuromotor deficits commonly observed in this disorder, it has been believed that patients with PD may be potential beneficiaries of WBV ([Lau et al. 2011](#)).

However, the reported findings were conflicting. A single session of WBV (five 1-minute bouts) has been demonstrated to be able to cause a significant improvement of gait that is measured using the TUG test in comparison to standing exercises ([Haas et al. 2006](#); [King et al. 2009](#)), but longer duration of WBV (3-5 weeks) did not result in better sensorimotor outcomes or significant results in comparison with physical therapy in body balance or signs and symptoms measured with the Unified Parkinson's Disease Rating Scale (UPDRS) ([Ebersbach et al. 2008](#); [Arias et al. 2009](#)). These results showed that it has not been confirmed whether the longer duration of WBV has a beneficial effect on signs and symptoms of Parkinson's disease ([Lau et al. 2011](#); [del Pozo-Cruz et al. 2012](#); [Sitjà et al. 2012](#)). And also, the mechanisms underlying the reported improvement in patients with PD induced by short-duration of vibration training are not well determined.

The nigrostriatal pathway is involved in the control of movement. We hypothesized that VT, no matter short or longer periods, should have a favorable effect on the pathway in PD brain if the training has a beneficial effect on sensorimotor outcomes. We therefore investigated the effects of 4 weeks of low amplitude vibration training with high or low frequency on the numbers of dopaminergic neurons in the substantia nigra by immunostaining and the levels of DA and BDNF in the striatum by HPLC and ELISA in the chronic MPTP lesion mouse. We demonstrated for the first time that longer duration (4 weeks) of vibration training could significantly increase the number of nigrostriatal DA neurons and the contents of striatal DA and BDNF in the MPTP mice, indicating that VT have a favorable effect on the nigrostriatal pathway in PD brain. Our findings implied that longer duration of VT could protect dopaminergic neurons from the MPTP-induced damage probably by upregulating BDNF and also provided evidence for the beneficial effect of longer duration of VT on PD at the cellular and molecular level.

## Materials and methods

### ***Materials***

MPTP·HCl (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine·HCl) and DA (dopamine) standard sample were purchased from Sigma Chemical, St. Louis, MO, USA. Mouse monoclonal anti-tyrosine hydroxylase and DAB kit were got from Abcam, Cambridge, UK. BDNF (brain-derived neurotrophic factor) ELISA protease assay kit was obtained from China Co. Ltd (an agent of R&D Systems), Shanghai, PRC. DA standard sample was dissolved in 0.1 M perchloric acid at an initial concentration of 1 mg/ml. The MPTP·HCl was dissolved in physiological saline (0.9%) at a concentration of 2.0 mg/ml.

### ***Mice and treatments***

Male C57BL mice weighing 25–30g were supplied by the Military Science Academy of Beijing, housed in pairs in stainless steel cages at  $22\pm 2^{\circ}$  C with a relative humidity of 50-70%, and provided free access to food (the Laboratory Rodent Diet; PMI Nutrition International Inc., Brentwood, MO) and distilled water at all times. Rooms were in a cycle of 12-hours light (0700–1900 h) and 12-hours darkness (from 1900–0700 h). The animals were randomly assigned to the following four groups: (1) Normal control group (n = 6), (2) MPTP group (n = 6), (3) MPTP+LAV-LF (low amplitude and low frequency vibration, n = 7), and (4) MPTP+ LAV-HF (low amplitude and high frequency vibration, n = 6). The mice in MPTP and normal control groups were given two intraperitoneally injections of MPTP (30 mg/kg) at 12 hours-intervals per day for 7 consecutive days or a comparable volume of 0.9% saline; respectively. The animals in MPTP+LAV-LF and MPTP+LAV-HF groups were treated with vibration training for four weeks, five days each week after the injection of MPTP. After received different treatments, the mice were decapitated and the brains were

removed for all relevant measurements. Beijing Laboratory Animal Welfare and Ethics Committee and The Animal Research Ethics Committees of Beijing Sports University approved the experimental procedures of this study.

### ***Kuribara's grid test***

Twenty minutes after the last injection of MPTP, all mice went through Kuribara's grid test as described by [Kuribara \*et al\* \(1977\)](#). The scoring scale is as follows: 0-4 seconds gets none point, 5-9 seconds 1 point, 10-14 seconds 2 points, 15-19 seconds 3 points, 20-24 seconds 4 points, 25-29 seconds 5 points and those above 30 seconds scores 6 points. Each mouse was tested three times and evaluated on an average point scale. A PD model is successfully built up only if the average point is below 3 points. Of all the 26 mice that received MPTP, only 19 of them successfully became PD mice. The score of each group in grid test before vibration training is listed in Table. 1.

### ***Vibration training***

A vibrational platform (Columbus Instruments, Columbus, OH, USA) was utilized for vibration training. The mice allowed roaming freely on the platform. Based on the findings of our preliminary experiments, vibration frequency was chosen at 10Hz (LF) or 30Hz (HF) with amplitude of 5 mm. The protocol was executed as the following: a session/day, 15 repeats/session, 1 min/repeat, with 1 min of rest in between. The mice in normal control and MPTP groups did not vibrate; however, they were transported daily to the platform so that they were exposed to the same environment as the vibration group of animals.

### ***Analysis of the number of TH-positive neurons in the substantia nigra***

Brains were cut into 25  $\mu\text{m}$  coronal sections with a freezing microtome (Thermo Scientific, Germany) and stored in cryoprotectant solution at  $-20\text{ }^{\circ}\text{C}$ . Every 6th sections containing

the substantia nigra region were subject to immunostaining. Briefly, sections were rinsed in phosphate buffered saline (PBS) and incubated with mouse monoclonal anti-tyrosine hydroxylase (1:3000) at 4°C for 12 hours. After washing with PBS for 3 times, the sections were incubated with the rabbit polyclonal anti-mouse secondary antibody (1:500) at room temperature for 1 hour. After rinsing in PBS, the immunocomplex was visualized using DAB kit. The total numbers of TH-positive cells were counted in the sections using the optical fractionator method.

### ***HPLC analysis of dopamine***

Striatal tissues for analysis were homogenized in 0.4 N perchloric acid and centrifuged at 12,000 g to separate precipitated protein. The protein pellet was resuspended in 0.5 N NaOH and the total protein concentration determined using a Biotek Model Elx800 microplate reader (Biotek Instruments Wincoski, VT) with KCjunior software. The concentrations of dopamine were assayed by HPLC with electrochemical detection as previously described ([Xie et al. 2003](#)). The electrochemical detector was an ESA model Coularray 5600A with a four-channel analytical cell with three set potentials at -100, 50, and 220 mV.

### ***Immunoassay of brain-derived neurotrophic factor (BDNF)***

BDNF levels in the striatum tissues were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). For extraction, tissue samples of the striatum were sonicated to homogeneity on ice in 10 vol/wt of an acidic extraction buffer [0.05 M sodium acetate (Merck, Darmstadt, Germany), pH 4.0, 1 M NaCl (Merck), 0.1% Triton X-100 (Sigma, Buchs, Switzerland), 1% BSA (Europa Bioproducts, Cambridge, UK), complete protease inhibitor cocktail (Roche, Mannheim, Germany)], and subsequently centrifuged for 30 min at 100,000 g and 4°C. The clear supernatants were collected, stored at -80°C, and

centrifuged again for 30 min at 20,000 g and 4°C immediately before use.

### **Statistical analysis**

Statistical analysis was performed using SPSS version 14.0 for Windows (SPSS, Chicago, IL) or InStat software (GraphPad Software). Differences in behavioral tests between groups were analyzed using repeated-measures ANOVA with the between subjects factors being lesion (saline or MPTP) and intervention and the within subject factor being time. For HPLC analysis and immunocytochemistry staining, a two-way ANOVA was performed to compare the different groups and examine for significant interactions. Within-subject effects were performed using the Huynh-Feldt correction for sphericity. For all analyses, a significance level of  $p < 0.05$  was used.

## **Results**

### ***Low amplitude vibration significantly increased the numbers of dopaminergic neurons in the substantia nigra of MPTP mice***

To find out the effects of low amplitude vibration on dopaminergic neurons in the brain, we measured the number of TH positive cells in the substantia nigra using immunohistochemical staining in mice received different treatments. It was found that MPTP treatment induced a significant reduce in the numbers of TH immunoreactive nerve fibers in mice ( $P < 0.01$ . vs. the control) (**Figure 1A and B**). However, the numbers of TH positive cells in the MPTP plus LAV-LF and the MPTP plus LAV-HF groups were significantly higher than those in the MPTP mice ( $p < 0.01$ ). No significant differences in TH positive cells were observed between the mice treated with MPTP plus low amplitude high frequency vibration and the mice received MPTP plus low amplitude low frequency



vibration. These findings indicated that low amplitude vibration, no matter with low or high frequency, could significantly increase the numbers of dopaminergic neurons in the substantia nigra of the MPTP mice.

***Low amplitude vibration significantly increased the levels of dopamine in the striatum of MPTP mice***

HPLC analysis demonstrated that the 4 weeks of low amplitude vibration induced a significant effect on the contents of dopamine in the striatum of the MPTP mice (**Figure 2**). MPTP treatment led to a significant reduction in dopamine content ( $128.33 \pm 15.68$  ng dopamine/ml) compared with the control (saline) group ( $260.56 \pm 24.44$  ng dopamine/ml), which corresponded to 73% depletion. Treatment with low amplitude vibration induced a significant increase in the contents of striatal dopamine in the MPTP mice, showing a beneficial effect of low amplitude vibration on striatal dopamine in MPTP mice. The contents of striatal dopamine in the MPTP+LAV-LF ( $204.97 \pm 25.11$  ng dopamine/ml) and MPTP+LAV-HF ( $206.37 \pm 8.99$  ng dopamine/ml) mice were significantly higher than those in the mice treated with MPTP only. This showed that low amplitude vibration could lead to about 52% recovery of striatal dopamine in MPTP mice. No significant differences were found in striatal dopamine levels between the MPTP plus LAV-LF and MPTP plus LAV-HF mice, suggesting that the frequency of vibration was not an important factor in the determination of effects of low amplitude vibration on striatal dopamine.

***Low amplitude vibration significantly increased the contents of BDNF in the striatum of MPTP mice***

To determine whether BDNF plays a role in the survival of dopaminergic neurons, we measured the contents of BDNF in the striatum using ELISA. In consistent with the effects of MPTP and low amplitude vibration on the numbers of dopaminergic neurons in the

substantia nigra and the contents of striatal dopamine, MPTP also induced a significant reduction in the levels of BDNF compared with the control (saline) mice ( $P < 0.05$ ). In addition, low amplitude vibration led to a significant enhancement in levels of BDNF in the MPTP mice (**Figure 3**). The BDNF contents in the MPTP plus LAV-LF group were higher than those in the MPTP plus LAV-HF mice although there was no significant difference between these two groups.

### ***Analysis of the correlation relationship***

The analysis on the relationship between the contents of BDNF in striatum and the numbers of TH positive cells in the substantia nigra was conducted by plotting the values for these two measurements against one another. A positive correlation was found between these two measurements in the Control ( $r = 0.78$ ,  $p < 0.01$ ); MPTP ( $r = 0.78$ ,  $p < 0.01$ ), MPTP+LAV-LF ( $r = 0.74$ ,  $p < 0.01$ ) and MPTP+LAV-HF ( $r = 0.84$ ,  $p < 0.01$ ) groups. The analysis on the relationship between the numbers of TH positive cells in the substantia nigra and the concentrations of dopamine in the striatum also showed the existence of a positive correlation between these two measurements in the Control ( $r = 0.78$ ,  $p < 0.01$ ); MPTP ( $r = 0.91$ ,  $p < 0.01$ ), MPTP+LAV-LF ( $r = 0.83$ ,  $p < 0.01$ ) and MPTP+LAV-HF ( $r = 0.84$ ,  $p < 0.01$ ) groups.

## **Discussion**

At present, it is unknown whether the longer duration of WBV has a beneficial effect on signs and symptoms of Parkinson's disease ([Lau \*et al.\* 2011](#); [del Pozo-Cruz \*et al.\* 2012](#); [Sitjà \*et al.\* 2012](#)). Although a single session of WBV (five 1-minute bouts) has been demonstrated to be able to cause a significant improvement of gait that is measured using

the TUG test ([Haas et al. 2006](#); [King et al. 2009](#)), the relevant mechanisms has not been determined. In the present study, we therefore investigated effects of 4 weeks (longer duration) of low amplitude vibration with low or high frequency on the dopaminergic neurons and the DA and BDNF contents in the brain of a MPTP Mouse Model of Parkinson's disease. We demonstrated for the first time that longer duration of low amplitude vibration could induce a significant increase in the numbers of dopaminergic neurons in the substantia nigra as well as the levels of dopamine and BDNF in the striatum of the MPTP mice. Our findings implied that longer duration of VT could protect dopaminergic neurons from the MPTP-induced damage probably by upregulating BDNF and also provide evidence at the cellular and molecular level for the existence of the beneficial effects of longer duration of VT on PD.

The significant increase in the numbers of dopaminergic neurons in the substantia nigra in the MPTP mice treated with low amplitude vibration implied that low amplitude vibration has the role to protect dopaminergic neurons from the MPTP-induced damage. Our findings also showed that there was a significant increase in BDNF in the striatum in MPTP mice treated with low amplitude vibration. BDNF is a small dimeric protein that is particularly abundant in the hippocampus and cerebral cortex. This endogenous “neuroprotectant” ([Deogracias et al. 2012](#)) is anterogradely transported from the hippocampus and cerebral cortex to its striatal target via the corticostriatal afferents ([Altar et al. 1997](#); [Huang and Reichardt 2001](#); [Baquet et al. 2004](#)). This factor has been shown to promote the survival of all major neuronal types affected in PD and other neurodegenerative diseases ([Baquet et al. 2004](#); [Zuccato and Cattaneo 2007](#)). The decreased neurotrophins including BDNF has been demonstrated in the nigrostriatal region of postmortem brains and/or in the ventricular or lumbar cerebrospinal fluid (CSF) of the PD patients and also the MPTP- and 6-OHDA animal PD models ([Nagatsu and Sawada 2005](#)). And the decreased BDNF has also been

considered as one of causes for the degeneration of the DA neurons in the substantia nigra pars compacta and the loss of nerve terminals accompanied by DA deficiency in the striatum that are responsible for most of the movement disturbances in the PD patient ([Nagatsu et al. 2000](#)). Therefore, it is highly likely that protective effects of vibration training on dopaminergic neurons in the PD mice might be at least partly due to the significant increase in BDNF in the striatum. This possibility was also strongly supported by the existence of a positive correlation between the contents of BDNF in striatum and the numbers of TH positive cells in the substantia nigra.

Tyrosine hydroxylase is an enzyme responsible for the rate-limiting step in dopamine biosynthesis ([Cartier et al. 2010](#); [Prandovszky et al. 2011](#)). The loss of dopaminergic or TH positive neurons in substantia nigra is always accompanied by the decreased DA content in striatum in the PD patients ([Toulouse and Sullivan 2008](#); [Hilker et al. 2012](#)). In addition, the analysis on relationship between the numbers of TH positive cells in the substantia nigra and the concentrations of dopamine in the striatum demonstrated the existence of a positive correlation between these two measurements in all four groups. The positive correlation and also the reduction in TH positive neurons with DA contents in the PD patients suggested that the significant increase in the levels of dopamine in the striatum in the MPTP mice treated with low amplitude vibration probably resulted from the increased numbers of dopaminergic neurons in the substantia nigra.

Our data showed that four weeks of vibration training almost completely prevented the MPTP-induced loss of DA neurons in the substantia nigra and reduction of DA levels in the striatum. In addition to BDNF, we did not examine the contents of other neurotrophic factors such as nerve growth factor (NGF), cytokines including tumor necrosis factor (TNF)-alpha and transforming growth factor (TFG)-alpha and the activities of caspase-1 and caspase-3

in the MPTP mice treated with different treatments. To better understanding the mechanisms underlying the improvement in sensorimotor function induced by short- or longer-duration of vibration training, further investigations on the changes of these parameters are absolutely needed in the MPTP mice treated with or without vibration training.

In the present study, we also found that there were no significant differences in the numbers of dopaminergic neurons and the levels of dopamine and BDNF in the brain between the MPTP mice treated with LAV-LF or MPTP+ LAV-HF. This indicated that frequency is not a key factor under our experimental conditions. The findings also remained us to consider the possibility that the amplitude and/or frequency might be not the optimal in those studies where longer duration of WBV (3-5 weeks) did not result in a better sensorimotor outcome in the PD patients ([Ebersbach \*et al.\* 2008](#); [Arias \*et al.\* 2009](#)). Therefore, further studies about the effects of different amplitude and/or frequency and also duration on PD are also needed. These studies will provide important information for the establishment of the clinical efficacy of vibration training in improving sensorimotor function in patients with PD.

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## **Author Contributions**

L. Z. and Z.M.Q. conceived, organized and supervised the study; L.X.H., S.N. H., L.G., L.L. and Y.L. performed the experiments; L.Z. and Z.M.Q. contributed to the analysis and interpretation of data, prepared and wrote the manuscript.

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## Legends of Figures

**Figure 1. Low amplitude vibration significantly increased the numbers of dopaminergic neurons in the substantia nigra of MPTP mice.** After the designed treatments, the mice were decapitated and perfused, the brains were removed, and the numbers of TH positive cells in the substantia nigra were then determined using immunohistochemical staining as described in “Methods and materials”. A: A representative immunohistochemical staining, Scale bars = 200  $\mu$ m, B: TH positive cells. Data were presented as mean  $\pm$  SEM (the control: n = 6; MPTP: n = 6; MPTP+LAV-LF (low amplitude and low frequency vibration): n = 7, and MPTP+ LAV-HF (low amplitude and high frequency vibration): n = 6). \*\*P<0.01 versus the corresponding value.

**Figure 2. Low amplitude vibration significantly increased the levels of dopamine in the striatum of MPTP mice.** After the completion of all experimental procedures, the mice were decapitated and perfused, the brains were removed, and the contents of striatal dopamine (ng/ml) were assayed by HPLC analysis as described in “Methods and materials”. Data were presented as mean  $\pm$  SEM (the control: n = 6; MPTP: n = 6; MPTP+LAV-LF (low amplitude and low frequency vibration): n = 7, and MPTP+ LAV-HF (low amplitude and high frequency vibration): n = 6). \*\*P<0.01 versus the corresponding value.

**Figure 3. Low amplitude vibration significantly increased the contents of BDNF in the striatum of MPTP mice.** After the completion of all experimental procedures, the mice were decapitated and perfused, the brains were removed and the contents of BDNF (pg/ml) in the striatum were determined using ELISA analysis as described in “Methods and materials”. Data were presented as mean  $\pm$  SEM (the control: n = 6; MPTP: n = 6;

MPTP+LAV-LF (low amplitude and low frequency vibration): n = 7, and MPTP+ LAV-HF (low amplitude and high frequency vibration): n = 6). \*P<0.05 versus the corresponding value.

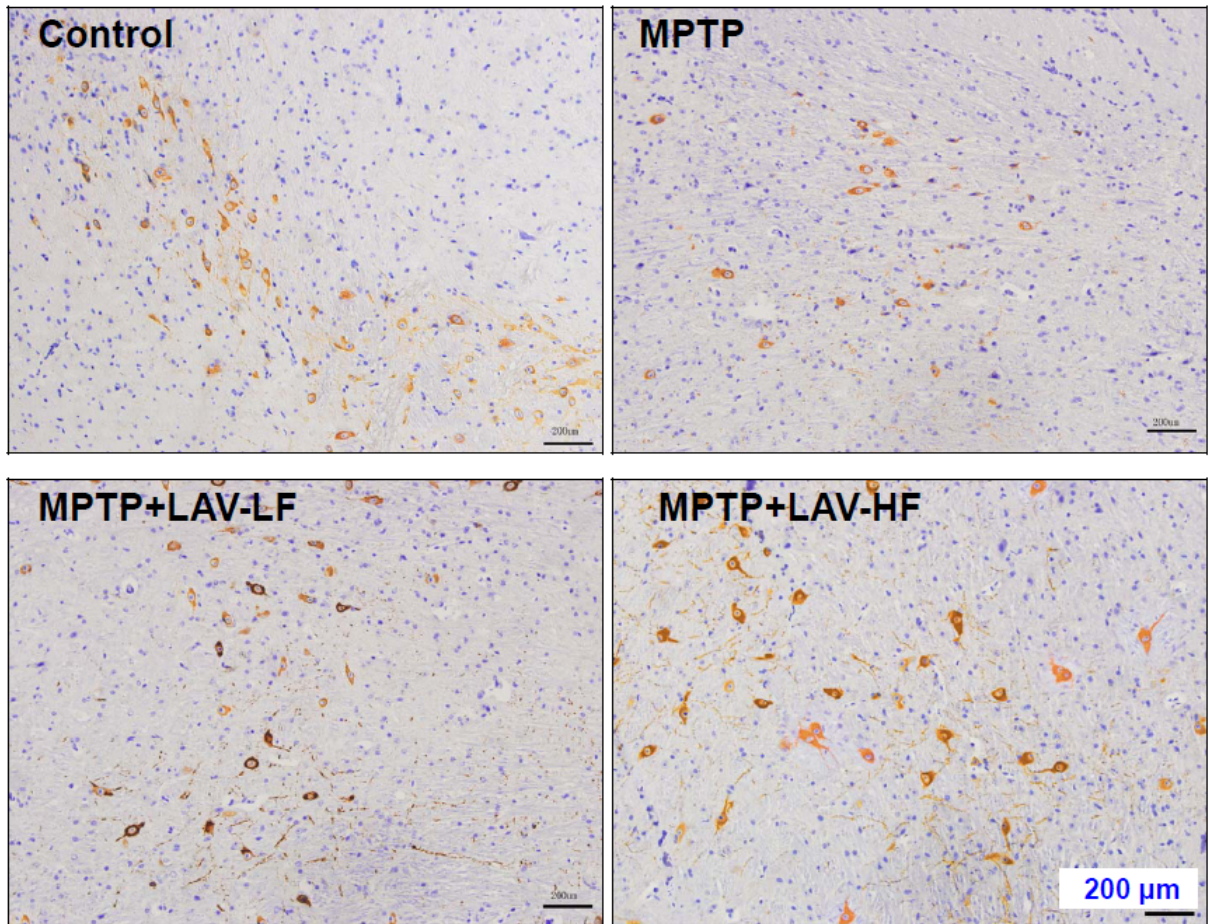
**Table 1. Scores of Kuribara's grid test**

| Group       | n | Score (point) | P (vs. Control) |
|-------------|---|---------------|-----------------|
| Control     | 6 | 6.00±0.00     |                 |
| MPTP        | 6 | 1.50±1.29     | < 0.01          |
| MPTP+LAV-LF | 7 | 1.40±1.52     | < 0.01          |
| MPTP+LAV-HF | 6 | 1.33±1.17     | < 0.01          |

All mice went through Kuribara's grid test twenty minutes after the last injection of MPTP before vibration training was conducted. Data were expressed mean ± S.E.M..

Figure1

**A**



**B**

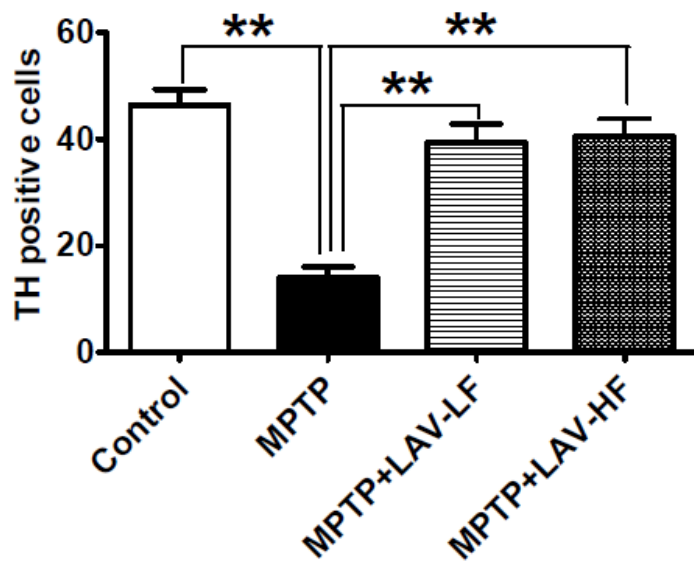


Figure 2

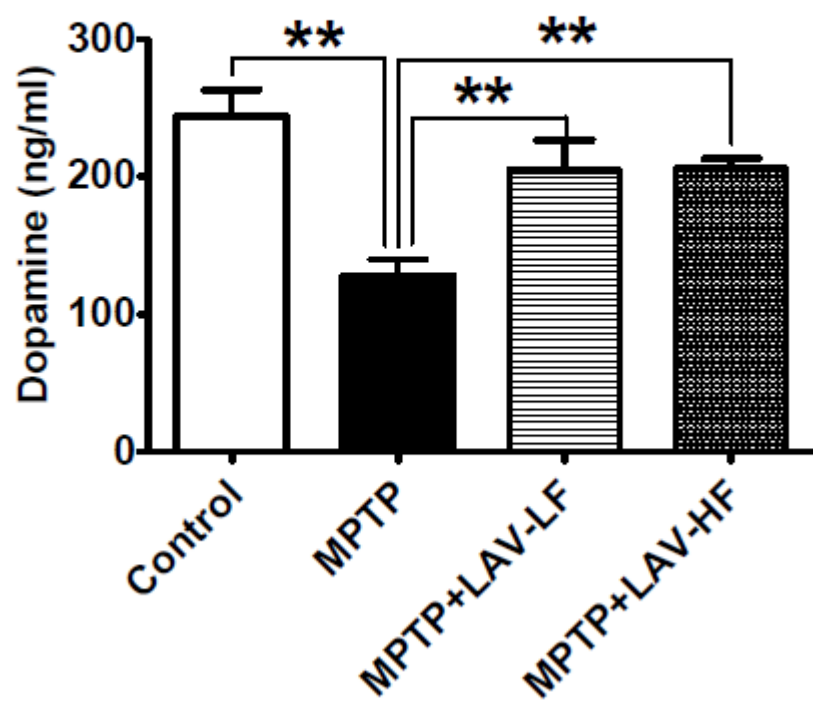


Figure 3

