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# Sinapic Acid Ameliorates REV-ERB α Modulated Mitochondrial Fission against MPTP-Induced Parkinson's Disease Model

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## Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide, and accumulating evidence indicates that mitochondrial dysfunction is associated with progressive deterioration in PD patients. Previous studies have shown that sinapic acid has a neuroprotective effect, but its mechanisms of action remain unclear. The neuroprotective effect of sinapic acid was assayed in a PD mouse model generated by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as well as in SH-SY5Y cells. Target protein expression was detected by western blotting. Sinapic acid treatment attenuated the behavioral defects and loss of dopaminergic neurons in the PD models. Sinapic acid also improved mitochondrial function in the PD models. MPTP treatment increased the abundance of mitochondrial fission proteins such as dynamin-related protein 1 (Drp1) and phospho-Drp1 Ser616. In addition, MPTP decreased the expression of the REV-ERB α protein. These changes were attenuated by sinapic acid treatment. We used the pharmacological REV-ERB α inhibitor SR8278 to confirmation of protective effect of sinapic acid. Treatment of SR8278 with sinapic acid reversed the protein expression of phospho-Drp1 Ser616 and REV-ERB α on MPTP-treated mice. Our findings demonstrated that sinapic acid protects against MPTP-induced PD and these effects might be related to the inhibiting abnormal mitochondrial fission through REV-ERB α.

**Key Words:** Parkinson disease, Sinapic acid, Mitochondrial fission, MPTP, REB-ERB α

## INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease and is characterized by the progressive loss of dopaminergic neurons in the midbrain, especially in the substantia nigra pars compacta (SNpc), and by the formation of α-synuclein aggregates called Lewy bodies (Li *et al*., 2021a). Mitochondrial dysfunction is considered a key pathogenic event of PD and contributes to the degeneration of dopaminergic neurons by activating inflammation and oxidative stress. Several mechanisms, especially mitochondrial fission and fusion, provide quality control through the recovery and/or elimination of damaged mitochondria, but an imbalance of these mechanisms could lead to the development of disease (Shirihai *et al*., 2015). The PD model induced by N-methyl-4-phenylpyridinium ($MPP^+$) and/or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is one of the most adequate experimental models. $MPP^+$/MPTP selectively inhibits complex I in the mitochondrial electron transport chain, which consequently leads to oxidative stress and impaired calcium homeostasis. These events induce the degeneration of dopaminergic neurons via necrosis or apoptosis (Rudenok *et al*., 2020). Various studies have shown that MPTP changes mitochondrial quality control mechanisms, including fission and fusion. The carboxyl terminus of Hsp70-interacting protein was shown to attenuate $MPP^+$/MPTP-treated injury by inhibiting the expression of Drp1, which plays a central role in regulating mitochondrial fission (Hu *et al*., 2021). Overexpression of mitofusin 2 (MFN2), a mitochondrial fusion protein, decreased oxidative stress and inflammation in an MPTP mouse model (Zhao *et al*., 2021). Thus, modulation of mitochondrial fission and fusion might be a promising therapeutic candidate for PD.

The term "circadian rhythm" refers to the physiologic, metabolic, and behavioral rhythms that follow a daily cycle. When

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circadian rhythms are disrupted, the regulation of sleep-wake cycles and immunity is disrupted, increasing susceptibility to sleep disturbances, infections, and inflammatory disease (Carter *et al*., 2016). Circadian rhythms are regulated by an autoregulatory transcriptional feedback loop including the proteins REV-ERB α and REV-ERB β, which form a supporting loop to stabilize the main loop by regulating the expression of circadian rhythm-related proteins. The protein expression of Bmal1, the core circadian rhythm component, is controlled by Rev response elements in a Baml1-Rev feedback loop (Ercolani *et al*., 2015). Recently, components of the supporting loop have been recognized as being important for the development of disease. SR9009, an agonist of REV-ERB α, improved cardiac function by regulating inflammation and cardiac remodeling (Stujanna *et al*., 2017). In an animal model of Alzheimer's disease, SR9009 also decreased cognitive deficits and the β-amyloid burden (Roby *et al*., 2019). Furthermore, REV-ERB α was shown to modulate mitochondrial biogenesis via the Stk11-AMPK-SIRT1-PGC1 signaling pathway (Woldt *et al*., 2013). Nevertheless, the relationship between REV-ERB α and mitochondrial fission is not fully understood.

Sinapic acid (SA) is a hydroxycinnamic acid-derived polyphenol with 3,5-dimethoxyl and 4-hydroxyl substitutions in the phenyl group of cinnamic acid. It is widely found in various plant-derived foods, such as fruits, vegetables, cereals, and oilseed crops. We found that SA in *Polygala tenuifolia* (Willd.) is a major compound of WIN-1001X. Our previous study demonstrated that WIN-1001X, which was derived from a modified version of the Korean traditional herbal formula 'Chungsimyeoldatang' composed of a mixture of three ingredients, *Polygala tenuifolia* (Willd.), *Dimocarpus longan* Lour, and *Angelica tenuissima* Nakai, showed antiparkinsonism effects by modulating autophagic activity. One of the major compounds of *Polygala tenuifolia* (Willd.), onjisaponin B, also showed increased autophagy activation (Li *et al*., 2021b). However, SA did not show autophagy activation (Supplementary Fig. 1). For this reason, we tested the other action mechanism of SA. Several studies have suggested that SA has anti-inflammatory, antioxidant, antimicrobial, and neuroprotective effects (Shahmohamady *et al*., 2018; Li *et al*., 2019). Kim *et al*. (2010) discovered that SA could attenuate kainic acid-induced hippocampal neuronal damage by suppressing reactive gliosis. In addition, SA also exerted anxiolytic-like effects by acting on GABAA receptors and potentiating $Cl^-$ currents (Yoon *et al*., 2007). Recently, Zare *et al*. (2015) showed that SA inhibited the loss of dopaminergic neurons in a 6-OHDA-induced PD rat model by decreasing oxidative stress and lowering nigral iron levels. However, the mechanism of its neuroprotective effects against PD have not been investigated. In this study, we elucidated the possible underlying mechanism of SA in PD by using *in vivo* and *in vitro* methods.

## MATERIALS AND METHODS

### Materials

Sinapic acid (SA, Cat# D7927), MPTP (Cat# M0896) and $MPP^+$ (Cat# D048) were obtained from Sigma–Aldrich (St. Louis, MO, USA). SR8278 (SR, Cat# 4463) and GSK4112 (GSK, Cat# 3663) were purchased from Tocris Bioscience (Bristol, UK). DMEM and FBS were obtained from HyClone (Logan, UT, USA). Trypsin-EDTA and a mixture of penicillin and streptomycin were purchased from Gibco-BRL (Grand Island, NY, USA). Rabbit anti-TH (Cat# 2792S), REV-ERB α (Cat# 13418S), p-Drp1 Ser616 (Cat# 3455S), p-Drp1 Ser637 (Cat# 4867S), and GAPDH (Cat# 2118S) were purchased from Cell Signaling Technology Inc (Boston, MA, USA). Anti-rabbit (Cat# 7074S) and mouse (Cat# 7076S) horseradish peroxidase (HRP)-linked IgG antibodies were also obtained from Cell Signaling Technology Inc. The mouse anti-Drp1 antibody (Cat# sc-271583) was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). The rabbit anti-OPA1 antibody (Cat# ab157457) and MFN2 (Cat# ab56889) were purchased from Abcam (Cambridge, UK).

### Cell culture and treatment

SH-SY5Y cells (ATCC, Cat# CRL-2266) were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin and streptomycin at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. The cells were incubated for 1 h prior to $MPP^+$ treatment with SA, SR8278 (SR, REV-ERB α antagonist) and GSK4112 (GSK, REV-ERB α agonist), and the cells were harvested after 24 h. The stock solutions of each reagent were diluted to the final concentrations in medium prior to use.

### Cell viability assay

Cell viability was detected by using an MTT assay kit (EZ-Cytox kit, Cat# EZ-3000, DAEILLAM Co, Ltd, Seoul, Korea) according to the manufacturer's instructions. Briefly, SH-SY5Y cells were seeded at a density of $6\times10^4$ cells/well in 96-well culture plates and incubated with each reagent and EZ-Cytox solution for 1 h at 37°C. The absorbance of the culture plate was measured at 450 nm with a microplate reader (TECAN, Männedorf, Switzerland).

### Animals

Male C57BL/6 mice (weight 25-28 g, 7 weeks old, Orient Bio Inc, Seongnam, Korea, MGI Cat# 5656552) were kept in a fully automatic temperature- and humidity-controlled room (22 ± 3°C, 50%, 12 h light and dark cycle) with free access to food and water. All animal experiments were performed in accordance with guidelines established by the Korea Institute of Science and Technology Animal Care Committee and were approved by the Korea Institute of Science and Technology Animal Care and Use Committee (KIST-2018-077). All experiments were also performed in accordance with the ARRIVE guidelines.

The mice were randomly divided into 5 groups in each animal experiment. The doses of SA were selected according to the results from previous studies (Kim *et al*., 2010; Lee *et al*., 2012). The first study included a vehicle-treated control group (1, Control), 20 mg/kg SA-treated control group (2, SA20), vehicle-treated MPTP group (3), 10 mg/kg SA-treated MPTP group (4, SA10), and 20 mg/kg SA-treated MPTP group (5, SA20). In the second study, the 5 groups were the vehicle-treated control group (1, control), vehicle-treated MPTP group (2), 5 mg/kg ropinirole-treated MPTP group (3, RO), 20 mg/kg SA-treated MPTP group (4, SA), and both 20 mg/kg SA- and 25 mg/kg SR-treated MPTP group (5, SA+SR). Mice were injected with 30 mg/kg MPTP (i.p.) dissolved in PBS on five consecutive days with or without SA (p.o.), SR and ropinirole (i.p.), which were administered 30 min before the MPTP injection. SA and other reagents were treated from seven days before the MPTP injection. SR was intraperitoneally administered 30

min before SA treatment in the SA and SR combination group. The dose of SA was selected based on previous neurological studies, and all reagents were dissolved in PBS except for SR, which was dissolved in 5:5:90 DMSO/cremophor/PBS vehicle in accordance with a previous studies (Kim *et al*., 2010; Lee *et al*., 2012; Welch *et al*., 2017). After the behavioral tests, the mice were euthanized with $CO_2$ inhalation, and SNpc tissues were then collected and stored at –80°C.

### Motor coordination measurements

Seven days after the last MPTP injection, the behaviors of the mice were assessed using the rotarod and pole tests. Motor performance was evaluated using a rotarod apparatus. The mice were trained for 3 min 3 times a day for 3 consecutive days. The rotarod speed was gradually increased from 2 rpm to 16 rpm on all training days. On the test day, mice were subjected to the behavior ability test in 3 trials with the same procedure on the last training day, and the average of 3 trials per test was considered the final score. For the pole test, mice were placed on the top of the vertical pole (55 cm height, 8 mm diameter) before starting the measurement. The time until the mice descended to the floor completely was recorded as the time to turn (T-turn).

### Preparation of samples

For other biomarker measurements, lysates were obtained from SH-SY5Y cells and mouse brains. The cells were collected after treatment and lysed with RIPA buffer (Cell Signaling Technology Inc.) supplemented with a protease inhibitor cocktail (Roche, Penzberg, Germany) according to the manufacturer's instructions. Half of the mouse brain tissues, especially SNpc tissues, were homogenized using the IKA RW 20 digital homogenizer (IKA, Staufen, Germany) with PRO-PREP (iNtRON Biotechnology Inc., Seongnam, Korea) and then centrifuged at 13,000 rpm for 30 min at 4°C.

### Western blot analysis

The protein of lysates from cells and mouse brains were quantified by the Bradford method and then separated by 8-15% SDS–PAGE. After transferring the proteins onto polyvinylidene difluoride (PVDF, Millipore Corp., Billerica, MA, USA) membranes, the membranes were incubated with 5% skim milk or 3% BSA. The membranes were then sequentially incubated with the primary antibodies (1:1,000) overnight and with HRP-conjugated secondary antibodies (1:5,000) for 1 h. Immunoreactive bands were detected with a SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) using the LAS-4000 mini system (Fujifilm, Tokyo, Japan). The intensities of the bands were normalized to that of GAPDH using Multi Gauge software (Fujifilm).

### Mitochondrial glutamate dehydrogenase activity

Glutamate dehydrogenase (GDH) activity was measured using a commercial assay kit (Cat# K729, Biovision, CA, USA) according to the manufacturer's instructions. Because GDH was released from cell due to cell injury, the medium GDH activity was measured in the *in vitro* model. In the case of animal experiments, the GDH activity was measured in the brain for degree of damage. The cell medium and lysates were mixed with reaction reagents, and the absorbance of each sample was then detected at 450 nm. To calculate the GDH activity, these mixtures were incubated for 30 min, and the absorbance was measured again.

### ATP concentration measurement

The concentration of ATP was measured by a commercial ATP assay kit (Cat# K354, Biovision). After the deproteinization of lysates using a Deproteinization Sample Preparation Kit (Cat# K808, Biovision) to rapidly block ATP consumption, deproteinized lysates and reagents were mixed and plated on a 96-well plate. The plate was incubated for 30 min at room temperature, and the absorbance was then measured at 570 nm.

### Electron microscopy

Brain tissues were fixed with 2.5% glutaraldehyde solution (pH 7.3) and then postfixed in 2% osmium tetroxide. The samples were dehydrated with a series of ethanol and propylene oxide solutions. Subsequently, the samples were embedded in epoxy resin. After ultrathin sectioning (60-70 nm) and counterstaining with uranyl acetate and lead citrate, the samples were detected using scanning electron microscopy (SEM) (Teneo Volume Scope Microtome, FEI, Hillsboro, OR, USA)

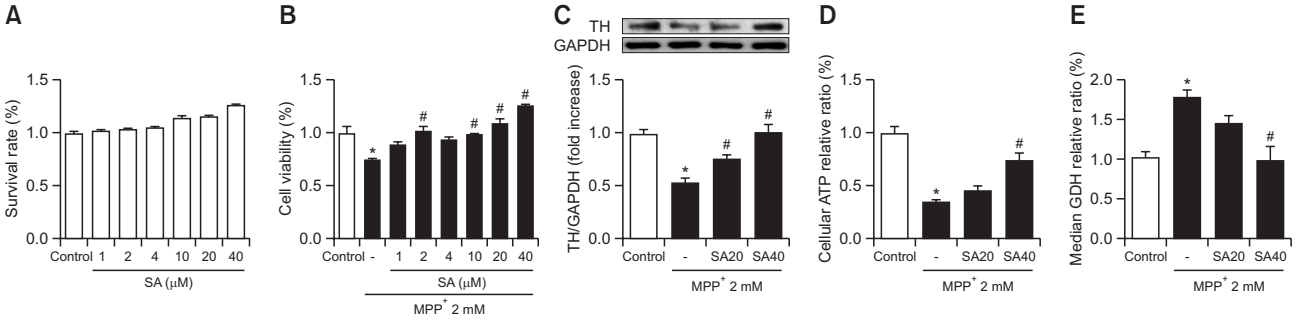


**Fig. 1.** Effect of sinapic acid (SA) on N-methyl-4-phenylpyridinium ($MPP^+$)-treated SH-SY5Y cells. (A) Effect of SA on the viability of SH-S5Y5 cells (n=5). (B) Evaluation of the dose-dependent effects of SA on $MPP^+$-treated cells (n=5). (C) The protein expression of tyrosine hydroxylase (TH) was measured by western blot after treatment with both $MPP^+$ and SA (n=5). (D) The cellular ATP content and (E) median glutamate dehydrogenase (GDH) activity were measured using a commercially available assay kit to examine mitochondrial function (n=5). Full-length blots are shown in the supplementary information. The results are presented as the mean ± SEM. *$p$<0.05 compared to the control group, $^{\#}p$<0.05 compared to the $MPP^+$ group.

and transmission electron microscopy (TEM) (Tecnai F20 G2, FEI).

### Data and statistical analysis

The sample sizes of cells (n=5) and animals (n=6) were selected based on the statistical analysis of at least 5 per group using randomization and blinded analysis, where n= number of independent values and not technical replicates. Data were analyzed with Prism 7.0 software (GraphPad Software, Inc., San Diego, CA, USA) using one-way ANOVA followed by the Bonferroni test for multiple comparisons. The results are expressed as the mean ± SEM.

## RESULTS

### Effect of SA on N-methyl-4-phenylpyridinium ($MPP^+$)-induced cytotoxicity in human SH-SY5Y neuroblastoma cells

First, we investigated the cytotoxicity of SA in SH-SY5Y cells using an MTT assay. SA showed no cellular toxicity at a variety of concentrations (from 1 to 40 μM) (Fig. 1A). To examine the effect of SA on $MPP^+$-induced cytotoxicity, we treated cells with SA at a concentration of 40 μM with or without 2 mM $MPP^+$. Fig. 1B shows that the cell survival rate was significantly decreased by 2 mM $MPP^+$. However, SA treatment dose-dependently protected the inhibitory effect of $MPP^+$ on the survival rate of SH-SY5Y cells. To further investigate the protective effects of SA, we measured the level of tyrosine hydroxylase (TH), which is an indicator of the abundance of dopaminergic neurons. As shown in Fig. 1C, the protein expression of TH was significantly inhibited by approximately 51% in the $MPP^+$ group compared to the control group. This decrease was attenuated by SA in a dose-dependent manner. Cellular ATP levels and GDH activity are usually detected for the measurement of mitochondrial function. GDH localizes in the mitochondrial matrix, and leakage of GDH from mitochondria indicates disruption of mitochondrial membrane integrity. Several studies have shown that the loss of GDH activity is associated with mitochondrial function, as with a reduction in ATP levels (Holownia *et al*., 1994; Lee *et al*., 2019). After $MPP^+$ treatment, the cellular ATP level was significantly decreased by approximately 36% compared to that in the control group, while the SA 40 μM treatment group showed recovery of the cellular ATP level (Fig. 1D). The median GDH activity in the $MPP^+$ group was markedly increased by 1.7-fold compared to that in the control group, and 40 μM SA attenuated this increase (Fig. 1E). To investigate how SA protected against $MPP^+$-induced neurotoxicity, we measured the protein expression of REV-ERB α, a circadian clock component, as a potent regulatory mechanism of mitochondrial fission. The protein expression of REV-ERB α was significantly decreased by 72% in the MPTP group, while SA attenuated REV-ERB α protein expression (Fig. 2A). To determine whether the protective effect of SA against $MPP^+$-induced neurotoxicity is dependent on REV-ERB α, we used the REV-ERB α agonist and antagonist with SA. As shown in Fig. 2B and 2C, treatment with both the REV-ERB α antagonist and SA diminished the protein expression of TH and REV-ERB α, but agonist treatment with SA elicited no change compared with treatment with SA only.

### Effect of SA on MPTP-induced neurotoxicity in C57BL/6 mice

We also determined the effect of SA on the MPTP-induced PD mouse model according to the experimental scheme (Fig. 3A). First, motor dysfunction was assessed using the rotarod and pole tests. In the rotarod test, the latency to fall was markedly decreased in the MPTP group (51.7 ± 11.3 s) compared with the control group (123.8 ± 14.3 s). After 10 and 20 mg/kg SA treatment, the latency to fall was increased by 2.2- and 2.3-fold, respectively, compared with that in the MPTP only group (Fig. 3B). MPTP treatment also impaired performance on the pole test (12.2 ± 1.4 s) compared with that in the control group (7.5 ± 0.3 s). However, 10 and 20 mg/kg SA treatment improved the performances of the mice by approximately 86 and 72%, respectively (Fig. 3C). Similar to the *in vitro* experiment, we detected the protein expression of TH. Several studies have shown that TH levels are reduced in the SNpc regions of MPTP-treated mice, recapitulating the main neuropathological characteristic of PD (Laloux *et al*., 2008; Hayashi *et al*., 2013). In this study, the protein expression of TH in the SNpc was significantly reduced by 39% in MPTP-treated mice compared with control group mice. SA at 20 mg/kg protected against the loss of dopaminergic neurons (Fig. 3D). In the mitochondrial function assay of ATP levels and GDH activity, these two factors were decreased by 83% and 55% in the MPTP group compared with the control group, respectively.

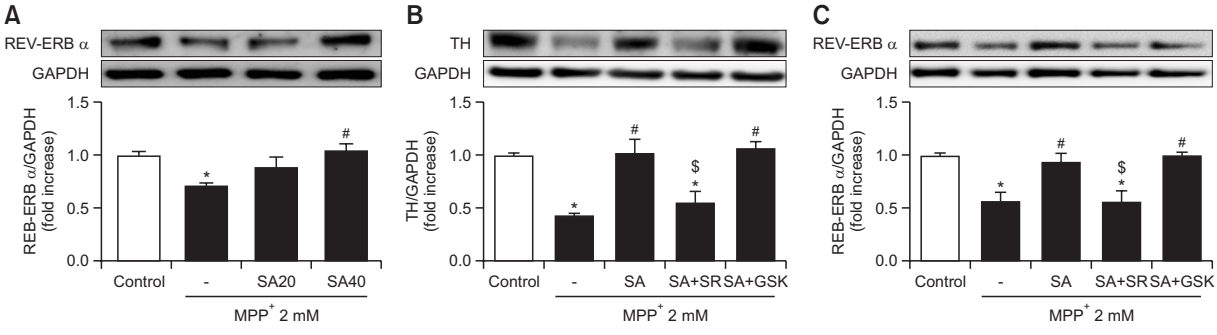


**Fig. 2.** Effect of sinapic acid (SA) on N-methyl-4-phenylpyridinium ($MPP^+$)-treated SH-SY5Y cells exposed to the REV-ERB α antagonist SR8278 and the REV-ERB α agonist GSK4112. (A) The protein expression of REV-ERB was measured by western blot after treatment with both $MPP^+$ and SA (n=5). (B, C) The protein expression of tyrosine hydroxylase (TH) and REV-ERB α was measured by western blot after treatment with $MPP^+$, SA, SR and GSK (n=5). Full-length blots are shown in the supplementary information. The results are presented as the mean ± SEM. *$p$<0.05 compared to the control group, $^{\#}p$<0.05 compared to the $MPP^+$ group, $^{\$}p$<0.05 compared to the SA group.

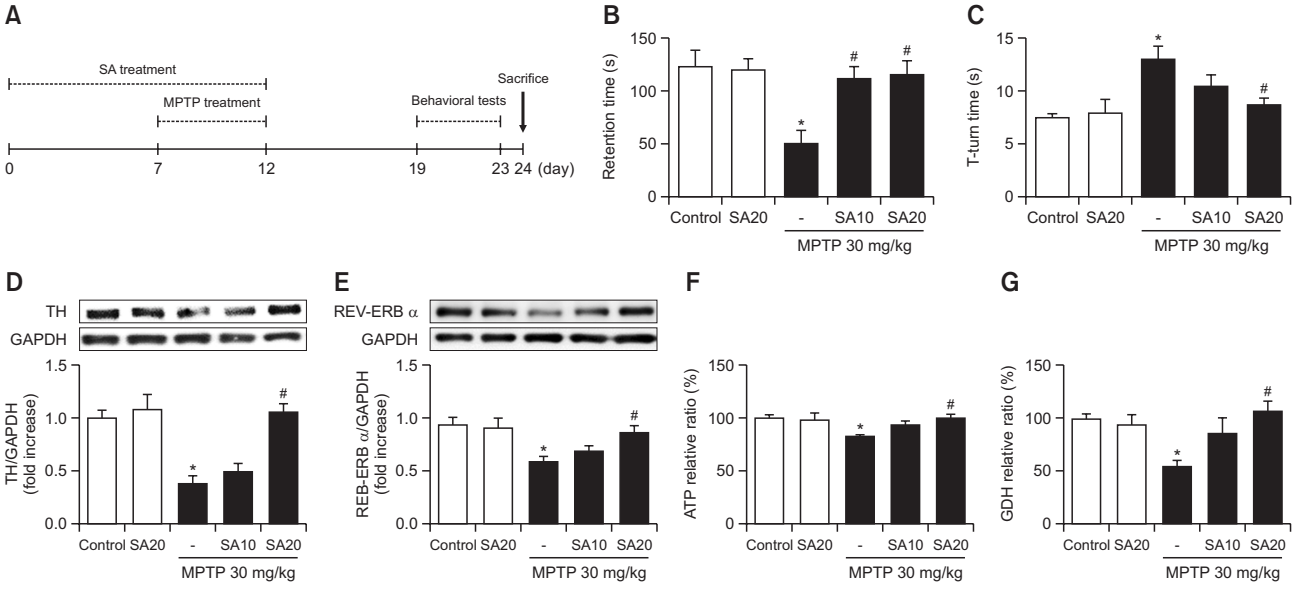


**Fig. 3.** Effect of sinapic acid (SA) on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. (A) Experimental procedure and drug administration scheme. (B, C) After 5 days of SA treatment with or without MPTP, the motor functions of the mice were examined using the rotarod and pole tests (n=6). (D, E) The protein expression of tyrosine hydroxylase (TH) and REV-ERB was measured by western blot after treatment with both MPTP and SA (n=6). (F, G) The ATP content and glutamate dehydrogenase (GDH) activity were measured using a commercially available assay kit to examine mitochondrial function (n=6). Full-length blots are shown in the supplementary information. The results are presented as the mean ± SEM. *$p$<0.05 compared to the control group, $^{\#}p$<0.05 compared to the MPTP group.

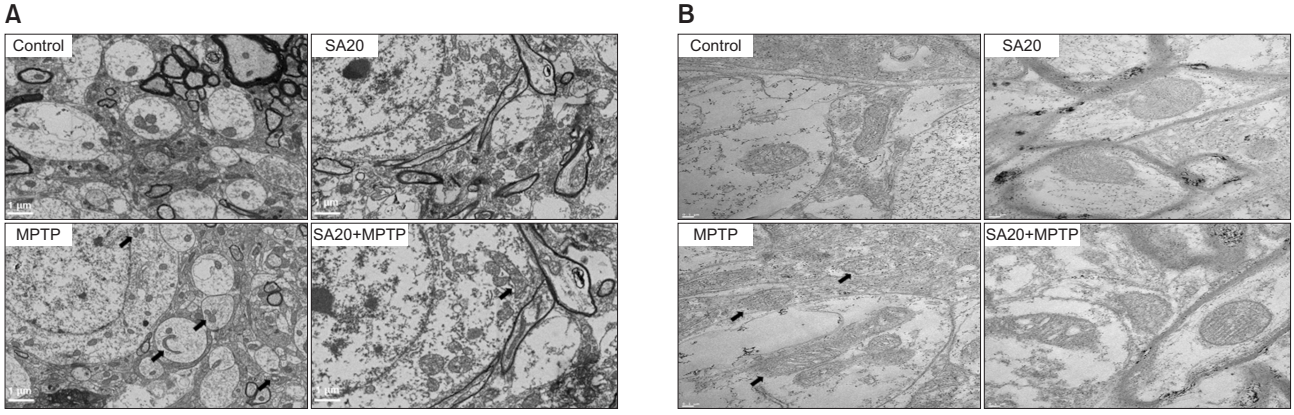


**Fig. 4.** (A, B) Mitochondria were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (n=3). After MPTP treatment, several mitochondria showed irregular shape (swollen condition, long and ring shapes; pointed by the arrow) in images (Scale bars=(A): 1 μm; (B): 0.1 μm).

The protein expression of REV-ERB was also similar to that observed *in vitro*. The protein expression of REV-ERB α in the MPTP group was approximately 64% lower than that in the control group, while 20 mg/kg SA attenuated this decrease (Fig. 3E). SA at 20 mg/kg also improved these changes (Fig. 3F, 3G). In addition, TEM analysis showed that MPTP treatment induced abnormal mitochondrial conditions such as mitochondria swelling, long- and ring-shaped mitochondria. However, the abnormal state of mitochondria was recovered by SA treatment (Fig. 4).

### Effects of SA on the expression of mitochondrial fission and fusion proteins in C57BL/6 mice with MPTP-induced neurotoxicity

Mitochondria easily maintain their homeostasis through several maintenance mechanisms, such as mitochondrial fission and fusion. However, excessive and aberrant mitochondrial fission promotes mitochondrial fragmentation and dysfunction, which are associated with the pathologies of numerous neurodegenerative diseases (Qi *et al*., 2013). In this study, MPTP treatment tended to increase the Drp1 protein expression, while SA treatment reversed this increase (Fig. 5A). Interestingly, the expression of the activated form of Drp1, phospho-Drp1 Ser616, was significantly increased by 1.8-fold

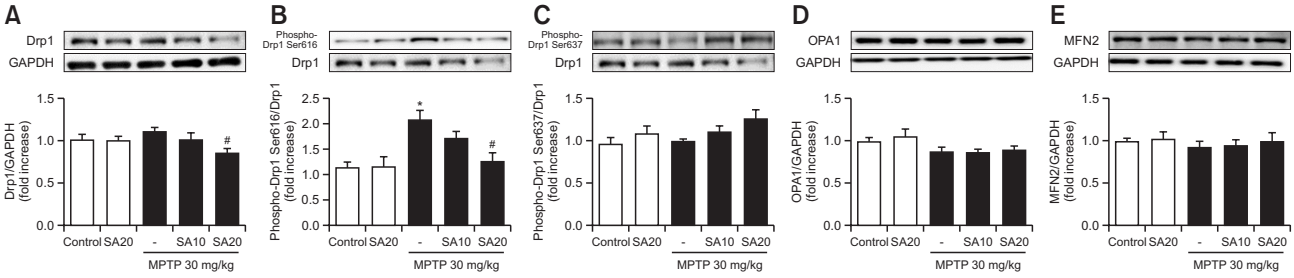


**Fig. 5.** Effect of sinapic acid (SA) on mitochondrial fission and fusion in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. (A-E) The expression of mitochondrial fission-related proteins, including Drp1, phospho-Drp1 Ser616, and phospho-Drp1 Ser637, and mitochondrial fusion-related proteins, including mitofusin 2 (MFN2) and OPA1, was measured by western blot after treatment with both MPTP and SA (n=6). Full-length blots are shown in the supplementary information. The results are presented as the mean ± SEM. **p*<0.05 compared to the control group, #*p*<0.05 compared to the MPTP group.

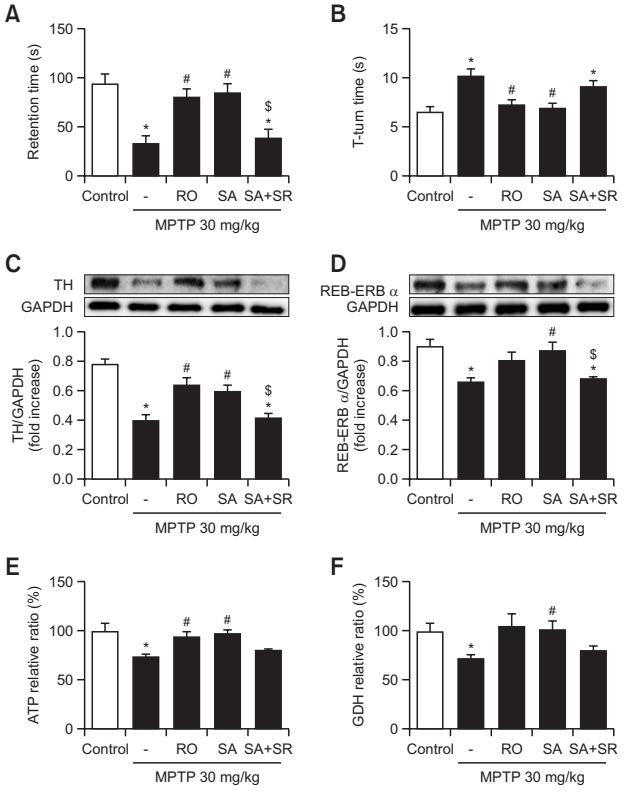


**Fig. 6.** Effect of sinapic acid (SA) on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice through the inhibition of REV-ERB α. (A, B) After 5 days of SA and SR8278 (SR) treatment with MPTP, the motor functions of the mice were examined using the rotarod and pole tests (n=6). (C, D) The protein expression of tyrosine hydroxylase (TH) and REV-ERB α was measured by western blot after treatment with MPTP, SA and SR (n=6). (E, F) The ATP content and glutamate dehydrogenase (GDH) activity were measured using a commercially available assay kit to examine mitochondrial function (n=6). Full-length blots are shown in the supplementary information. The results are presented as the mean ± SEM. **p*<0.05 compared to the control group, #*p*<0.05 compared to the MPTP group, $*p*<0.05 compared to the SA group.

in MPTP-treated mice compared with control group mice, but that of the inactivated form of Drp1, phospho-Drp1 Ser637, was not altered (Fig. 5B, 5C). This increase was attenuated by SA. However, the levels of OPA1 and MFN2, mitochondrial fusion proteins, were not changed in any of the groups (Fig. 5D, 5E).

### Effect of SA on MPTP-induced neurotoxicity in C57BL/6 mice through inhibition of REV-ERB α

Next, we investigated whether REV-ERB α was directly associated with the protective effect of SA on mice with MPTP-induced neurotoxicity. We also used ropinirole as a positive control to confirm the pathological changes after MPTP treatment. Previous study demonstrated that ropinirole have protective effect against mitochondrial dysfunction through attenuating mitochondrial ROS and blocking the mitochondrial permeability transition pore in ischemia/reperfusion neural damaged rats (Andrabi *et al*., 2020). This is an example of what ropinirole and SA have similar effect about mitochondrial protection, but it is different from the inhibiting excessive mitochondrial division, which is considered the mechanism of SA action. SR8278 (SR), a REV-ERB antagonist, was employed to inhibit the protein expression of REV-ERB α. In the motor function test, cotreatment with SA and SR markedly decreased motor performances on the rotarod and pole tests compared with those in the SA treatment group (Fig. 6A, 6B). In addition, the protein expression of TH and REV-ERB α in the SNpc was markedly decreased by approximately 70 and 78% in the SA and SR cotreatment groups compared with that in the SA group, respectively (Fig. 6C, 6D). The ATP level and GDH activity were also decreased by approximately 88% and 83%, respectively, in the SA and SR cotreatment groups compared with the SA group (Fig. 6E, 6F). Consistent with the previous data, cotreatment with SA and SR showed a tendency to increase the protein expression of Drp1 compared with that in the SA treatment group (Fig. 7A). In addition, phospho-Drp1 Ser616 levels were significantly increased by 1.2-fold in both SA- and SR-treated mice compared with mice in the SA group, but the level of phospho-Drp1 Ser637 was not altered (Fig. 7B, 7C). Ropinirole also showed a protective effect similar to that of SA, improving behavioral defects and mitochondrial function. However, ropinirole had a tendency to decrease Drp1 Ser616 protein expression, indicating that SA is more directly associated with mitochondrial fission proteins.

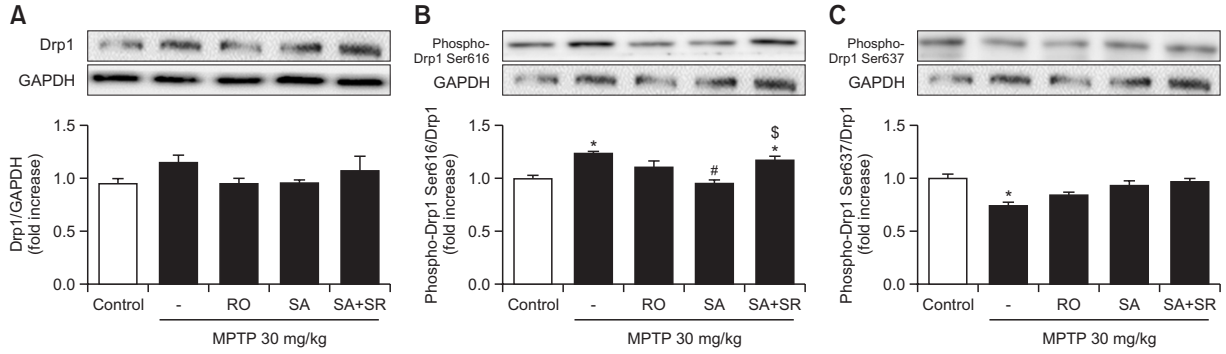


**Fig. 7.** Effect of sinapic acid (SA) on mitochondrial fission in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice through inhibition of REV-ERB α. (A-C) The protein expression of Drp1, phospho-Drp1 Ser616, and phospho-Drp1 Ser637 was measured by western blot after treatment with MPTP, SA and SR (n=6). Full-length blots are shown in the supplementary information. The results are presented as the mean ± SEM. *$p$<0.05 compared to the control group, $^{\#}p$<0.05 compared to the MPTP group, $^{\$}p$<0.05 compared to the SA group.

## DISCUSSION

The protective effects of SA against neurodegenerative diseases and other diseases, such as obesity and osteoarthritis, have been studied, but no mechanistic studies have investigated the effect of SA on circadian rhythms and mitochondrial function (Kim *et al*., 2010; Zare *et al*., 2015; Li *et al*., 2019; Yang *et al*., 2019). The present study demonstrated that SA ameliorates the neurotoxicity induced by MPTP and its metabolite $MPP^+$ through REV-ERB α-modulated mitochondrial fission.

Mitochondrial dysfunction is closely linked to the pathogenesis of PD, and the maintenance of mitochondrial function is considered a potential cure for the disease (Biorklund *et al*., 2020). MPTP is one of most commonly used chemical reagents for the PD model, and its treatment causes dopaminergic neuronal degeneration in the SNpc and behavioral abnormalities, which are based on mitochondrial damage (Zhou *et al*., 2020). In the present study, treatment with MPTP and its metabolite $MPP^+$ increased dopaminergic neuronal damage in mice and cells by decreasing ATP levels and altering GDH activity, thereby decreasing TH protein expression and mitochondrial damage. However, SA treatment restored TH protein expression and mitochondrial function. In addition, SA treatment restored the phosphofructokinase (PFK) and pyruvate kinase (PK) concentrations in MPTP-treated mice (Supplementary Fig. 3), indicating that SA affected not only mitochondrial function but also neuronal cell conditions. SA also improved the behavioral performances of MPTP-treated mice on both tests. Zare *et al*. (2015) reported that SA showed neuroprotective potential against 6-OHDA in a PD animal model by regulating oxidative stress and lowering nigral iron levels. Our results confirmed that SA protects against dopaminergic neuronal cell damage and mitochondrial function.

Damaged mitochondria are regulated by several maintenance mechanisms, such as mitophagy, mitochondrial biogenesis, and mitochondrial fission and fusion, all working in coordination. Mitochondrial fission is considered the first step in the maintenance of mitochondrial homeostasis. However, excessive mitochondrial fission induces cell injury through ATP depletion, ROS generation and apoptosis activation, which are common causes of the development of neurodegenerative diseases (Suen *et al*., 2008; Roe and Qi, 2018). Drp1 is considered the most important protein in the fission process, and its activity is controlled by the phosphorylation of serine 616 and serine 637. An impaired balance between these two types of phosphorylation results in excessive mitochondrial fragmentation and neuronal cell death (Cho *et al*., 2013). Recently, accumulating evidence has demonstrated that inhibition of Drp1 and/or phospho-Drp1 Ser616 ameliorates neurotoxicity and deficits in dopamine release in PD animal models (Park *et al*., 2019; Mishra *et al*., 2020). Bido *et al*. (2017) showed that treatment with Mdivi-1, a putative inhibitor of Drp1, decreased mitochondrial dysfunction and oxidative stress in α-synuclein-overexpressing rats. Our findings demonstrated that MPTP-induced mitochondrial fission was significantly weaker after SA treatment due to the decreased protein expression of phospho-Drp1 Ser616 and was correlated with the recovery of mitochondrial function. Therefore, our data suggested that SA attenuated excessive mitochondrial fission, especially the reduction in Drp1 phosphorylation at serine 616.

Circadian rhythms, which control the repeated approximately 24-hour sleep-wake cycle, manage various physiological functions, such as antioxidant and inflammatory responses, by changing gene expression (Zheng *et al*., 2017). Thus, disruption of the circadian cycle is considered to be a key factor in several diseases, including cancer, metabolic diseases, and neurodegenerative diseases (Xie *et al*., 2019). The most well-known clinical characteristic of an abnormal circadian cycle is sleep disruption. For this reason, disruption of the circadian cycle is widely accepted as a novel risk factor for the development of PD because sleep and circadian disorders are easily discovered in PD patients (Videnovic and Golombek, 2013). Several studies using animal PD models showed dopaminergic neuronal death and deregulated circadian symptoms, including behavioral and physiological outputs (Hayashi *et al*., 2013; Wang *et al*., 2018). Furthermore, MPTP treatment of environmental circadian disruption induced by long-term exposure to a 20:4 light/dark cycle exacerbated motor and cognitive deficits by inducing excessive neuronal cell loss and the neuroinflammatory response (Lauretti *et al*., 2017). In addition, MPTP intoxication led to sleep disorders, including sleep episodes in the daytime and sleep fragmentation at night, whereas melatonin and L-dopa significantly improved these sleep symptoms in MPTP-treated monkeys (Belaid *et*

*al*., 2015). Collectively, these studies suggest that an abnormal circadian rhythm and PD are closely related. REV-ERB α is responsible for the stability of the circadian rhythm by regulating circadian-related protein expression and is considered to be involved in the development of PD. Deletion of REV-ERB α contributed to exacerbated motor deficits and dopaminergic neuronal loss in a 6-OHDA-treated PD mouse model (Kim *et al*., 2018). In this study, SA treatment restored the protein level of REV-ERB  in MPTP-treated mice. Our data suggested that SA treatment influences the recovery of circadian cycle disruption by regulating REV-ERB α.

Although disruption of the circadian cycle and its contribution to the onset and development of PD were found in previous studies, whether disruption of the circadian cycle contributes to the progression of PD remains controversial. To adapt to the constant changes in the environment caused by daily changes during the day and night, a continuous supply of energy is essential for maintaining and improving cell function (Goede *et al*., 2018). Thus, the regulation of mitochondrial function is considered a possible candidate for this argument. Interestingly, several studies have suggested that mitochondrial functions and morphologic changes are dependent on a viable circadian clock. *Bmal1* knockout mice showed mitochondrial defects, accompanied by morphological changes and functional abnormalities, which led to severe, progressive, age-dependent heart failure (Kohsaka *et al*., 2014). Altered mitochondrial gene expression and increased mitochondrial oxidative stress were observed in aged mice with mutations in the *clock* gene (Gong *et al*., 2015). Moreover, Woldt *et al*. (2013) demonstrated that genetic deletion of REV-ERB α in mice resulted in the impairment of mitochondrial content and oxidative function; however, the amount of mitochondria and respiratory capacity were improved after treatment with the vector expressing REV-ERB α. To determine whether the protective effect of SA is mediated by mitochondrial fission through the regulation of REV-ERB α, we utilized an antagonist and agonist of REV-ERB α together with SA in both *in vitro* and *in vivo* PD models. The protective effect of SA disappeared after cotreatment with SA and REV-ERB  antagonists, as shown by decreased mitochondrial function and TH protein expression, while SA and agonist cotreatment had no effect on the *in vitro* model. These *in vivo* results obtained using an antagonist with SA indicated that the recovery effect of SA was diminished, as shown by decreased behavioral function and TH protein expression. In addition, mitochondrial fission was boosted after cotreatment with SA and the antagonist. Our data also showed that the protein level of Drp1 driven by the plasmid was decreased after treatment with a REV-ERB recombinant protein (Supplementary Fig. 4). Together, these data support a prominent role of SA in the regulation of mitochondrial fission via the REV-ERB α protein.

In this study, we demonstrated that SA was pharmacologically activated in a neurotoxin-induced PD model through the regulation of REV-ERB protein expression and revealed a novel role of REV-ERB in managing mitochondrial homeostasis by maintaining the balance between two types of Drp1 phosphorylation. Even though we didn't confirm whether SA directly affect to mitochondria and the protein expression of REV-ERB α, SA might be related to the recovering of abnormal mitochondrial condition and regulating of REV-ERB α expression. Together, these results highlight the potential of SA as an option for the prevention and treatment of PD.

## CONFLICT OF INTEREST

The authors have no competing financial interest to declare.

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