



Neuroprotective changes in degeneration-related gene expression in the substantia nigra following acupuncture in an MPTP mouse model of Parkinsonism: Microarray analysis

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the death of dopamine-generating cells in the substantia nigra (SN). Acupuncture stimulation results in an enhanced survival of dopaminergic neurons in the SN in Parkinsonism animal models. The present study investigated changes in gene expression profiles measured using whole transcript array in the SN region related to the inhibitory effects of acupuncture in a chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Parkinsonism model. In this model, acupuncture stimulation at GB34 and LR3 attenuated the decrease in tyrosine hydroxylase in the SN region; stimulation at non-acupoints did not suppress this decrease. Gene array analysis revealed that 22 (10 annotated genes: *Cdh1*, *Itih2*, *Mpzl2*, *Rdh9*, *Serpig1*, *Slc6a13*, *Slc6a20a*, *Slc6a4*, *Tph2*, and *Ucma*) probes that were up-regulated in MPTP animals relative to controls were exclusively down-regulated by acupuncture stimulation. In addition, 17 (two annotated genes: *4921530L21Rik* and *Gm13931*) probes that were down-regulated in MPTP animals compared to controls were exclusively up-regulated by acupuncture stimulation. These findings indicate that the 39 probes (12 annotated genes) affected by MPTP and acupuncture may be responsible for the inhibitory effects of acupuncture on degeneration-related gene expression in the SN following damage induced by MPTP intoxication.

Keywords: Parkinson's disease, gene array, differentially expressed genes, neurodegeneration, acupoints.

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by major behavioral symptoms, which include tremor, akinesia, bradykinesia, and stiffness (Jankovic, 2008). PD results from the death of dopaminergic neurons in the substantia nigra (SN; (Goto *et al.*, 1989; Chauhan *et al.*, 2001; Kim *et al.*, 2003; Fernandez-Espejo 2004), which plays an important role in several brain functions, particularly, motor planning, eye movement, learning, reward-seeking and addiction. Not surprisingly, gress movement dysfunction caused by damage to this area is a symptom of PD.

Using a 6-hydroxydopamine (6-OHDA)-induced Parkinsonism model, a previous study found that acupuncture stimulation at acupoints GB34 and LR3 results in the enhanced survival of dopaminergic neurons in the SN and a ~87.7% improvement in motor dysfunction (Park *et al.*, 2003). Similar finding were found following the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), where the acupuncture-induced enhancement of synaptic dopamine availability may play a critical role in the observed motor function improvement (Kim *et al.*, 2011). Acupuncture stimulation at the same acupoints also attenuates the decrease in tyrosine hydroxylase (TH) immunoreactivity (IR) and generates neuroprotective effects in the SN in an MPTP-induced mouse model of Parkinsonism (Kang *et al.*, 2007). The mechanisms underlying the neuroprotective effects of acupuncture on the SN are thought to be in part related to decreased microglial activation and reduced inflammatory responses (Kang *et al.*,

2007); these, in turn, inhibit ferric iron and ferritin heavy chain deposition (Choi *et al.*, 2009).

Several genes that are affected by MPTP and stimulation at acupoints may be responsible for the inhibitory effects of acupuncture on MPTP-induced degeneration in striatal regions (Choi *et al.*, 2011b). Additionally, gene expression in thalamic regions damaged by MPTP intoxication may be affected, at least in part, by acupuncture at specific acupoints (Yeo *et al.*, 2013). Changes in gene expression profiles in the SN following acupuncture stimulation were identified in an acute Parkinsonism model over three days (Hong *et al.*, 2010); however, no such changes have been demonstrated in a chronic Parkinsonism animal model. Because PD is a chronic neurodegenerative disorder, changes in gene expression in the SN in a chronic Parkinsonism animal model should be investigated to identify the neuroprotective mechanisms underlying acupuncture treatment.

Thus, we investigated changes in gene expression profiles in the SN region in a relatively chronic Parkinsonism model and identified the genes related to the inhibitory effect of acupuncture on alterations in gene expression induced by MPTP intoxication.

Materials and Methods

MPTP model of Parkinsonism

Six-week-old male inbred C57BL/6 mice (20–22 g; Samtaco, Korea) were divided into the following four groups: control (CTL); MPTP-treatment only (MPTP); MPTP and acupuncture treatment at GB34 and LR3 (MPTP-A); and MPTP and acupuncture treatment at non-acupoints (MPTP-NA). Mice in the control group ($n = 9$) were injected intraperitoneally with saline 0.9% (100 μ L) once daily for four weeks, while mice in the MPTP group ($n = 9$) were injected intraperitoneally with MPTP-HCl (20 mg/kg of free base) in saline 0.9% (100 μ L) at 24-h intervals over four weeks to produce a sustained chronic model of Parkinsonism (Bezard *et al.*, 1997; Kuhn *et al.*, 2003; Choi *et al.*, 2011a; Yeo *et al.*, 2013). On the day following the final MPTP treatment, mice were anesthetized using 16.5% urethane and perfused transcardially with cold 0.05 M sodium-phosphate buffer to enable immunohistochemical evaluation. The Kyung Hee University Animal Experimentation Committee approved all animal protocols used in this study. Reagents used but not mentioned were purchased from Sigma (USA).

Acupuncture administration

Acupuncture was performed manually 2 h after the first MPTP injection and at 48-h intervals thereafter for a total of 14 sessions. The acupuncture procedure (acupoints: GB34 [Yanglingquan] and LR3 [Taichong]; non-acupoints: both sides of the hips) was performed as reported previously (Park *et al.*, 2003; Kang *et al.*, 2007; Choi *et al.*,

2011b). Briefly, mice in the acupoints group were immobilized by hand 2 h following MPTP administration, and the acupuncture needles were inserted bilaterally to depths of 1 mm at acupoint LR3 and 3 mm at acupoint GB34 before being turned at a rate of two spins per second for 15 s, as reported previously (Kang *et al.*, 2007). In the non-acupoints group, the needles were inserted to a depth of 3 mm on both sides of the hips, and a procedure identical to that described above was performed.

Immunohistochemistry

After four weeks, the brains were removed, post-fixed in 0.05 M sodium-phosphate buffer containing 4% paraformaldehyde for 12 h at 4 °C, rinsed with 0.05 M sodium-phosphate buffer, dehydrated with sucrose for 12 h at 4°C, and then cryosectioned. Coronal sections of the brains (30- μ m thickness) were cut using a cryomicrotome. Immunohistochemical analyses were carried out using an ABC kit and a Mouse on Mouse (M.O.M) immunodetection kit (Vector Laboratories, CA) using a modification of the avidin-biotin-peroxidase method. Briefly, sections encompassing the entire striatal and SN regions were incubated in phosphate-buffered saline (PBS; pH 7.4) with 3% H₂O₂, exposed to 3% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS for 1 h, and then treated with an avidin-biotin blocking kit (Vector Laboratories). When using the mouse anti-TH antibody, sections were treated with an M.O.M. mouse Ig-blocking reagent (Vector Laboratories) at room temperature for 1 h prior to incubation with the primary antibody. Thereafter, each section was stained overnight at 4 °C with a mouse anti-TH antibody (1:1,500; Santa Cruz Biotechnology, USA) to identify dopaminergic neurons in the striatal and SN regions. The sections were then sequentially treated with a biotinylated anti-mouse IgG followed by an avidin-biotin-peroxidase complex and developed using a diaminobenzidine-hydrogen peroxide solution (0.003% 3,3-diaminobenzidine and 0.03% hydrogen peroxide in 0.05 M Tris, pH 7.0).

Western blotting

The bilateral striatal and SN regions were homogenized in 20 mM HEPES-KOH buffer (pH 7.5) with 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail. After centrifugation at 15,000 \times g for 10 min, soluble supernatant samples of equal protein concentration (30 μ g total protein) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad; USA). The membranes were blocked with 5% skim milk in 0.1% Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.5] and 150 mM NaCl containing 0.1% Tween-20; TBST) at room temperature for 1 h and then incubated with mouse anti-TH (1:2,000) and mouse anti-actin (1:5,000, Santa Cruz Biotechnology) antibodies. After being washed with 0.1%

TBST, the membranes were incubated with an anti-mouse IgG-peroxidase antibody (1:2,000, Bio-Rad), and the antigen-antibody complexes were visualized using the Pierce ECL western blotting substrate (Thermo Scientific, USA).

RNA extraction and microarray analysis

Total RNA was extracted from the bilateral SN tissue of each group ($n = 2$; both groups) using an RNeasy Plus Mini kit (QIAGEN, USA) according to the manufacturer's instructions. Isolated RNA quality was estimated and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). An aliquot (300 ng) of total RNA was subjected to an Affymetrix GeneChip[®] Mouse Gene 1.0 ST Array (genome-wide expression profiling chip; 28,853 genes of 35,557 probes; Affymetrix, USA) according to the GeneChip Whole Transcript (WT) Sense Target Labeling Assay manual, as reported previously (Choi *et al.*, 2011a; Hwang *et al.*, 2009; Lin *et al.*, 2010). Briefly, the procedures were carried out as follows: mixing of T7-(N)6 primer and poly-A RNA controls with 300 ng of total RNA isolated from the SN of each group; synthesis of first-cycle, first-strand complementary DNA (cDNA) and then second-strand cDNA; synthesis of first-cycle cRNA and cleanup of cRNA; synthesis of second-cycle, single-strand cDNA (ss cDNA) and cleanup of ss cDNA; fragmentation of ss cDNA and labeling of the fragmented ss cDNA; hybridization of labeled ss cDNA to the GeneChip; and finally, the staining, washing, and scanning of the GeneChip using a Fluidics 450 station and the GeneChip Operating Software (GCOS, Affymetrix).

Microarray data analysis

The usefulness of the signal intensities was first evaluated by visual examination of the scanned images. Quality control of the scanned data was then conducted by confirming the order of the signal intensities of poly-A controls and hybridization controls using the Expression Console software (Affymetrix). Microarray data were analyzed using GenPlex ver. 3.0 (ISTECH, Korea, (An *et al.*, 2009; Choi *et al.*, 2011a; Hwang *et al.*, 2009; Yeo *et al.*, 2013). A total of eight CEL files (two CEL files generated from each group x four experimental groups) were uploaded and normalized under the following conditions: perfect match (PM)-only as a PM intensity adjustment; the Robust Multichip Analysis (RMA) quantification method as a probe set summarization algorithm for log transformation with base 2 (\log_2); and the quantile normalization method for evaluation of preliminary data quality in the preprocessing module, which functions as a data quality control, using the Expression Console software (Affymetrix). The mean signal intensities of the 28,853 genes were obtained from two chips from each group. Following normalization, the differentially expressed genes (DEGs) in the GeneChip that satisfied the conditions of the fold change cutoff (1.3) as well as the Student's *t*-test significance criterion ($p < 0.05$) were identified

using the DEG-finding module; *i.e.*, $\log_1.3/\log_2 = 0.379 <$ average \log_2 fold-change value (X) for the identification of up-regulated genes and $-\log_1.3/\log_2 = -0.379 >$ average \log_2 fold-change value (X) for the identification of down-regulated genes. A pathway analysis of all metabolic and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed using the pathway module, as described previously (Hwang *et al.*, 2009; Choi *et al.*, 2011a; Yeo *et al.*, 2013). A National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) accession number was assigned to the raw microarray data generated in this study.

Real time RT-PCR

For real-time reverse transcription polymerase chain reaction (RT-PCR) analyses, the total RNA (500 ng) of the samples (CTL, MPTP, and MPTP-A) was subjected to the reaction using a SuperScript First-strand Synthesis Kit (Invitrogen Life Technologies, USA) according to the manufacturer's instructions. The advanced relative expression levels of representative DEGs were monitored with a LightCycler 480 II Real Time PCR Instrument and the LightCycler 480 software ver. 1.5.0.39 (Roche Diagnostics; Germany) using a LightCycler 480 SYBR Green I Master (Roche Diagnostics). Mean crossing point (CP) values were obtained and the expression levels of the target genes in the various groups were then compared to those of the reference gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), using an advanced relative quantification method. The ratios of the concentrations of target genes to that of the reference gene were obtained; the primer sequences for each gene are shown in Table 1. The specificity of each primer set was confirmed by determining the melting temperature and size of each product by gel electrophoresis.

Statistical analysis

Statistical analyses were performed using the statistical package for the social sciences (SPSS) software, ver. 18. Values are means \pm SE of three independent determinations.

Table 1 - The sequences of primers.

Gene symbol	Gene accession No.	Primer sequences (5'→3')
Rdh9	NM_153133	F: gagcgtgttgggaacagag R: gtttctcatcactcgttg
Slc6a4	NM_010484	F: acctggactccattccac R: cctggagtcccttgactga
Tph2	NM_173391	F: gagcttgatgccaccat R: tggccacatccacaaaatac
Ucma	NM_001113558	F: cgtggaggaacagagatga R: aggccatcataatgccactg
GAPDH	NM_008084	F: gtcttcaccacatggagaagg R: tcatggatgaccttgccag

Results and Discussion

Chronic MPTP-induced Parkinsonism mouse model and the preventive effects of acupuncture

Because TH expression is significantly attenuated in the brains of PD patients (Pardridge, 2005) and in Parkinsonism animal models (Park *et al.*, 2003; Kang *et al.*, 2007; Choi *et al.*, 2009), we evaluated TH levels to confirm the establishment of a chronic MPTP-induced Parkinsonism mouse model and the mediating effects of acupuncture at GB34 and LR4. In the SN pars compacta (SNpc) and striatal regions, TH levels were noticeably reduced in the MPTP group (MPTP) relative to the control group (CTL; Figures 1a, b, e, f, i, and j). Acupuncture stimulation at GB34 and LR3 (MPTP-A) attenuated the decrease in TH in both areas (Figures 1c, g, and k), but acupuncture at non-acupoints (MPTP-NA) did not similarly influence TH (Figures 1d, h, and l). Immunohistochemical analyses using Western blots confirmed that the decrease in TH levels in the striatal and SNpc regions induced by MPTP intoxication was significantly inhibited by acupuncture at acupoints (MPTP-A) but not at non-acupoints (MPTP-NA; Figure 2). These results confirm that acupuncture stimulation suppresses the pathological change in TH levels induced by MPTP intoxication in the striatal and SNpc regions, of the mice in our study.

Changes in gene expression in the SN region following chronic MPTP intoxication

As shown in the box plot graph (Figure S1) normalization through the preprocessing module was successful. Of the 28,835 genes represented in the oligonucleotide array, genes were selected and evaluated if they displayed log₂-transformed mean signal intensities greater than 0.379 (1.3 fold change) and $p < 0.05$ by Student's *t*-test between the control and MPTP groups. Compared to the control, 244 up-regulated (Table S1) and 255 down-regulated (Ta-

ble S2) DEGs were identified in the SN region following chronic MPTP intoxication.

Changes in gene expression in the SN region following acupuncture

Of the 28,853 genes represented in the oligonucleotide array, those with log₂-transformed mean signal intensities greater than 0.379 (1.3-fold change) and a $p < 0.05$ by Student's *t*-test between the MPTP-A and MPTP groups or the MPTP-NA and MPTP groups were selected and evaluated. Compared to the MPTP group, genes in the SN region were regarded as DEGs following acupuncture stimulation at acupoints and non-acupoints, respectively.

Up-down-regulated genes following MPTP intoxication and acupuncture

Compared to the control, 22 (10 annotated genes) of the 244 probes were up-regulated in the SN region following MPTP exposure and exclusively down-regulated by acupuncture at acupoints but not non-acupoints ("Up-down"; Table 2).

Cadherin 1 (*Cdh1*) is a classic member of the cadherin superfamily that is thought to contribute to the control of synapse formation and synaptic transmission and plasticity (Angst *et al.*, 2001). *Cdh1* signaling is an important component of the regulation of GABAergic synapses in brain neurons (Fiederling *et al.*, 2011); indeed, elevated GABA levels have been detected in PD (Emir *et al.*, 2012). In the current study, *Cdh1* was up-regulated in the MPTP group compared to controls. However, acupuncture stimulation at GB34 and LR3 maintained *Cdh1* expression similar to levels in control animals, suggesting that the down-regulation of *Cdh1* expression by acupuncture may have a neuroprotective effect.

Solute carrier family 6 (neurotransmitter transporter, GABA), member 13 (*Slc6a13*) is a sodium- and chloride-dependent member of solute carrier family 6 (SLC6)

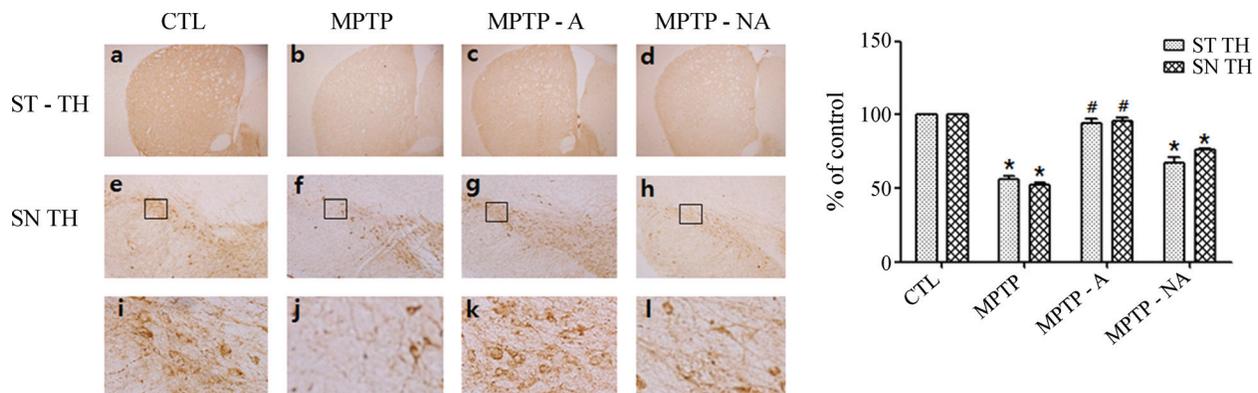


Figure 1 - TH expression is maintained by acupuncture in a chronic MPTP-induced Parkinsonism model. The striatum (ST, a-d) and substantia nigra (SN, e-l) were immunostained using an anti-tyrosine hydroxylase (TH; a-d, 40x; e-h, 100x; i-l, 400x) antibody. CTL, saline control; MPTP, MPTP treated only; MPTP-A, acupuncture treated at acupoints GB34 and LR3, with MPTP; MPTP-NA, acupuncture treated at non-acupoints, with MPTP. The TH density in each ST region was evaluated by measuring the densitometry in the same area (500 μm^2) and the number of TH-positive cells in the SNpc (200x400 μm) counted in the same area. A * $p < 0.05$ compared to the control and a # $p < 0.05$ compared to the MPTP were considered significant.

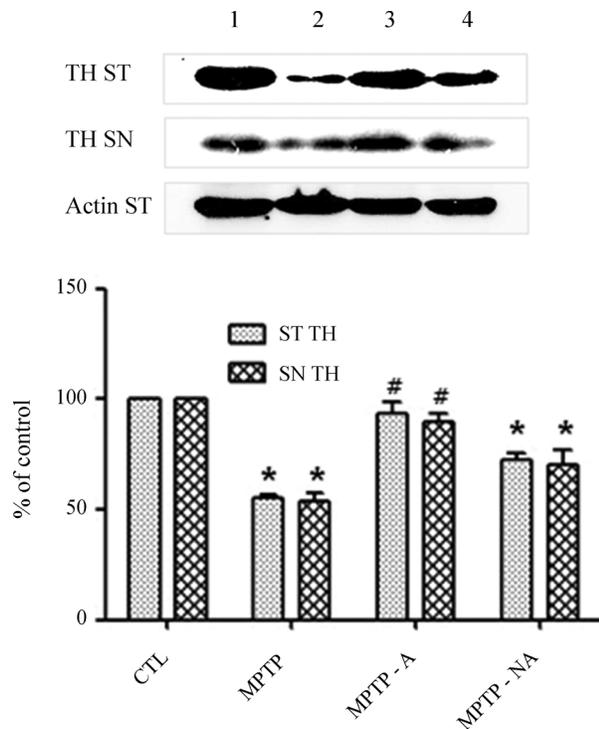


Figure 2 - Inhibition of the decrease in MPTP-induced TH expression by acupuncture at acupoints. Western blot determination of tyrosine hydroxylase (TH) expression levels in the striatum (ST) and substantia nigra (SN). Actin in the ST was used as a total protein loading control (30 μ g). Lane 1, CTL; Lane 2, MPTP; Lane 3, MPTP-A; and Lane 4, MPTP-NA. A * $p < 0.05$ compared to the control and a # $p < 0.05$ compared to the MPTP were considered significant.

that mediates the rapid removal of GABA and maintains low extracellular levels of this neurotransmitter (Chen *et al.*, 2004; Kanner, 2006). Evidence suggests that alteration of GABA transporters is linked to PD (Wang *et al.*, 2007). Accordingly, the overstimulation of GABA_A receptors can potentiate neuronal cell damage; GABA transport blockers are neuroprotective in these situations (Zeevalk and Nicklas, 1996, 1997), suggesting that overexpression of the GABA transporter can damage neurons. In the current study, *Slc6a13* was up-regulated in the MPTP group compared to the control group; therefore this may be the underlying cause of neuronal intoxication. However, acupuncture stimulation at GB34 and LR3 maintained *Slc6a13* gene expression at levels similar to those in control animals, suggesting that acupuncture has a neuroprotective effect.

Tryptophan hydroxylase 2 (Tph2), with a molecular weight of 56 kDa, is the predominant form of this enzyme present in brain extracts from the mesencephalic tegmentum, striatum, and hippocampus (Sakowski *et al.*, 2006). Tph2 initiates serotonin synthesis in mammals with Tph1 (Alenina *et al.*, 2009), and plays a critical role in the maintenance of brain serotonin homeostasis (Beaulieu *et al.*, 2008). Tph2-derived serotonin is involved in the regulation of behavior and autonomic pathways but is not essential for adult life (Alenina *et al.*, 2009). However, Tph2 is known to

be highly labile to oxidation (Kuhn *et al.*, 1980). Therefore, the oxidative processes that prevail in PD may cause a misfolding of Tph2 and result in the modification of serotonin function such as is seen in dopamine neurons. Tph2 oxidation inhibits its activity and leads to the formation of high-molecular-weight aggregates in a dithiothreitol-reversible manner (Kuhn *et al.*, 2011). Alteration of serotonin levels by Tph2 hyperinnervation affects the wiring of the brain and can produce long-lasting changes leading to the development of neurodevelopmental disorders such as PD (Migliarini *et al.*, 2012).

Myelin protein zero-like 2 (Mpzl2) is also known as Eva and Eva1. In human choroid plexus epithelial cells and a subset of CD4 T lymphocytes, Mpzl2 is expressed at high levels. Mpzl2 expressed in choroid plexus cells may regulate the permeability of the blood-cerebrospinal fluid (CSF) barrier (Chatterjee *et al.*, 2008), suggesting a novel mechanism of CNS immune surveillance regulation (Wojcik *et al.*, 2011). In the current study, Mpzl2 was up-regulated in the MPTP group compared to controls; however, acupuncture stimulation at GB34 and LR3 maintained Mpzl2 gene expression at levels similar to those in control animals. These results also suggest that acupuncture suppresses the Mpzl2 overexpression that can be caused by MPTP neuronal loss.

The serine (or cysteine) peptidase inhibitor, clade G, member 1 (Serping1) gene encodes the serine protease inhibitor (serpin) known as C1 inhibitor. The C1 inhibitor is important for regulation of several processes, including inflammation, that are involved in maintenance of blood vessels (Shagdarsuren *et al.*, 2008). Serping1 is up-regulated in the microglial pathway upon stimulation by interferon-gamma (IFN-gamma; Moran *et al.*, 2007) or lethal lipopolysaccharide-induced endotoxic shock (Liu *et al.*, 2007).

Inter-alpha trypsin inhibitor, heavy chain 2 (Itih2) has also been identified during inflammation (Scavenius *et al.*, 2011), and may contribute to the cascade of events leading to neuronal degeneration (Hirsch and Hunot 2009; Tufekci *et al.*, 2012). Therapeutic strategies aim to down-regulate these inflammatory processes and may slow the progression of PD (Hirsch and Hunot 2009). Therefore, the maintenance by acupuncture stimulation of expression levels of Itih2 and Serping1, which are involved in neuroinflammation, similar to those in control animals suggests that acupuncture may have a neuroprotective effect.

SLC6 (neurotransmitter transporter), member 20A (Slc6a20a) is also known as Xt3s1, Xtrp3s1, AU022428, and A730081N20Rik. Neurotransmitter transporters of the SLC6 family play an important role in the removal of neurotransmitters in brain tissue and in amino acid transport in epithelial cells (Kowalczyk *et al.*, 2005). Either the IMINO system or the imino acid carrier supports the main load of proline transport (Munck and Munck, 1994); this gene is expressed at high levels in the brain. More specifically, the IMINO system is present in the microglial cells of

Table 2 - List of (up-down) substantia nigral genes which were up-regulated in MPTP vs. control and down-regulated in MPTP-A vs. MPTP.

Gene symbol	Gene title	Probe ID (Gene accession No.)	Average log ₂ (fold change) in M vs. C (Up-regulated)	p-value ^a in M vs. C	Average log ₂ (fold change) in MA vs. M (Down-regulated)	p-value ^a in MA vs. M
Cdh1	cadherin 1	10575052 (NM_009864)	0.705	0.002	-0.502	0.004
Ith2	inter-alpha trypsin inhibitor, heavy chain 2	10480003 (NM_010582)	0.439	0.024	-0.392	0.036
Mpzl2	myelin protein zero-like 2	10584827 (NM_007962)	0.630	0.010	-0.884	0.027
Rdh9	retinol dehydrogenase 9	10367041 (NM_153133)	0.470	0.043	-0.539	0.048
Serp1	serine (or cysteine) peptidase inhibitor, clade G, member 1	10484463 (NM_009776)	0.467	0.042	-0.569	0.026
Sle6a13	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	10541318 (NM_144512)	0.611	0.011	-0.561	0.011
Sle6a20a	solute carrier family 6 (neurotransmitter transporter), member 20A	10597960 (NM_139142)	0.649	0.015	-0.594	0.004
Sle6a4	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (Sle6a4), mRNA	10378816 (NM_010484)	0.544	0.017	-1.233	0.015
Tph2	tryptophan hydroxylase 2	10372443 (NM_173391)	0.796	0.007	-1.582	0.006
Uona	upper zone of growth plate and cartilage matrix associated	10469058 (NM_001113558)	0.455	0.012	-0.607	0.033
		10338456 ^b	0.794	0.011	-0.505	0.046
		10338657 ^b	0.652	0.010	-0.617	0.044
		10339444 ^b	1.200	0.005	-1.107	0.033
		10342771 ^b	0.657	0.037	-0.457	0.004
		10343150 ^b	0.743	0.035	-0.512	0.024
		10343377 ^b	1.136	0.030	-0.594	0.015
		10343396 ^b	0.439	0.018	-0.383	0.027
		10343743 ^b	1.258	0.032	-1.296	0.007
		10344355 ^b	0.478	0.030	-0.425	0.020
		10423383 ^b	0.412	0.028	-0.402	0.004
		10594513 ^b	0.526	0.038	-0.434	0.028
		10598073 ^b	0.914	0.028	-1.013	0.014

^aDetermined using Student's *t*-test.^bGene accession No. was not determined.

the brain (Kowalczyk *et al.*, 2005), by which proline is taken up (Hubscher and Berkley, 1992; Mikulska and Lisowski, 2003). This, in turn, influences the proline level in extracellular fluid (ECF). Proline itself has been postulated to be neurotoxic when its extracellular concentration is increased in the brain (Cohen and Nadler 1997; Nadler *et al.*, 1988). In the current study, Slc6a20a expression in the SN was up-regulated in the MPTP-induced mouse model of PD. Thus, a disturbance in the uptake of proline influences the extracellular concentration of proline. Increasing proline levels in ECF may induce neuronal cell death, suggesting that down-regulation of Slc6a20a gene expression following acupuncture may have a neuroprotective effect.

SLC6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4) is also known as Htt, Sert, 5-HTT, or AI323329. Recently, it was reported that MPTP-induced dopaminergic denervation is followed by serotonergic hyperinnervation (Hebert *et al.*, 2005). In a 6-OHDA animal model of PD, the effects of destroying ascending dopamine pathways on extracellular levels of serotonin and serotonin innervation in the rat striatum were examined (Balcioglu *et al.*, 2003). Specifically, the Slc6a4 promoter may govern the genetic risk of PD (Albani *et al.*, 2009). In the current study, Slc6a4 was up-regulated in the MPTP group compared to controls, and acupuncture stimulation at GB34 and LR3 maintained Slc6a4 gene expression at levels similar to those in control animals. These results indicate that acupuncture may suppress the Slc6a4 overexpression caused by MPTP intoxication.

Upper zone of growth plate and cartilage matrix associated (Ucma) is a highly conserved tyrosine-sulphated secreted protein with a molecular weight of 17 kDa that is expressed by juvenile chondrocytes (Tagariello *et al.*, 2008). Ucma may be involved in the negative control of the osteogenic differentiation of osteochondrogenic precursor cells in peripheral zones of fetal cartilage and at the cartilage-bone interface (Surmann-Schmitt *et al.*, 2008). An imbalance in isoform expression may, therefore, be involved in skeletal pathology (Le Jeune *et al.*, 2010). PD patients have been reported to have a significantly increased risk of fracture (Sato *et al.*, 2001; Pouwels *et al.*, 2013), which may be caused by an imbalance in *Ucma* expression. In the current study, *Ucma* expression was up-regulated in the MPTP group compared to the control group, and acupuncture stimulation at GB34 and LR3 maintained *Ucma* expression at levels similar to those in control animals.

Retinol dehydrogenase 9 (Rdh9) is a short-chain dehydrogenase/reductase and converts 9-cis-retinol into 9-cis-retinal and 3 α -androstenediol into dihydrotestosterone (Zhuang *et al.*, 2002). Widespread Rdh9 function in steroid or retinoid metabolism begins mid-embryogenesis (Hu *et al.*, 2007), and may be an important link between neuroactive steroids and neurodegenerative disorders (Melcangi *et al.*, 2012). Moreover, testosterone has been reported to be neurotoxic to dopaminergic neurons (Cun-

ningham *et al.*, 2009). The up-regulation of Rdh9 in the MPTP-treated group relative to the control group is thought to be one aspect of this consequence. However, further evidence is needed to confirm this, and the maintenance of *Rdh9* expression following acupuncture may have neuroprotective effects.

Down-up regulated genes by MPTP and acupuncture

Of the 255 probes down-regulated in the SN region compared to controls, 17 (two annotated genes) were exclusively up-regulated following acupuncture at acupoints but not at non-acupoints (“Down-up”; Table 3).

The function of the RIKEN cDNA 4921530L21 gene (4921530L21Rik) has yet to be characterized. The function of the predicted gene 13931 (Gm13931) is known as olfactory receptor-related genes. The olfactory system may be particularly suitable route for the penetration of xenobiotic agents into the CNS, as was shown in an intranasal MPTP model (Prediger *et al.*, 2009, 2010). Thus, olfactory receptor-related genes may be related to the pathological mechanisms of MPTP neurotoxicity.

Signal pathway analysis

Pathway analyses for the 22 probes that were up-regulated by MPTP intoxication (*vs.* CTL) and down-regulated by acupuncture at acupoints (*vs.* MPTP), as well as for the 17 probes down-regulated by MPTP intoxication (*vs.* CTL) and up-regulated by acupuncture at acupoints (*vs.* MPTP; Table 4) were performed. *Cdh1*, which is “down-up” regulated by MPTP and acupuncture stimulation at acupoints, was involved in “thyroid, endometrial, bladder cancer”, “bacterial invasion of epithelial cells”, and “melanoma” at significant levels according to the overrepresentation analysis (ORA). However, “pathways in cancer”, “adherens junction” and “cell adhesion molecules (CAMs)” were not significant. *Tph2* was involved in “tryptophan metabolism” and “serotonergic synapse” at significant levels. *Slc6a4* was involved in “serotonergic synapse” at a significant level. *Serping1* was involved in “complement and coagulation cascades” and “pertussis” at significant levels. *Slc6a13* was involved in “GABAergic synapse”, albeit not significantly so. Gm13931 was involved in “olfactory transduction” and was “down-up” regulated by MPTP and acupuncture stimulation at acupoints, even if not significant according to the ORA.

These findings demonstrate that both MPTP and acupuncture at acupoints influence *Cdh1*, *Tph2*, *Slc6a4*, *Serping1*, *Slc6a13*, and *Gm13931* expression in the indicated pathways in the SN region.

Validation of gene expression changes in the SN region following MPTP and acupuncture at acupoints

To validate the microarray data, representative genes were selected and their expression levels in the SN region

Table 3 - List of (down-up) substantia nigral genes which were down-regulated in MPTP vs. control and up-regulated in MPTP-A vs. MPTP.

Gene symbol	Gene title	Probe ID (Gene accession No.)	Average log 2 (fold change) in M vs. C (Down-regulated)	p-value ^a in M vs. C	Average log2 (fold change) in MA vs. M (Up-regulated)	p-value ^a in MA vs. M
4921530L2IRik	RIKEN cDNA 4921530L21 gene	10416727 (BC049573)	-0.398	0.022	0.492	0.002
Gm13931	predicted gene 13931	10485792 (XM_003086873)	-0.711	0.015	0.611	0.048
		10338220 ^b	-0.566	0.021	0.605	0.007
		10338896 ^b	-0.829	0.024	0.806	0.028
		10339243 ^b	-1.417	0.009	1.176	0.042
		10340276 ^b	-0.982	0.001	0.894	0.002
		10341589 ^b	-0.693	0.043	1.286	0.017
		10342476 ^b	-0.936	0.036	1.254	0.046
		10342786 ^b	-0.634	0.032	0.536	0.018
		10342853 ^b	-1.097	0.011	0.701	0.034
		10343119 ^b	-0.899	0.011	1.229	0.003
		10343349 ^b	-1.936	0.042	1.311	0.016
		10343551 ^b	-2.031	0.023	1.863	0.024
		10343553 ^b	-1.053	0.022	1.442	0.002
		10343684 ^b	-1.027	0.042	0.757	0.043
		10344102 ^b	-1.306	0.015	0.454	0.042
		10344601 ^b	-0.523	0.036	0.473	0.036

^aDetermined using Student *t*-test.^bGene accession No. was not determined.

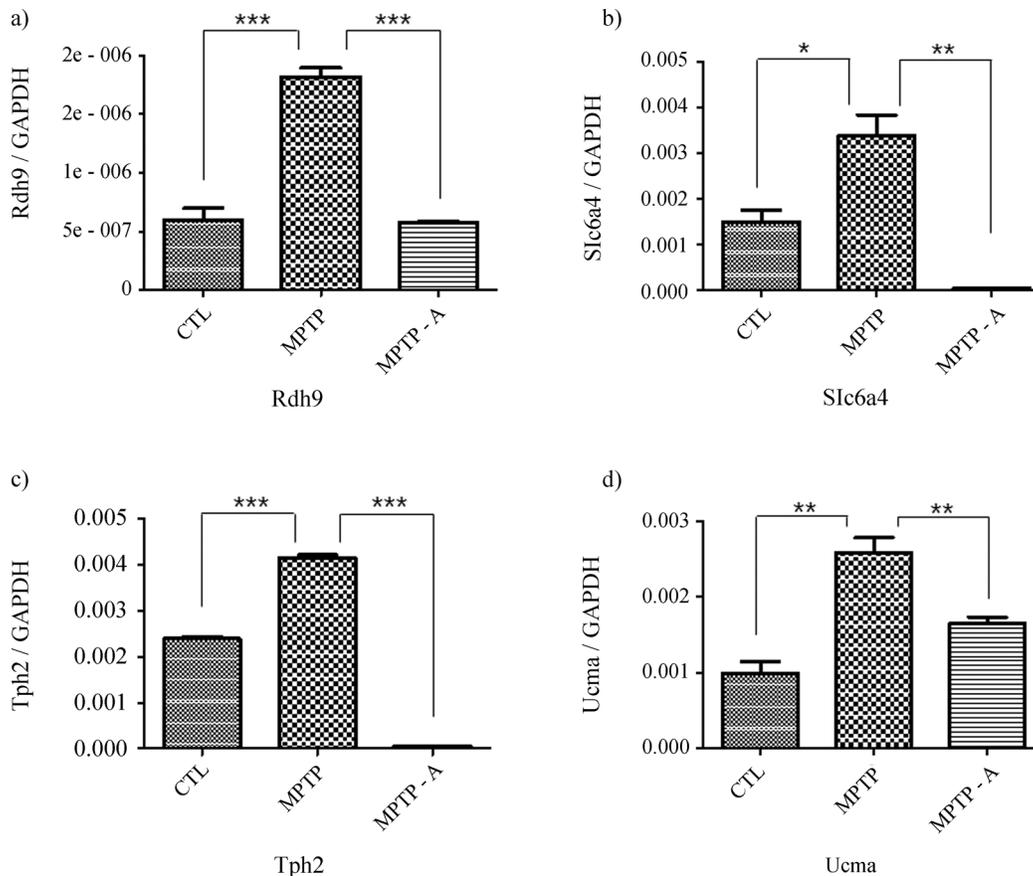


Figure 3 - Validation of the expression of representative genes by real-time RT-PCR. *Rdh9* (A), *Slc6a4* (B), *Tph2* (C) and *Ucma* (D), which were “up-down” regulated in the microarray data, were amplified by real-time RT-PCR and then quantified relative to the reference gene (*GAPDH*). Values are means \pm SE. * $p < 0.05$, ** $p < 0.005$, or *** $p < 0.0001$ were considered significant.

were evaluated by real time RT-PCR (Figure 3). Expression levels of *Rdh9*, *Slc6a4*, *Tph2* and *Ucma*, which were “up-down” regulated in the microarray analysis, also showed “up-down” regulation in real time RT-PCR (Figure 3). These findings demonstrate that the regulation patterns in the SN region regulated by MPTP and acupuncture at acupoints determined by real-time RT-PCR correlate with those determined using microarrays.

Acupuncture stimulation attenuates the reduction of TH induced by 6-OHDA intoxication (Park *et al.*, 2003) and MPTP (Kang *et al.*, 2007; Choi *et al.*, 2011b) in nigrostriatal dopaminergic neurons. Behavioral tests demonstrated that acupuncture stimulation improves motor dysfunction in a 6-OHDA Parkinsonism model by ~87.7% (Park *et al.*, 2003). The SN is a crucial aspect of the motor circuit (Brazhnik *et al.*, 2012; Gaugler *et al.*, 2012) and is one of the areas damaged most markedly in PD (Castro *et al.*, 2013; Fahim *et al.*, 2013), particularly relative to dystonia (Truong *et al.*, 2009).

The neuroprotective effects of acupuncture have been confirmed at a genetic level. The microarray results indicate that *Slc6a4* and *Tph2*, which are involved in PD, and *Mpz12* and *Serping1*, which are involved in inflammation,

are primary causes of neurodegenerative disorders (Drouin-Ouellet and Cicchetti, 2012; Pradhan and Andreasson 2013), and their expression can be maintained at similar to normal levels by acupuncture. Therefore, the effect of MPTP intoxication on gene expression in the SN region may be ameliorated by acupuncture at acupoints. This suggests an attenuating effect of acupuncture on the degeneration of dopaminergic neuron-like cells by MPTP in the SN. Previous research employing a mouse model of acute MPTP Parkinsonism has demonstrated that acupuncture treatment suppressed genes related to cytokine-cytokine receptor interaction and oxidative phosphorylation pathways (Hong *et al.*, 2010). Cellular responses to deleterious events like oxidative stress and cytokine receptor-mediated apoptosis might eventually lead to dopaminergic cell death and hence disease progression. Oxidative stress is an early event that may directly kill dopaminergic neurons (Hong *et al.*, 2010). Moreover, the current study showed that at the chronic stage, the expression of genes involved in PD was modulated by acupuncture stimulation. These results suggest that a variety of genes may be related to the effect of acupuncture on the treatment of PD.

Table 4 - Substantia nigral KEGG pathway list of the 22 probes which were up-regulated in MPTP vs. control and down-regulated only in MPTP-A vs. MPTP, and of the 17 probes which were down-regulated in MPTP vs. control and up-regulated only in MPTP-A vs. MPTP.

Regulation pattern	KEGG Pathway	Probe ID	Gene symbol or Gene Count	p-value
Up-Down-regulated	Thyroid cancer	10575052	Cdhl	0.021
	Pathways in cancer	10575052	Cdhl	0.197
	Endometrial cancer	10575052	Cdhl	0.037
	Bladder cancer	10575052	Cdhl	0.029
	Bacterial invasion of epithelial cells	10575052	Cdhl	0.048
	Adherens junction	10575052	Cdhl	0.054
	Melanoma	10575052	Cdhl	0.048
	Cell adhesion molecules (CAMs)	10575052	Cdhl	0.092
	Tryptophan metabolism	10372443	Tph2	0.028
	Complement and coagulation cascades	10484463	Serping1	0.048
	Pertussis	10484463	Serping1	0.047
	Serotonergic synapse	10372443;10378816	Tph2, Slc6a4	0.003
	GABAergic synapse	10541318	Slc6a13	0.057
	-No relevant Pathway Info-	10338456;10338657;10339444;10342771;10343150;10343377;10343396;10343743;10344355;10367041;10378816;10423383;10469058;10480003;10541318;10584827;10594513;10597960;10598073	19	1

^aDetermined using over representation analysis (ORA). A p-value < 0.05 was considered significant.

Further studies of the association between gene expression and motor function following acupuncture are warranted, as the investigated genes may mediate the protective effects of acupuncture on SN-mediated motor function. Moreover, the regulation of these genes following acupuncture and their influence on the SN via the involvement of abnormal motor circuits should be clarified.

Conclusions

We investigated gene expression changes in the SN using a whole-transcript GeneChip microarray following acupuncture stimulation at acupoints GB34 and LR3 in an MPTP-induced Parkinsonism model. Our data suggest that acupuncture at these acupoints attenuates the decrease in TH in the SN region, while acupuncture at non-acupoints did not suppress this decrease. Compared to the control group, 22 probes (10 annotated genes: *Cdh1*, *Itih2*, *Mpzl2*, *Rdh9*, *Serping1*, *Slc6a13*, *Slc6a20a*, *Slc6a4*, *Tph2*, and *Ucma*) were up-regulated in the MPTP group and were exclusively down-regulated by acupuncture at acupoints but not at non-acupoints. Additionally, compared to the control group, 17 probes (two annotated genes; *4921530L21Rik* and *Gm13931*) were down-regulated in the MPTP group and were exclusively up-regulated after acupuncture at acupoints but not at non-acupoints. Therefore, these 39 probes (12 annotated genes) may be responsible for the protective effect of acupuncture in the SN following MPTP-induced impairment.

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Supplementary Material

The following online material is available for this article:
Table S1 - Up-regulated genes in MPTP-treated substantia nigral region.
Table S2 - Down-regulated genes in MPTP-treated substantia nigral region.
Figure S1 - Box plot before and after normalization.
This material is available as part of the online version of this article from <http://www.scielo.br/gmb>.

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