Neuroprotective effects of lithium on a chronic MPTP mouse model of Parkinson's disease via regulation of α-synuclein methylation

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Received September 18, 2018; Accepted April 2, 2019

DOI: 10.3892/mmr.2019.10152

Abstract. The pathological process of Parkinson's disease (PD) is closely associated with the death of nigral neurons, for which an effective treatment has yet to be found. Lithium, one of the most widely certified anticonvulsant and mood-stabilizing agents, exhibits evident neuroprotective effects in the treatment of epilepsy and bipolar disorder. In the present study, the neuroprotective mechanisms by which lithium acts on a chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD were investigated by employing animal behavioral tests, immunohistochemistry, RT-PCR, and western blotting. The results revealed that, in open field tests, lithium treatment counteracted the reduction in movement distance as well as activity time induced by MPTP administration. The compound could also prolong the drop time of MPTP-treated mice in rotarod tests. Moreover, lithium treatment corrected the loss of nigral neurons, the increase of α-synuclein (SNCA) in substantia nigra as well as in the striatum of MPTP-treated mice, and decreased the methylation of SNCA intron 1 in DNA from the same regions. Furthermore, marked changes were observed in the expression of miRNAs including miR-148a, a potential inhibitor of DNMT1, in the MPTP-treated mice. These results suggested that the early application of lithium was important for alleviating the behavioral deficits experienced in the PD model, and that the neuroprotective action of lithium was achieved through a lithium-triggered miRNA regulation mechanism. Essentially, our findings indicated that lithium may be beneficial in the prevention and treatment of PD through the regulation of α-synuclein methylation.

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Key words: Parkinson's disease, DNA methylation, lithium, α-synuclein, nigral neuron

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide and affects 1% of the population over the age of 60 (1). This neurodegenerative disorder is characterized by the progressive loss of substantia nigra dopaminergic neurons as well as striatal projections, which causes the typical symptomatology, including bradykinesia, tremor, muscle rigidity, and postural instability (1). However, only 5-10% of PD cases are considered to involve genetic factors (1). Although recent studies have found that several factors play a key role in the neuronal pathogenesis of PD (2), the etiology of PD remains elusive. It is now considered that α-synuclein (SNCA), a major component of Lewy bodies, is one of the morphological markers of PD. Several site mutations in SNCA have been identified as the cause for early onset PD in a dominant mode of inheritance (3). Moreover, methylation of intron 1 of human SNCA results in reduction of gene expression in the brains of PD patients, suggesting that methylation of SNCA is correlative with PD pathogenesis (3). Despite the lack of any differences in the postmortem analysis of regional specific methylation at the anterior cingulate or putamen of the brains of PD patients and healthy individuals, methylation in the substantia nigra of PD patients was significantly and specifically decreased (4). Furthermore, single CpG analysis revealed a fluctuation in the methylation levels of various brain regions and LBD stages, which is suggestive of a potential role for DNA methylation of α-synuclein in the occurrence of PD (5).

One of the first-line antiepileptic drugs (AEDs) is lithium, a drug that is also used in the treatment of bipolar disorder (6). Similar to other anticonvulsants, lithium inhibits some of the functions of sodium and calcium channels (6). Despite the need for deeper investigation into its *in vivo* target, lithium treatment could induce a significant change in the activity of histone deacetylase (HDAC) as well as glycogen synthase kinase (GSK-3). Further studies have revealed that the inhibition of GSK-3β activity induced by lithium mimics resulted in a reduction of DNA methylation in neural stem cells (7).

Similar to valproic acid (VPA), lithium was recently demonstrated to be an effective regulator for the expression of several PD-related miRNAs (6). In neuroblastoma cells, lithium treatment attenuated the apoptosis induced by rotenone, an inhibitor of mitochondrial complex 1 that induces PD-like neurodegeneration *in vivo* (8). It has also been suggested that by enhancing the autophagic pathway that is associated with the degradation of aberrant accumulated α-synuclein protein in PD, lithium could, act as a neuroprotective agent in rotenone-induced SH-SY5Y cells as well as in MPTP-lesioned mice (6,8-10). Other studies reported that lithium could increase the expression of neurotrophins, which are involved in neural survival as well as plasticity, for example the nerve growth factors (NGF), brain-derived neurotrophic factor (BDNF), and the glial cell line-derived neurotrophic factor (GDNF) (11,12).

Herein, in order to detect the effects of lithium on the neurodegenerative symptoms using behavioral tests, a commonly used PD-like mouse model was employed, and the animals were fed chow with lithium carbonate for 5 weeks. Biochemical assays were conducted to explore the molecular events associated with PD pathogenesis and further elucidate the relationship between lithium and the development and progression of this disease.

Materials and methods

Animals. A total of 80 male C57BL/6 mice (7-8 weeks, ~25 g) were obtained from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). Each individual cage contained 4 mice, with free access to water and food. All of the animal rooms were maintained at a temperature of 21-23˚C and humidity of 40-60%, with a 12-h light-dark cycle. To develop the MPTP PD model, male C57BL/6J mice were injected intraperitoneally (i.p.) with 1-methyl-4-phenyl-1,2,3,6-tetrahydrapyridine (MPTP) at 20 mg/kg (body weight; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) twice a week, plus 250 mg/kg probenecid injected 0.5 h before. Administration of the treatment was carried out for 5 weeks with feeding of 0.2% lithium carbonate (Sigma-Aldrich; Merck KGaA) by chow. On the 36th day, the mice were subjected to behavioral tests. Upon completion, mice were anesthetized with 250 mg/kg avertin intraperitoneally and perfused transcardially with sterile saline. Brains were quickly harvested, and one hemibrain was immediately frozen in liquid nitrogen for biochemical studies while the other hemibrain was post-fixed in 4% paraformaldehyde for immunohistochemical stains. All animal procedures complied with the current ethical considerations of the Shanghai University of Traditional Chinese Medicine's Animal Ethics Committee, which is in accordance with the National Research Council criteria. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai University of Traditional Chinese Medicine and were performed in accordance with the relevant guidelines and regulations as well as approved by the guidelines on ethical standards for investigation of PD in conscious animals. Animals were sedated or anesthetized using carbon dioxide prior to cervical dislocation. Cervical dislocation to euthanize mice was performed by trained research personnel after the approval of the IACUC of Shanghai University of Traditional Chinese Medicine's Animal Ethics Committee and the method was performed in accordance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2013 Edition).

Behavioral tests

Open-field test. The open field activity was assessed in a 27x27x38 cm chamber with 50 lux (lx) illumination. Fifteen minutes of free movement was tracked by TruScan Open Field version 2.04 software 2.04 (Coulbourn Instruments, Holliston, MA, USA). Total movement times and distances were scored to assess locomotor activity while a percentage of movement time in the margin area (the area within 8.76 cm from the chamber wall) was calculated for scrutinizing the autonomous activity (13).

Rotarod performance test. Rotarod training was performed concurrently for 10 min during 5 consecutive days: On the first day, the mice were placed on the rotating rod at 1.5 x g for 5 min. Every 30 sec, the rod speed was increased by 0.4 x g up to 5.5 x g, and then maintained at this speed for 1 min. On the second day, a 1.5 x g speed was used for 1.5 min. The rod speed was increased by 0.4 x g every 30 sec until it reached 7.3 x g, where it was maintained for 10 min. On the third, fourth, and fifth days, the latencies of falling off the rod with a linear increase in rod speed from 1.5 x g up to 15 x g for 5 min were measured 3 times and averaged. The actual test protocol was the same for the last 3 days of the training protocol.

Bisulfite conversion and methylation-specific-PCR (MSP). For the bisulfite modification, the EZ DNA Methylation-Gold™ Kit (Zymo Research Corp., Irvine, CA, USA) was used. In this technique, unmethylated cytosines are converted to uracil while methylated cytosines remain unchanged in bisulfite-treated DNA. Thus, the methylation status of the DNA sample can be analyzed by PCR amplification, called 'methylation specific-PCR.' DNA (~1 μg) was used for bisulfite conversion, and then MSP was carried out using specific primers for both bisulfite converted methylated and unmethylated DNA samples in a total 25 μl mix containing 1X PCR buffer, 15 mM $MgCl_2$, 200 μM of each dNTP, 0.4 μM of each primer, <1 μg template DNA, and 2.5 U HotStarTaq DNA Polymerase (Qiagen, Inc., Valencia, CA, USA). After the initial denaturation step at 94˚C for 15 min, 35 cycles were performed for the regions of SNCA using the following conditions: denaturation at 94˚C for 1 min, at annealing temperature for each PCR bisulfide conversion-specific primer pair for 1 min, extension at 72˚C for 1 min, and then final extension at 72˚C for 10 min. Bisulfite-treated DNA was amplified using primers SNCA-PromF, 5'-AAAATTTTGAAGATA TTTGAATTAAAG-3' and SNCA-PromR, 5'-CTAATCCTC CTCCTTCTCCTTCTC-3'; SNCA-IntF, 5'-GGAGTTTAA GGAAAGAGATTTGATT-3' and SNCA-IntR, 5'-CAAACA ACAAACCCAAATATAATAA-3', specifically designed for the bisulfite-treated DNA. The PCR products SNCA (-926/-483; intron 1) were cloned into pMD 18-T Vector (Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer's instructions. Plasmid DNA was isolated from at least 10 clones per region (Wizard Plus SV Minipreps; Promega Corporation, Madison, WI, USA), sequenced using vector-specific primers and the Big Dye Terminator v1.1

Cycle Sequencing Kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Quality control for DNA methylation was performed using BiQ (software tool for DNA methylation analysis; http://biq-analyzer.bioinf.mpi-inf.mpg.de/).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA, including mRNA and miRNA, was isolated from samples using an RNeasy Mini Kit (Qiagen, Inc.). Next, cDNA was synthesized using a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.) and subsequently used as templates for qPCR. The primers were designed using Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA). Primers of real-time PCR were SNCA F, 5'-GGACCAGTT GGGCAAGAATG-3' and R, 5'-GGGCACATTGGAACT GAGCAC-3'; GAPDH F, 5'-CGGAGTCAACGGATTTGG TC-3' and R, 5'-TTCTCCATGGTGGTGAAGAC-3'; miR-148a F, 5'-TCAGTGCACTACAGAACTTTGT-3' and R, 5'-GCT GTCAACGATACGCTACG-3'; U6 F, 5'-CTTCGGCAGCAC ATATAC-3' and R, 5'-GAACGCTTCACGAATTTGC-3'. qPCR was performed in triplicate with SYBR-Green (Takara Biotechnology Co., Ltd.) on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction parameters were set as follows: 95˚C for 5 min, followed by 40 cycles of 10 sec for 95˚C and 1 min for 60˚C. For miRNA RT-qPCR, 1 mg of isolated RNA was reverse-transcribed with stem-loop primers from a BioTNT miRNA qPCR Detection Primer Set (BioTNT Biotechnologies Co, Ltd., Shanghai, China). All reactions were repeated in triplicate. The relative mRNA and miRNA expression levels were separately analyzed using the $2^{-\Delta\Delta Cq}$ method (14) with GADPH and U6 as the endogenous controls.

miRNA expression microarrays. Total RNA (including miRNAs) was isolated from the brain tissue of the substantia nigra using TRIzol® (Thermo Fisher Scientific, Inc.) with slight modification. Polyacryl carrier was added to improve RNA recovery. The global miRNA expression profiles were obtained using the Agilent SurePrint Mouse miRNA Microarrays (Agilent Technologies, Inc., Santa Clara, CA, USA) containing 1,881 mouse miRNAs based on Sanger miRBase release 12.0. RNA samples were processed, labeled, and hybridized onto the microarrays according to the manufacturer's protocols. Microarray slides were then scanned using the G3 High Resolution Scanner, and microarray data were extracted by Feature Extraction software version 10.7.3.1 (Agilent Technologies, Inc.).

Immunohistochemistry. Animals were anaesthetized with ether and perfused through the heart with 4% paraformaldehyde in 0.1 mM phosphate buffer pH 7.4. Brains were removed, post-fixed overnight in the same fixative, and then washed in buffered 18% sucrose until they sunk. Sections were cut with a freezing microtome at 30-μm thickness, and then permeabilized for 20 min with phosphate-buffered saline (PBS) containing 0.1% Triton X-100, before a 20-min incubation in methanol containing 0.3% H_2O_2 to quench endogenous peroxidase activity. Subsequently, the sections were incubated for 30 min in PBS containing 2% normal goat serum to block non-specific binding sites. For immunohistochemical localization of tyrosine hydroxylase (TH), the TH antibody was used at a dilution of 1:1,000 (cat. no. sc-136100, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Antibody exposure was performed overnight at 4˚C, followed by a 90-min incubation at room temperature with a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody (cat. no. A0216, Beyotime Institute of Biotechnology, Haimen, China). The immunoreaction was visualized using DAB Horseradish Peroxidase Color Development Kit (Beyotime Institute of Biotechnology). Digital images were captured using a Leica DM2000 Microscopic imaging system, and TH-positive neuronal counts and the integrated optical density value (IOD) of α-synuclein-positive fibers were estimated within the substantia nigra by ImageJ software (NIH) as previously described (15).

Western blotting. The substantia nigra of wild-type, MPTP-treated, and lithium-treated mice (n=3), were dissected, pooled, and homogenized in lysis buffer (Beyotime Institute of Biotechnology) on ice plus 1:100 volume of phenylmethylsulfonyl fluoride (PMSF), before final centrifugation at 14,000 x g for 5 min to remove debris. The BCA kit (Beyotime Institute of Biotechnology) was used to assess the protein concentrations. After heating at 100˚C with loading buffer for 4 min, the proteins (30 μg) were resolved by SDS-PAGE (12% for a-synuclein, 6% for DNMT1) and transferred to nitrocellulose membranes (Amersham; GE Healthcare, Chicago, IL, USA) at 200 mA for 40 min. Tris-buffered saline and Tween-20 (TBST) containing 5% skimmed milk powder was used to block the membranes at room temperature for 1 h, and then membranes were washed with TBST prior to incubation with antibodies against α-synuclein (ab27766, 1:2,000; Abcam, Cambridge, MA, USA) and DNMT1 (ab19905, 1:2,000; Abcam) overnight at 4˚*C*. After washing thrice with TBST, the membranes were incubated with horseradish peroxidase conjugated anti-rabbit (cat. no. A0208) or anti-mouse IgG (cat. no. A0216, Beyotime Institute of Biotechnology) at a dilution of 1:2,000 at room temperature for 45 min. After washing for three further times with TBST, the proteins were visualized using an enhanced chemiluminescence kit (EMD Millipore, Billerica, MA, USA), and then quantified using Quantity One software version 4.4.6 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), normalized to GAPDH.

Statistical analysis. The raw data were analyzed by Origin 8.5 software (OriginLab, Northampton, MA, USA). Results of data analysis were expressed as the means ± standard error of the mean (SEM) with the number of experiments indicated in the figure legends. Behavioral experiments were statistically analyzed by one-way ANOVA. Tukey's test was employed to compare the differences between all the groups. For all statistical tests, a value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Lithium improves behavioral performance in an MPTP-induced Parkinson mouse model. To examine the effects lithium had on MPTP-induced Parkinson mice, behavioral tasks were performed after 5 weeks of lithium treatment.

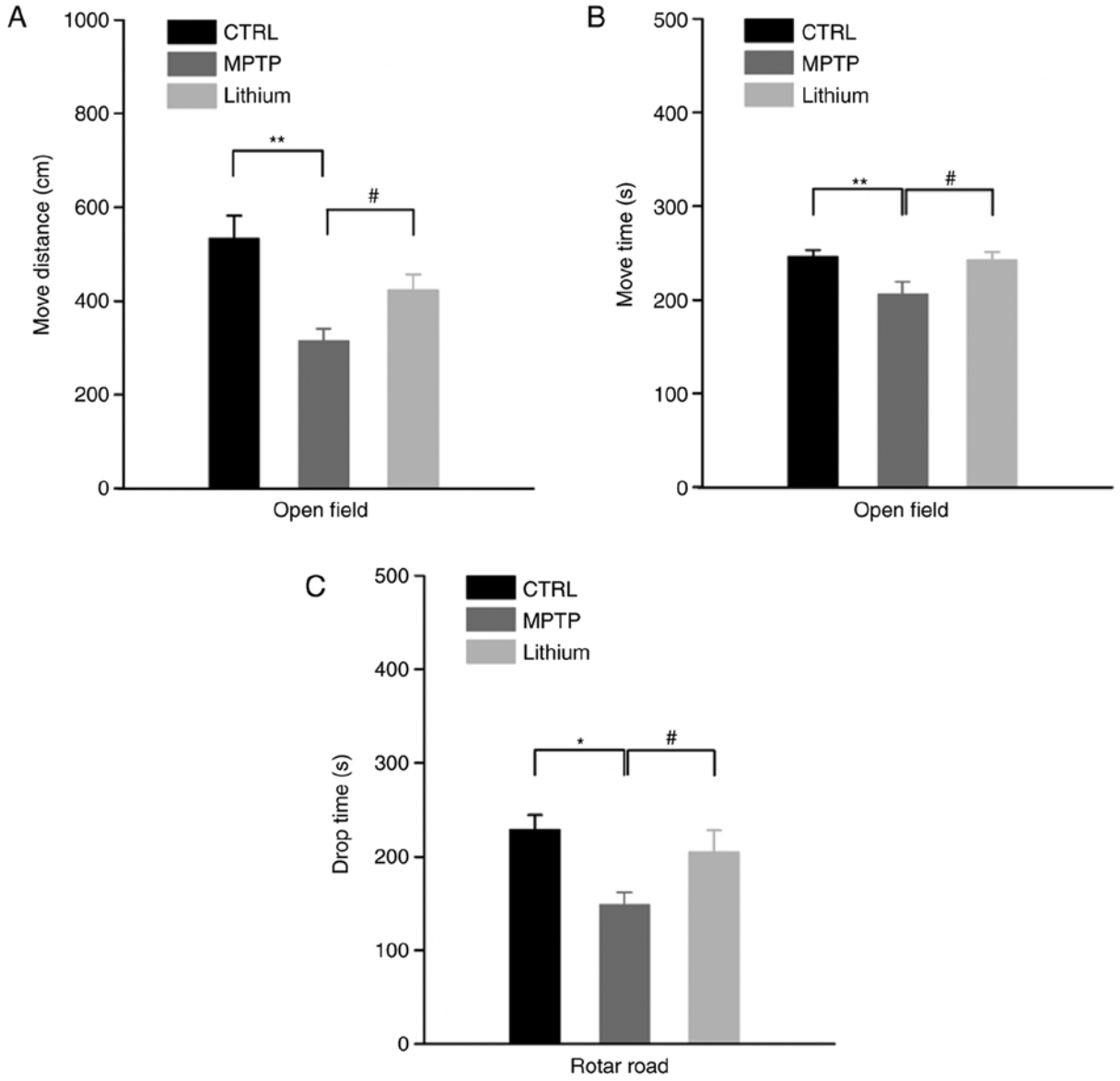

Figure 1. Effects of lithium on behavioral performance of an MPTP-induced PD model. (A and B) Results of behavioral tests revealed that the locomotor activity of MPTP-treated mice was significantly reduced (*P<0.05 and **P<0.01, n=10) and lithium treatment satisfactorily relieved this alteration ($^{\#}$P<0.05, n=10). (C) In rotarod tests, the compound also prolonged the drop time of MPTP-treated mice (*P<0.05, MPTP group vs. the control group, n=10; $^{\#}$P<0.05, lithium group vs. the MPTP group, n=10). MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease.

The results revealed a significant reduction in the locomotor activity in the MPTP-treated mice and lithium treatment relieved this effect satisfactorily. As revealed in Fig. 1A and B, the average movement distance was 534.23±48.01 cm and the average activity time was 246.33±6.48 sec. Following impairment, the movement distance and activity time values were reduced to 315.09±25.59 cm (MPTP group vs. CTRL group, P<0.01) and 194.80±40.79 sec (MPTP group vs. CTRL group, P<0.01), respectively, however, after lithium administration for 5 weeks, those values rebounded to 423.40±33.21 (lithium group vs. MPTP group, P<0.05) cm and 242.75±8.05 sec (lithium group vs. MPTP group, P<0.05), respectively. Furthermore, in rotarod tests, the compound also prolonged the drop time of MPTP-treated mice, resulting in an increase in the drop time for MPTP-treated mice from 148.59±13.03 sec (MPTP group vs. CTRL group, P<0.05) to 204.70±23.27 sec (lithium group vs. MPTP group, P<0.05) bringing it close to that of the control group results 228.62±15.79 sec (lithium group vs. CTRL group, P>0.05; Fig. 1C).

Lithium alleviates typical pathological alterations in the substantia nigra region of MPTP-impaired mice. A reduction in TH-positive neurons and an increase in expression of α-synuclein are both characteristic of PD pathology. Corresponding pathological changes were also observed in MPTP-impaired mice, and lithium treatment was able to mitigate these alterations as well. Using immunohistochemical staining, a significant loss of TH^{+} neurons in the substantia nigra regions of MPTP-impaired mice at 6 weeks (48.60±0.022% reduction; P<0.01 compared to control) was revealed. Conversely, the number of TH^{+} neurons rebounded considerably after lithium treatment (32.20±0.029% increased; P<0.01 compared to MPTP group; Fig. 2). In addition, the

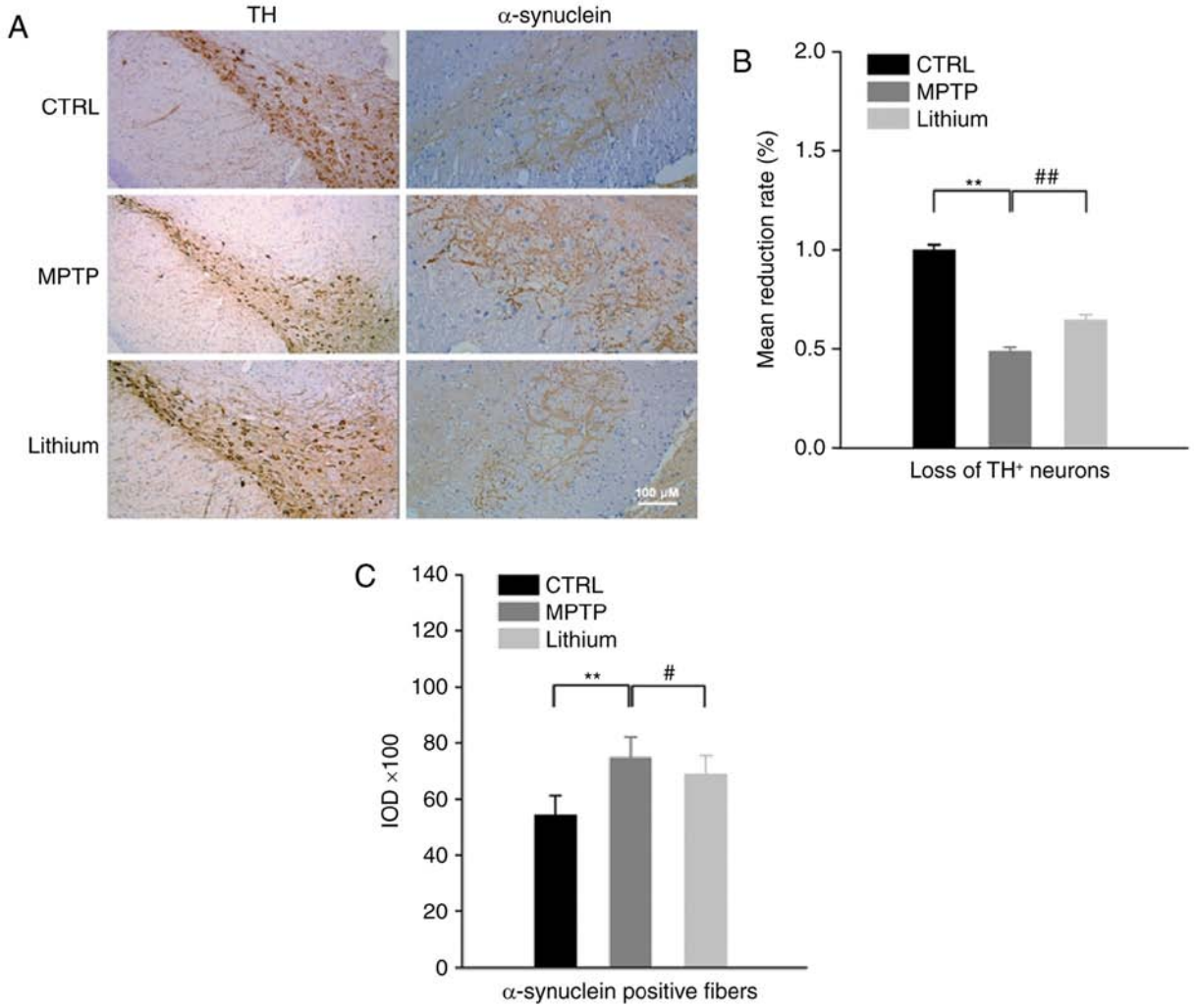

Figure 2. Lithium alleviates typical pathological alterations in the substantia nigra region. (A and B) Significant loss of TH^+ neurons in the substantia nigra region of MPTP-impaired mice at 6 weeks (48.60±0.022% reduction; $^{**}P<0.01$ compared to control). The number of TH^+ neurons rebounded considerably (32.20±0.029% increased; $^{\#\#}P<0.01$ compared to the MPTP group). (A and C) The integrated IOD of α-synuclein-positive fibers in MPTP-impaired mice increased from 54,370±699 to 74,790±736 (37.5% increase; $^{**}P<0.01$ compared to control), and decreased to 68,958±654 after lithium treatment (7.78% decrease; $^{\#}P<0.05$ compared to the MPTP group). MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; IOD, optical density value.

integrated optical density value (IOD) of α-synuclein-positive fibers in MPTP-impaired mice increased from 54,370±699 to 74,790±736 (37.5% increased; P<0.01 compared to the control), however, after lithium treatment it decreased to 68,958±654 (11.3% increased; P<0.05 compared to the MPTP group; Fig. 2).

Lithium increases the methylation of SNCA intron 1 in the substantia nigra of MPTP-impaired mice. To investigate whether epigenetic changes may contribute to the dysregulation of SNCA expression in the MPTP-induced PD animal model, DNA from the substantia nigra regions of each group was analyzed. Significantly fewer methylated CpG sites in the DNA of PD mice were revealed. There was a significant decrease in the mean methylation rate of SNCA (-926/-483; intron 1) in PD mouse substantia nigra (2.05±0.41%, P<0.01) when compared with that determined in the control group (3.62±0.58%). Although the methylation rate was lower (2.38±0.46%) in the lithium-treated group when compared to the control group, the opposite was revealed with comparison to the MPTP group, which resulted in a significant increase in the methylation rate (P<0.05; Fig. 3).

Lithium alters the miRNA and protein expression profiles in the substantia nigra of MPTP-impaired mice. The role of miRNAs in the pathogenesis of Parkinsonism has attracted increasing attention. Furthermore, the recent discovery of miRNA expression and regulation has led to the consideration of lithium as a novel mechanism of neuroprotection. SurePrint Mouse miRNA Microarrays (Agilent Technologies, Inc.) were utilized to profile changes in the miRNA expression of MPTP-impaired mice. The results revealed alterations in the expression levels of 39 miRNAs >1.5-fold in the substantia nigra of PD mice (data not shown). The results also revealed an upregulation in the expression of miR-148a in PD mice, an effect which was reversed after lithium treatment, resulting in a downregulation of this particular miRNA. RT-qPCR confirmed this result (Fig. 4A). Previous research has indicated that miR-148a could be considered as a potential inhibitor of DNMT1, a DNA methylase, and thus play a role in the establishment and regulation of tissue-specific patterns of methylated cytosine residues. Further RT-qPCR results revealed decreased expression of DNMT1 in the substantia nigra of PD mice compared to the control, and a

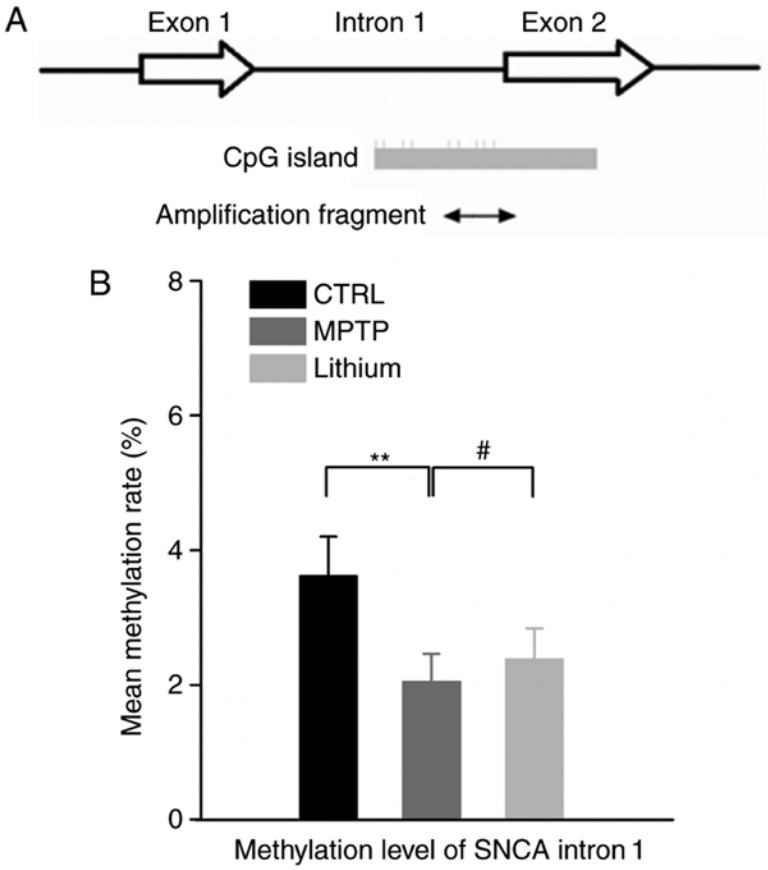

Figure 3. Enhancement of lithium on the methylation of SNCA intron 1 in substantia nigra. (A and B) Bisulfite specific cloning-based sequencing revealed that the mean methylation rate of SNCA (-926/-483; intron 1) in PD mouse substantia nigra decreased significantly when compared with control mice (**P<0.01). In the lithium-treated group, although the methylation rate remained lower compared to the control group, the opposite was revealed in a comparison with the MPTP group, with a significant increase in the methylation rate ($^{\#}$P<0.05). SNCA, α-synuclein; PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

significant difference was recorded when compared with the lithium-treated group (Fig. 4B). These results were also confirmed by western blot analysis, which presented a significant reduction in the expression of DNMT1 protein in PD mice compared with the control group. Furthermore, lithium treatment resulted in a significant increase in DNMT1 expression in PD mice (Fig. 4C).

Aberrant aggregation of α-synuclein is considered to be the major neuropathological characteristic of PD, and α-synuclein has been detected in insoluble inclusions within Lewy bodies and neurites. In the present study, a significant increase was observed in the expression of α-synuclein in the substantia nigra of mice. However, the increase of α-synuclein in PD mice could be attenuated through administration of lithium (Fig. 4D).

Discussion

Lithium mood stabilizers have long been used to treat epilepsy as well as bipolar disorder (BD), a mental illness that causes deficits in cellular plasticity as well as resiliency (8). Recent studies indicate that lithium could also be effective as a treatment for neurodegenerative diseases through diverse mechanisms. For example, pretreatment with lithium resulted in an obvious decrease in the size of quinolinic acid (QA)-induced lesions of striatum and loss of striatal medium-sized neurons in a rat excitotoxic model (16). In tauopathy mouse models, chronic treatment with lithium decreased the phosphorylation of tau as well as neuronal degeneration mediated by GSK-3 (17), findings which were corroborated with the research that lithium could increase neurogenesis in the rat hippocampus (18). An increasing number of studies have illuminated the beneficial effects of lithium on PD. Lithium chloride could promote dopaminergic differentiation of human immortalized RenVm cells (neuronal stem cell) by increasing expression of tyrosine hydroxylase and β-catenin marker (19). Moreover, low-dose lithium treatment could also prevent motor impairment, as demonstrated by the open field test, pole test, and rearing behavior. In parkin mutant transgenic mice, lithium prevented parkin-induced dopaminergic striatal degeneration, striatal astrogliosis, and microglial activation (20). The present results revealed that lithium treatment significantly counteracted the reduction in the movement distance as well as activity time of MPTP-treated mice in open field tests. Additionally, it prolonged the drop time of MPTP-treated mice in rotarod tests, which may be relative to its neuroprotective effects on nigral neurons. Clinical studies have revealed that low-dose lithium adjunct therapy may reduce off-time in Parkinson's disease.

It is well known that α-synuclein is a key component of Lewy bodies found in PD patients (21,22), of which site

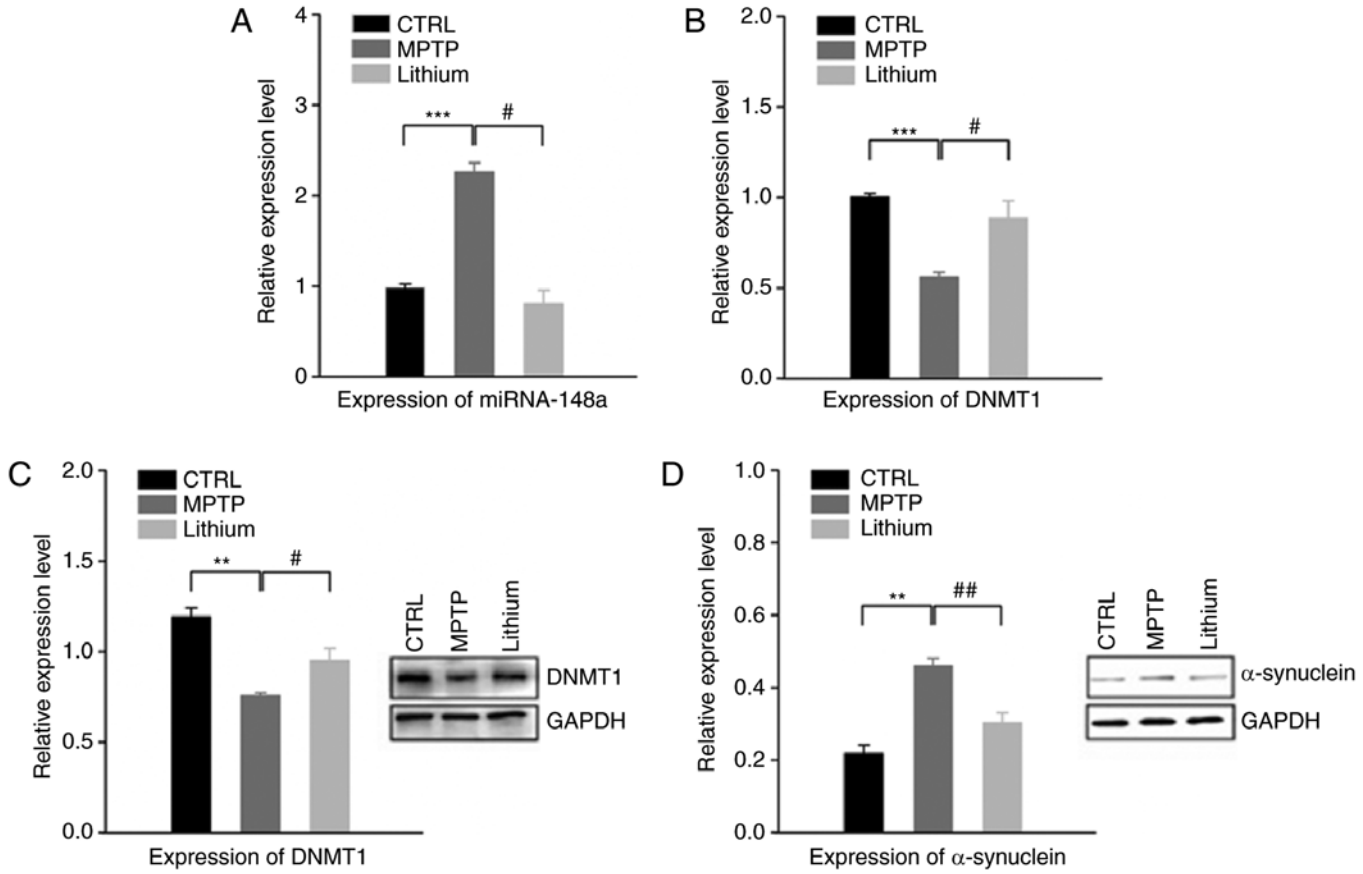

Figure 4. Effects of lithium on the regulation of the miRNA and protein expression profiles in substantia nigra. (A) Results of RT-qPCR assays revealed an upregulation in the expression level of miRNA-148a in the substantia nigra of PD mice compared to that of the control group. However, miRNA-148a expression was downregulated following lithium treatment. (B) The expression level of DNMT1 decreased in the substantia nigra of PD mice compared with the control group, and increased significantly in the lithium-treated group (vs. MPTP group, $^{\#}P<0.05$). (C) These results were further confirmed by western blot analysis, which revealed a reduction in DNMT1 in the substantia nigra of PD mice, and a significant difference between the MPTP and lithium group. (D) The expression of α-synuclein significantly increased in the substantia nigra of PD mice. This effect was attenuated by oral administration of lithium. $^{**}P<0.01$ and $^{***}P<0.001$, as indicated; $^{\#}P<0.05$ and $^{\#\#}P<0.01$, as indicated. PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

mutations and multiplications induce familial parkinsonian syndromes with high penetrance (23). Previous studies suggest that the gene SNCA equally harbors significant risk haplotypes for sporadic PD (24) and that MPTP treatment significantly decreases the DNA methylation of SCNA CpGs. In the present study, the effects of chronic lithium treatment were investigated on the neurodegenerative phenotype of an MPTP-induced mouse model of Parkinson's disease. In order to determine whether or not lithium could counteract this alteration, bisulfite specific cloning-based sequencing was performed to analyze the methylation status of SCNA intron 1. Our findings indicated that lithium could effectively prevent the decrease in DNA methylation. We then set out to discriminate the upstream modulator elements of SCNA DNA methylation.

Abundant in all multicellular organisms, miRNAs are 21-24 nucleotides long non-protein-coding RNAs. They are of importance in translational repression as well as mRNA degradation through binding either to the 3'-untranslated regions (3'-UTRs) of mRNAs (25), predominantly, or to coding regions (26). This mechanism allows miRNA-regulating drugs to play a crucial role in regulating the functions of the nervous system. For example, muscarinic M1-receptor knockout mice exhibited mania-like behavioral deficits (e.g., hypersensitivity to amphetamine-induced hyperlocomotion), and lithium treatment normalized these behavioral deficits in part through the enhancement of the M1-receptor-ERK pathway signaling (27). To further investigate whether or not lithium could act effectively by this method in the MPTP-induced PD mouse model, SurePrint Mouse miRNA Microarrays (Agilent Technologies, Inc.) were utilized to profile changes in the miRNA expression of MPTP-impaired mice. It was revealed that, in the substantia nigra of PD mice, 39 miRNAs exhibited altered expression levels at greater than 1.5-fold (data not shown). It was observed that miR-148a was upregulated in PD mice, however lithium treatment could reduce this altered level of expression. DNMT1, a maintenance DNA methylation enzyme, was predicted as a potential target gene of miR-148a, which was revealed to be correlated with DNA hypomethylation as well as α-synuclein expression (28). Based on these results, we could postulate that lithium achieved its protective effect against PD-like neurodegenerative symptoms, at least partially, by reducing the expression miR-148a and alleviating the suppression of DNMT1, eventually resulting in a reduction in the generation of neurotoxic α-synuclein. Further experiments are required to determine whether or not miR-148a could in fact bind directly to the 3'-UTR of DNMT1 *in vivo*.

Acknowledgements

The authors would like to thank Professor Dazheng Wu (Putuo Hospital, Shanghai University of Traditional Chinese Medicine) and Associate Professor Peihao Yin (Putuo Hospital, Shanghai University of Traditional Chinese Medicine) for their guidance on the experiments and manuscript.

Funding

The present study was supported by the National Natural Science Foundation of China (nos. 81202814 and 81603410), the Shanghai Municipal Commission of Health and Family Planning (20124Y116, 20184Y0086), and the Key Speciality Program (no. 2016102A) of Putuo Hospital, Shanghai University of Traditional Chinese Medicine.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

QZ and JT designed the study, performed part of the experiments, interpreted the data and performed the data analysis. JC, YZ, QX, and YB performed part of the experiments. HL, QZ, and JT interpreted the data, drafted the manuscript and revised it critically for intellectual content. All authors read and approved the final version of the manuscript prior to submission.

Ethics approval and consent to participate

All of the experimental animal protocols complied with the current ethical considerations of Shanghai University of Traditional Chinese Medicine's Animal Ethics Committee, which is in accordance with the National Research Council criteria. All animal experiments and procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai University of Traditional Chinese Medicine and were performed in accordance with the relevant guidelines and regulations, as well as approved by the guidelines on ethical standards for investigation of experimental pain in conscious animals.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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