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Original Research

Effects and Mechanisms of Melatonin Receptor Agonist and Antagonist on Disease Progression in a 6-OHDA-Induced Parkinson's Disease Rat Model

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Abstract

Background: Parkinson's disease (PD) is characterized by progressive dopaminergic neurodegeneration. Melatonin (MLT) is implicated in neuroprotection, yet the effects of modulating its receptors remain unclear. This study investigated the impact of the MLT receptor agonist agomelatine (AG) and antagonist luzindole (LU) on motor behavior, serum MLT levels, and dopaminergic neuron survival in a 6-hydroxydopamine (6-OHDA) rat model of PD. **Methods**: A PD model was induced by stereotaxic injection of 6-OHDA into the medial forebrain bundle. Rats received intraperitoneal AG or LU for 2 or 4 weeks. Motor function was assessed using the apomorphine-induced rotation test. Tyrosine hydroxylase (TH) and MLT receptor (MEL-1A/B) expression in the substantia nigra and striatum were evaluated by immunohistochemistry and Western blot. Serum MLT concentrations were measured using ELISA. Pearson's correlation analysis was performed to examine associations among serum MLT levels, TH expression, and motor performance. **Results**: AG significantly improved motor function, increased serum MLT levels, and enhanced TH expression in PD rats. LU also mitigated motor deficits and preserved dopaminergic neurons, despite reducing serum MLT levels. Correlation analysis revealed a dynamic temporal relationship between MLT levels, behavioral outcomes, and dopaminergic neuron survival, indicating that MLT signaling may differentially influence PD pathology at various stages. **Conclusions**: Both AG and LU demonstrated neuroprotective potential in 6-OHDA–induced PD rats. AG may exert its effects by enhancing endogenous MLT signaling, while LU may protect neurons by modulating excessive MLT activity. These findings highlight the complex regulatory role of the MLT pathway in PD progression and suggest stage-dependent therapeutic benefits of MLT receptor modulators.

Keywords: Parkinson's disease; melatonin; melatonin receptor agonist; melatonin receptor antagonist; tyrosine hydroxylase; neuroprotection

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized primarily by the progressive dopaminergic neuron loss in the substantia nigra pars compacta (SNc) of the midbrain, leading to dopamine depletion in the striatum and resulting in a spectrum of motor dysfunction [1]. Although the exact pathogenesis of PD remains unclear, mounting evidence suggests that oxidative stress, mitochondrial dysfunction, and neuroinflammation are vital for its progression [2,3]. The unilateral midbrain lesion model induced by 6-hydroxydopamine (6-OHDA) is a widely used and well-established animal model that closely mimics the pathological and behavioral characteristics of PD [4].

Melatonin (MLT), a pineal gland-secreted hormone, is broadly involved in regulating circadian rhythms, antioxidant defense, and neuroprotection. Recent studies have reported alterations in MLT levels in patients with PD, which may be closely associated with disease progression [5,6]. However, the precise role of MLT and its receptors, melatonin receptor 1A (MT1) and melatonin receptor 1B (MT2), in PD pathogenesis remains controversial [7].

Agomelatine (AG), an MLT receptor agonist with an additional 5-hydroxytryptamine 2C (5-HT2C) receptor antagonist, ameliorates motor and affective disturbances in various neuropsychiatric disorders [8,9]. Luzindole (LU), a non-selective MLT receptor antagonist, is commonly used to study the physiological effects of disrupted MLT signaling [10–12]. However, some studies have examined the relationship between MLT and PD. The specific effects of AG and LU on dopaminergic neurodegeneration and motor dysfunction in PD remain unclear [10–12].

In this study, we established a 6-OHDA-induced rat model of PD to systematically assess the time-dependent effects of AG and LU on motor behavior, serum MLT levels, and the nigrostriatal dopaminergic system, to explore the potential therapeutic value of targeting MLT signaling in PD.

2. Materials and Methods

2.1 Animals

All surgical procedures were performed under pentobarbital anesthesia, with efforts made to minimize animal suffering. Male specific pathogen-free (SPF) Sprague-Dawley (SD) rats (280–300 g) were obtained from the Laboratory Animal Center of Fujian Medical University (Fuzhou, Fujian, China). Rats were housed in SPF conditions under a 12 h light/dark cycle at 23 ± 2 ℃ and 55 ± 5% humidity, with free access to food and water.

2.2 Experimental Groups

The rats were randomly assigned to two main groups based on the treatment duration (2 and 4 weeks), with each group further subdivided into six subgroups: Control (Cont), Control + LU, Control + AG, PD, PD + LU, and PD + AG. The Control group received injections of normal saline (NS).

2.3 6-OHDA Lesion

The rats were anesthetized with 2% sodium pentobarbital (40 mg/kg, vehicle: ddH_2O, CAS: 57-33-0, Sigma-Aldrich, Taufkirchen, Bavaria, German) and placed in a stereotaxic apparatus (SR-6R-HT, Narishige, Tokyo, Japan). After sterilizing the scalp with 75% ethanol (LIRCON, Dezhou, Shandong, China), the skin was incised to expose the bregma. According to a rat brain atlas, 6-OHDA (8 µg/2 µL, CAS: 28094-15-7, Sigma-Aldrich) was injected into the right medial forebrain bundle (MFB) using the following coordinates: anteroposterior: –4.4 mm, mediolateral: –1.4 mm, dorsoventral: +8.5 mm. The 6-OHDA solution was slowly injected using a microinjection pump (2 µL over 8 min), and the needle was left in place for 5 min before being withdrawn at a rate of 5 mm/min. The control rats received an equivalent volume of saline containing 0.2% ascorbic acid (CAS: 50-81-7, Sigma-Aldrich).

2.4 Apomorphine (APO)-Induced Rotation Test

At 2- or 4-week post-lesion, rats were intraperitoneally injected with apomorphine (APO, 0.5 mg/kg, CAS: 41372-20-7, Sigma-Aldrich) to induce rotational behavior. Rats that consistently rotated contralaterally at a rate of $\geq$7 turns/min ($\geq$210 turns in 30 min) were considered a successful PD model.

2.5 Immunohistochemistry

The SD rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital. After deep anesthesia was confirmed, euthanasia was performed by cervical dislocation in accordance with animal welfare and ethical guidelines. All procedures were carried out under pentobarbital anesthesia, and every effort was made to minimize pain and distress, following the ARRIVE guidelines. Rats were sacrificed under deep anesthesia, and midbrain tissues containing the substantia nigra were collected and fixed in 4% paraformaldehyde (CAS: 30525-89-4, Xilong Scientific, Shantou, Guangdong, China) for 24 h. After dehydration, clearing with xylene (CAS: 1330-20-7, Xilong Scientific), and paraffin (39601006, Leica, Shanghai, China) embedding, sagittal sections (5 µm thick) were prepared through the SNc. After deparaffinization and antigen retrieval, the sections were blocked with 5% normal goat serum (ZLI-9021, ZSGB-BIO, Beijing, China) for 30 min and incubated overnight at 4 ℃ with an anti-tyrosine hydroxylase (TH) antibody (1:300, #58844, Cell Signaling Technology, Danvers, MA, USA). The next day, the sections were washed with phosphate-buffered saline, incubated with secondary antibodies (1:1000, SAP-9100, ZSGB-BIO), and visualized using 3,3′-Diaminobenzidine (DAB, SAP-9100, ZSGB-BIO). The entire section was scanned using a Motic digital microscope (12025323, Motil BA600-4, Motic, Xiamen, Fujian, China).

2.6 Western Blot

Midbrain substantia nigra tissues were harvested rapidly, homogenized in lysis buffer (MA0151, MeilunBio, Dalian, Liaoning, China), and centrifuged at 12,000 rpm for 15 min. Protein concentrations in the supernatants were determined using the bicinchoninic acid (BCA) protein assay (MA0082, MeilunBio). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PG12, Epizyme, Shanghai, China) and transferred to polyvinylidene difluoride (PVDF, IPVH00010, Merckmillipore, Darmstadt, German) membranes, which were blocked with 5% skim milk for 1 h. Membranes were then incubated overnight at 4 ℃ with primary antibodies against TH (1:1000, #58844, Cell Signaling Technology), MLT receptor (MEL-1A/B, 1:1000, sc-390328, Santa Cruz Biotechnology, Shanghai, China), and β-actin (1:3000, A17910, Abclonal, Hubei, Wuhan, China). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:3000, LF102, Epizyme) at room temperature for 1 h. Protein bands were visualized using enhanced chemiluminescence and analyzed using ImageJ software (1.52a, National Institutes of Health, Bethesda, MD, USA) for densitometry.

2.7 ELISA

Serum MLT levels were determined using a rat enzyme-linked immunosorbent assay (ELISA) kit (Rat MT ELISA Kit, E-EL-R0031, Elabscience, Wuhan, Hubei, China) following the manufacturer's protocol. Absorbance was measured at 450 nm, and concentrations were calculated from the standard curve.

2.8 Statistical Analysis

Data were analyzed using GraphPad Prism software (version 9.0, GraphPad Software, San Diego, CA, USA)

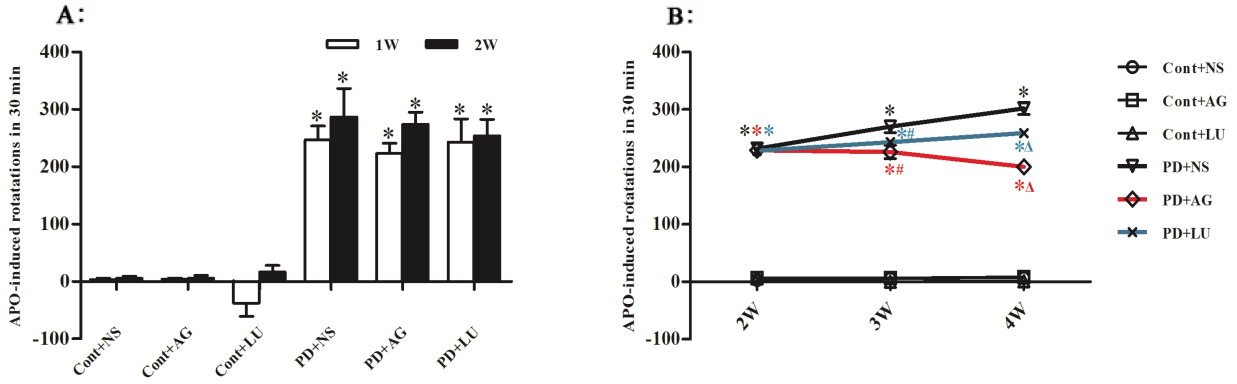

Fig. 1. Effects of MLT receptor agonist and antagonist on APO-induced rotational behavior in rats. (A) Number of APO-induced rotations in each group after 1 and 2 weeks of treatment with MLT receptor agonist or antagonist. *$p < 0.01$ versus respective control groups; n = 6. (B) Number of APO-induced rotations after 2, 3, and 4 weeks of treatment. *$p < 0.01$ versus control group at each time point; #$p < 0.05$, versus PD + NS group at week 3; △$p < 0.01$ versus PD + NS group at week 4; n = 6. MLT, melatonin; APO, apomorphine; Cont, control group; NS, normal saline; AG, agomelatine; LU, luzindole; PD, Parkinson's disease model group; w, week.

and expressed as mean ± standard deviation. One-way analysis of variance was used to compare differences among groups, followed by Fisher's Least Significant Difference Test (LSD). A $p < 0.05$ was considered statistically significant.

3. Results

3.1 APO-Induced Rotational Test

3.1.1 Number of Rotations After 1 and 2 Weeks of Treatment

After 1 week of treatment, the number of rotations in PD + NS, PD + AG, and PD + LU groups was significantly higher than that in the control group ($p < 0.01$). After 2 weeks of treatment, the rotational counts in PD + NS, PD + AG, and PD + LU groups remained significantly higher than those in the control group ($p < 0.01$). Statistically non-significant differences were observed among control subgroups or among PD subgroups at either 1 or 2 weeks (Fig. 1).

3.1.2 Number of Rotations After 2, 3, and 4 Weeks of Treatment

At week 2, the rats in PD + NS, PD + AG, and PD + LU groups exhibited significantly more rotations than those in the control group ($p < 0.01$). At week 3, the rotational behavior remained significantly increased in these PD groups compared to controls ($p < 0.01$). Compared with the PD + NS group, PD + AG and PD + LU groups revealed a reduction in rotational counts by 16.28% ± 4.54% and 10.23% ± 2.22%, respectively ($p < 0.05$). At week 4, all PD groups exhibited significantly higher rotation numbers than the control group ($p < 0.01$). However, PD + AG and PD + LU groups exhibited a marked reduction by 33.81% ± 2.10% and 14.25% ± 3.37%, respectively, relative to the PD + NS group ($p < 0.01$) (Fig. 1).

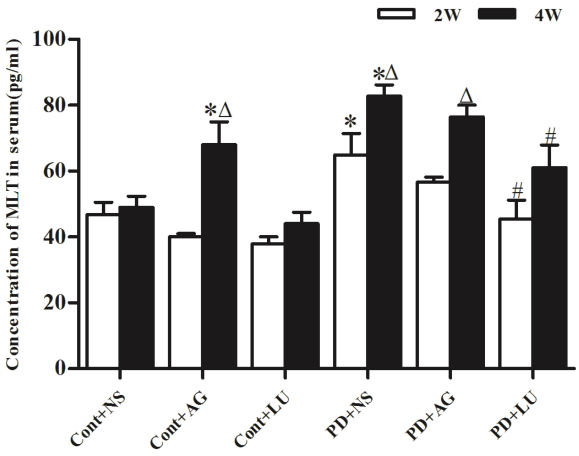

Fig. 2. Serum MLT concentrations in rat cardiac blood after 2 and 4 weeks of treatment with MLT receptor agonists and antagonists, measured by ELISA. * indicates $p < 0.05$, compared with the corresponding Cont + NS group (for PD + NS and Cont + AG groups); # indicates $p < 0.05$, compared with the corresponding PD + NS group (for the PD + LU group); △ indicates $p < 0.05$, compared with the same group at 2 weeks. n = 6/group. ELISA, enzyme-linked immunosorbent assay.

3.2 Serum MLT Levels and Striatal MEL-1A/B Protein Expression in Rats

3.2.1 Serum MLT Concentration in Rats (pg/mL)

As presented in Fig. 2, after 2 weeks of treatment, the serum MLT concentration in the PD + NS group was significantly higher than that in the control group ($p < 0.05$). In contrast, the PD + LU group revealed a significantly lower concentration compared to the control ($p < 0.05$). After 4 weeks of treatment, the serum MLT concentrations in Cont + AG and PD + NS groups were significantly elevated compared to the control group ($p < 0.05$). The PD + LU group

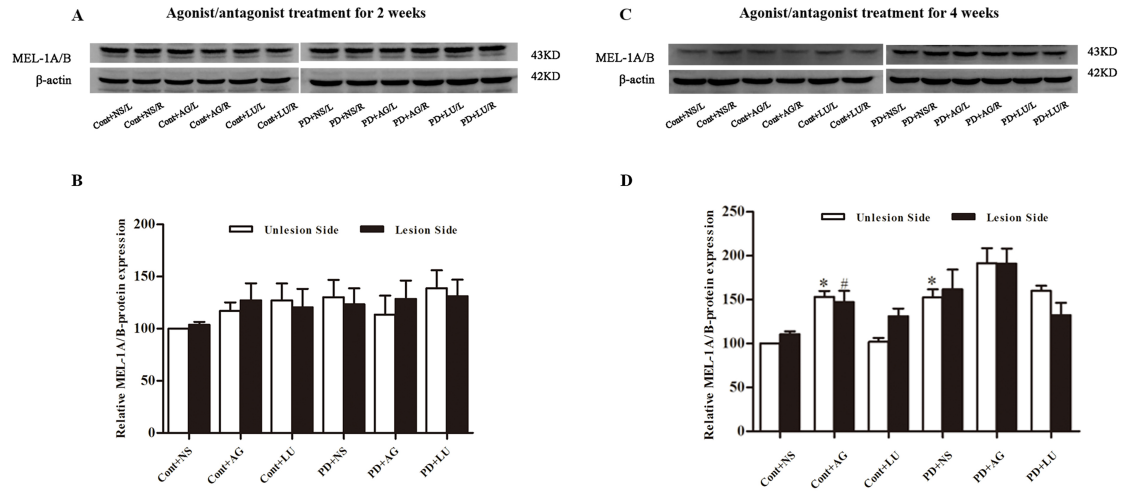

Fig. 3. Effects of MLT receptor modulation on MEL-1A/B expression levels in the striatum. (A,B) MEL-1A/B protein expression in the striatum of rats after 2 weeks of treatment with MLT receptor agonists and antagonists. (A) Representative Western blot results of MEL-1A/B expression in the striatum. (B) Relative protein expression levels were calculated using the ipsilateral striatum of the control group as baseline (100%). The ratio of (band intensity/internal control) for each group was normalized to that of the control group. n = 6. Cont, control group; PD, Parkinson's disease model group; L, ipsilateral striatum; R, lesioned striatum. (C,D) MEL-1A/B protein expression in the striatum of rats after 4 weeks of treatment with MLT receptor agonists and antagonists. (C) Representative Western blot results of MEL-1A/B expression in the striatum. (D) Relative protein expression levels were calculated using the ipsilateral striatum of the control group as baseline (100%). The ratio of (band intensity/internal control) for each group was normalized to that of the control group. * indicates $p < 0.01$ compared with the ipsilateral side of the Cont + NS group (for Cont + AG and PD + NS groups); # indicates $p < 0.01$, compared with the lesioned side of the Cont + NS group (for the Cont + AG group). n = 6.

revealed a 26.29% ± 10.46% reduction in MLT levels compared to the PD + NS group ($p < 0.05$). After 4 weeks of treatment, the serum MLT levels in Cont + AG, PD + NS, and PD + AG groups increased by 41.20% ± 6.14%, 21.53% ± 9.66%, and 25.85% ± 3.82%, respectively, compared to their levels at 2 weeks ($p < 0.05$).

3.2.2 MEL-1A/B Protein Expression in the Striatum

MEL-1A/B protein expression level in the ipsilateral striatum of control rats was set as the baseline (100%). Relative protein expression was calculated as the ratio of (experimental group band intensity/internal control) to (control group band intensity/internal control). After 2 weeks of treatment, there were statistically non-significant differences in MEL-1A/B protein expression in the striatum among the groups (Fig. 3A,B).

After 4 weeks of treatment, MEL-1A/B protein expression in the ipsilateral striatum was significantly increased by 34.74% ± 2.73% in the Cont + AG group and 34.52% ± 4.08% in the PD + NS group than the Cont + NS group ($p < 0.01$). On the lesioned side, MEL-1A/B expression in the Cont + AG group was 24.85% ± 6.71% higher than that in the Cont + NS group ($p < 0.01$). However, treatment with LU did not significantly change MEL-1A/B expression compared with the PD + NS group ($p > 0.05$) (Fig. 3C,D; The original Western blot images can be found in the **Supplementary Materials**).

3.3 Survival of Midbrain Dopaminergic Neurons and Striatal TH Protein Expression in PD Model Rats

3.3.1 Immunohistochemical Assessment of Dopaminergic Neuron Survival in the SNc

After 2 weeks of treatment (Fig. 4A,C), the number of TH-positive cells on the lesioned side of PD + NS, PD + AG, and PD + LU groups was significantly reduced by 89.04% ± 1.52%, 76.38% ± 1.15%, and 81.89% ± 1.15%, respectively, than the corresponding sides in Cont + NS, Cont + AG, and Cont + LU groups ($p < 0.001$). Compared with the ipsilateral side, the lesioned side of PD + NS, PD + AG, and PD + LU groups exhibited a reduction in TH-positive cells by 88.91% ± 1.90%, 76.53% ± 2.13%, and 80.71% ± 1.00%, respectively ($p < 0.01$). The number of TH-positive cells on the lesioned side of the PD + AG group was 52.77% ± 7.81% higher than that of the PD + NS group ($p < 0.01$). Similarly, the PD + LU group revealed a 39.08% ± 6.06% increase in TH-positive cells on the lesioned side compared to the PD + NS group ($p < 0.05$). The location of the SNc was identified according to the stereotaxic atlas (Fig. 4B).

After 4 weeks of treatment (Fig. 4A,D), the number of TH-positive cells on the lesioned side in PD + NS, PD + AG, and PD + LU groups decreased by 91.14% ± 1.43%, 60.79% ± 2.39%, and 75.46% ± 1.13%, respectively, compared with the corresponding sides in Cont + NS, Cont + AG, and Cont + LU groups ($p < 0.001$). Compared with the

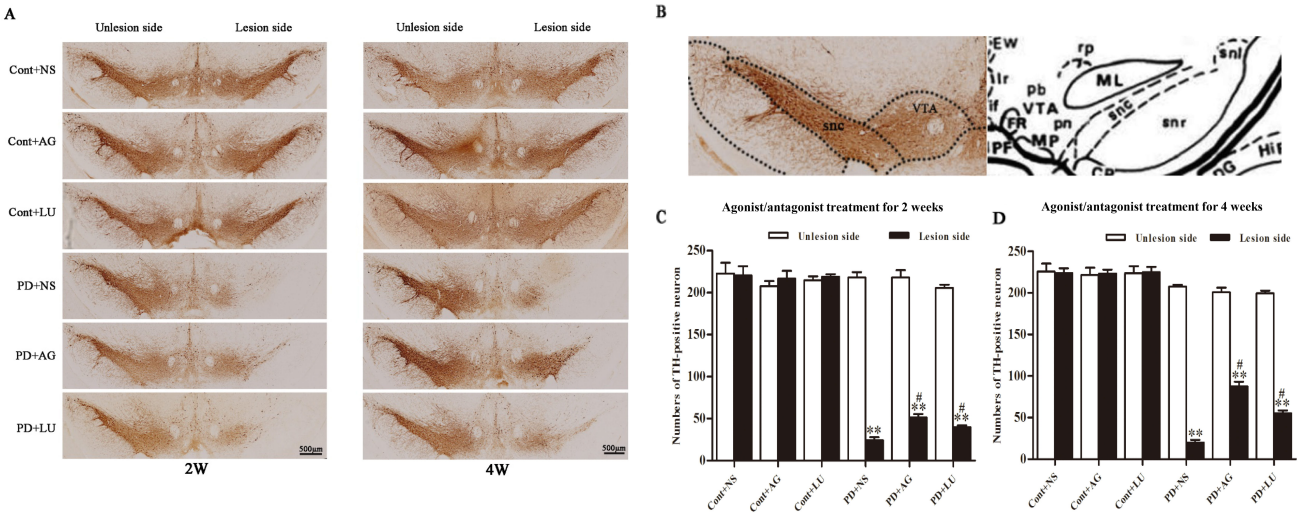

Fig. 4. Effects of MLT receptor agonist and antagonist on dopaminergic neuronal survival in the bilateral SNc of rats. (A) Tyrosine hydroxylase (TH) immunohistochemical staining of the SNc in each group after 2 and 4 weeks of treatment. Scale bar = 500 μm. (B) Schematic diagram indicating the locations of SNc and ventral tegmental area in the midbrain. (C,D) Quantification of TH-positive neurons in the bilateral SNc after 2 weeks (C) and 4 weeks (D) of treatment. ** indicates $p < 0.01$ for comparisons between the lesioned side of each PD group and the corresponding lesioned side of the Cont group, and between the lesioned and unlesioned sides within each PD group. # indicates $p < 0.01$ for comparisons between the lesioned sides of PD + AG and PD + LU groups and that of the PD + NS group; n = 6.

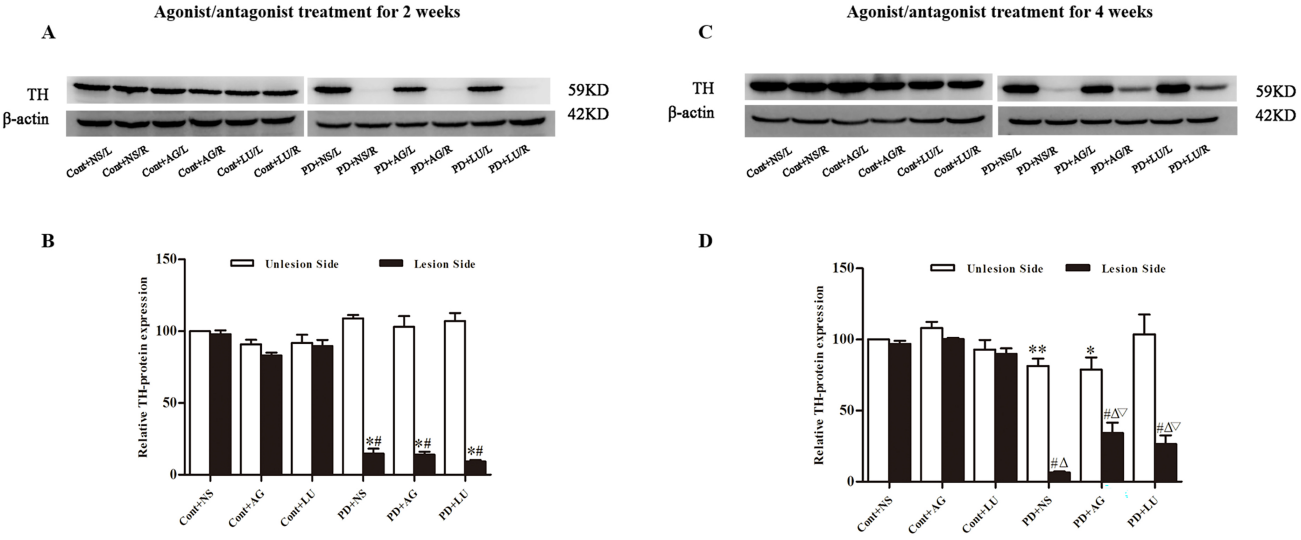

Fig. 5. Striatal TH protein expression after 2 and 4 weeks of treatment with MLT receptor ligands. (A,B) TH protein expression in the striatum of rats after 2 weeks of treatment with an MLT receptor agonist or antagonist. Thirty minutes after 6-hydroxydopamine (6-OHDA) lesioning, the rats received daily intraperitoneal injections of the indicated drugs for 2 weeks. (A) Representative Western blot analysis of the TH protein expression in the striatum. (B) Relative expression was calculated by normalizing the band intensity to the internal control and then expressing it as a percentage of the intact side of the control group (set to 100%). *$p < 0.01$ versus the lesioned side of the corresponding control group; #$p < 0.01$ versus the intact side of the same treatment group. n = 6 per group. (C,D) TH protein expression in the striatum of rats after 4 weeks of treatment with MLT receptor agonist or antagonist. Thirty minutes after 6-OHDA lesioning, the rats received daily intraperitoneal injections of the indicated drugs for 4 weeks. (C) Representative Western blot analysis of the TH protein expression in the striatum. (D) Relative expression was calculated as described above. **$p < 0.01$ versus intact side of Cont + NS group; *$p < 0.05$ versus intact side of Cont + AG group; #$p < 0.01$ versus lesioned side of corresponding control group; △$p < 0.01$versus intact side of the same treatment group; ▽$p < 0.01$ versus PD + NS group lesioned side. n = 6 per group.

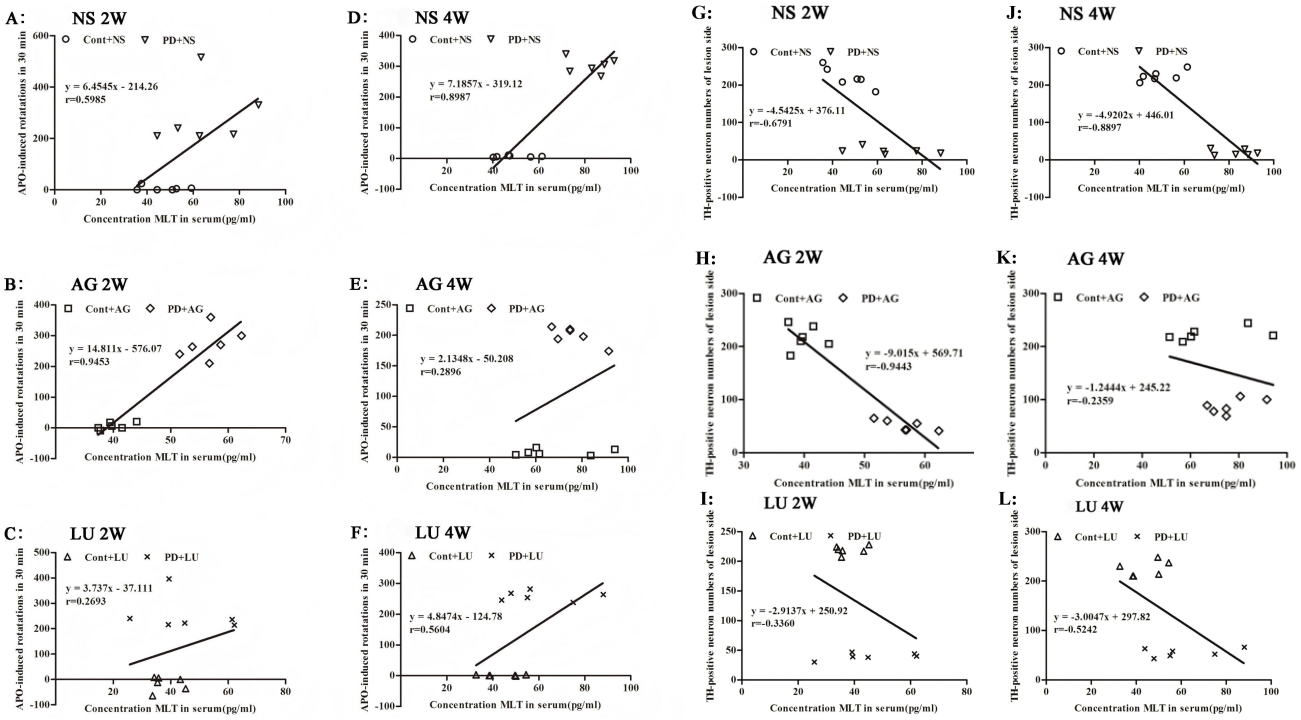

Fig. 6. Correlations between serum MLT levels and behavioral and histological parameters. (A–F) Correlation between serum MLT levels and APO-induced rotations after 2 and 4 weeks of treatment with MLT receptor agonists or antagonists. (A) Two weeks after the NS injection, a significant correlation was observed between control and PD groups ($p < 0.05$, n = 12). (B) Two weeks after the AG injection ($p < 0.0001$, n = 12). (C) Two weeks after LU injection, a non-significant correlation was observed ($p > 0.05$, n = 12). (D) Four weeks after the NS injection ($p < 0.0001$, n = 12). (E) Four weeks after the AG injection ($p > 0.05$, n = 12). (F) Four weeks after the LU injection ($p > 0.05$, n = 12). The Pearson correlation coefficients (r) are presented in the Figure. (G–L) Correlation between serum MLT levels and TH-positive cell number in the lesioned SNc after 2 and 4 weeks of treatment with MLT receptor agonists or antagonists. (G) Two weeks after the NS injection ($p < 0.05$, n = 12). (H) Two weeks after the AG injection ($p < 0.0001$, n = 12). (I) Two weeks after the LU injection ($p > 0.05$, n = 12). (J) Four weeks after the NS injection ($p < 0.001$, n = 12). (K) Four weeks after the AG injection ($p > 0.05$, n = 12). (L) Four weeks after the LU injection ($p > 0.05$, n = 12). Pearson's correlation coefficients (r) are presented in the Figure.

ipsilateral side, the lesioned side of PD + NS, PD + AG, and PD + LU groups revealed a reduction in TH-positive neurons by 90.45% ± 1.71%, 56.43% ± 2.93%, and 72.35% ± 0.02%, respectively ($p < 0.001$). The number of TH-positive neurons on the lesioned side of the PD + AG group was 77.33% ± 3.56% higher than that of the PD + NS group ($p < 0.001$). Similarly, the lesioned side of the PD + LU group exhibited a 64.05% ± 7.13% increase in TH-positive neurons compared to the PD + NS group ($p < 0.001$).

3.3.2 Expression of TH Protein in the Striatum of Rats

After 2 weeks of drug administration, the relative expression level on the intact side of the control group was set as 100%. The relative TH protein expression was calculated as the ratio of (band intensity/internal control) in the experimental group to that in the control group (Fig. 5A,B). On the lesioned side, TH protein levels in PD + NS, PD + AG, and PD + LU groups were significantly reduced by 84.87% ± 3.63%, 83.24% ± 2.26%, and 89.78% ± 1.36%, respectively, compared with the corresponding sides of Cont + NS, Cont + AG, and Cont + LU groups ($p < 0.01$). Furthermore, the lesioned side in PD + NS, PD + AG, and PD + LU groups revealed reductions of 86.40% ± 3.19%, 86.49% ± 1.00%, and 91.44% ± 1.08%, respectively, than their own intact sides ($p < 0.01$).

After 4 weeks of drug administration, the TH protein expression on the intact side in the PD + NS group was reduced by 18.73% ± 5.25% compared to that in the Cont + NS group ($p < 0.01$), and the PD + AG group exhibited a 27.08% ± 8.38% reduction compared to the Cont + AG group ($p < 0.05$) (Fig. 5C,D; The original Western blot images can be found in the **Supplementary Materials**). On the lesioned side, TH expressions in PD + NS, PD + AG, and PD + LU groups were significantly decreased by 93.32% ± 0.68%, 65.89% ± 7.34%, and 70.53% ± 6.35%, respectively, compared to the corresponding lesioned sides of Cont + NS, Cont + AG, and Cont + LU groups ($p < 0.01$). Compared to their own intact sides, the lesioned sides of PD + NS, PD + AG, and PD + LU groups revealed reductions of 92.03% ± 1.18%, 56.58% ± 4.72%, and 74.44% ± 3.86%, respectively ($p < 0.05$). Notably, TH expression on the lesioned side in PD + AG and PD + LU groups was

significantly increased by 81.10% ± 5.38% and 75.58% ± 10.66%, respectively, compared to the PD + NS group ($p < 0.01$).

3.4 Correlation Analysis

3.4.1 Correlation Between Serum MLT Levels and APO-Induced Rotational Behavior

Pearson correlation analysis was performed to assess the relationship between serum MLT levels and APO-induced rotational behavior (Fig. 6A–F). Two weeks after drug administration, a significant positive correlation was observed between serum MLT levels and APO-induced rotation number in PD rats treated with NS (r = 0.5985, $p < 0.05$, n = 12) and a strong positive correlation in those treated with AG (r = 0.9453, $p < 0.0001$, n = 12). However, a non-significant correlation was observed in PD rats treated with LU (r = 0.2693, $p > 0.05$, n = 12). After 4 weeks of treatment, a strong positive correlation between MLT levels and APO-induced rotation was still observed in the PD + NS group (r = 0.8987, $p < 0.0001$, n = 12), whereas non-significant correlation was detected in PD + AG (r = 0.2896, $p > 0.05$, n = 12) or PD + LU groups (r = 0.5604, $p > 0.05$, n = 12).

3.4.2 Correlation Between Serum MLT Levels and TH-Positive Cell Number

Pearson correlation analysis was used to assess the relationship between serum MLT levels and TH-positive cell number in the SNc (Fig. 6G–L). After 2 weeks of treatment, a significant negative correlation was observed between MLT levels and TH-positive cell number in the lesioned SNc of PD rats treated with NS (r = –0.6791, $p < 0.05$, n = 12), and a strong negative correlation was observed in the PD + AG group (r = –0.9443, $p < 0.0001$, n = 12). A non-significant correlation was observed in the PD + LU group (r = –0.3360, $p > 0.05$, n = 12). After 4 weeks of treatment, the serum MLT level remained negatively correlated with the number of TH-positive cells in the PD + NS group (r = –0.8897, $p < 0.001$, n = 12), while non-significant correlation was observed in either PD + AG (r = –0.2359, $p > 0.05$, n = 12) or PD + LU (r = –0.5242, $p > 0.05$, n = 12) groups.

4. Discussion

This study identified that in the PD group, the number of APO-induced rotations gradually increased from 2 to 4 weeks after modeling. During this period, serum MLT levels progressively increased, whereas dopaminergic neurons in the lesioned SNc progressively decreased. Following the administration of an MLT receptor agonist, PD rats exhibited a marked reduction in APO-induced rotations between weeks 2 and 4. Concurrently, serum MLT levels increased, the number of dopaminergic neurons in the lesioned SNc increased significantly, and TH protein expression in the lesioned striatum was elevated. After treatment with an MLT receptor antagonist, PD rats also revealed a reduction in APO-induced rotations between weeks 2 and 4. However, this was accompanied by a decrease in the serum MLT levels. Despite this, there was an increase in dopaminergic neurons in the lesioned SNc and TH protein expression in the lesioned striatum.

APO-induced rotational behavior is widely used as a standard method for evaluating the unilateral 6-OHDA lesion model [13,14]. In this study, we observed that administration of either an MLT receptor agonist or antagonist alone within 2 weeks of model induction had a non-significant effect on APO-induced rotational behavior in either control or PD rats. However, after 3 and 4 weeks of treatment with the MLT receptor agonist AG, the number of rotations in PD rats was reduced by approximately 16% and 34%, respectively, compared to the untreated PD group. This indicates that treatment with an MLT receptor agonist for more than 3 weeks can significantly improve motor symptoms in PD rats. Avila *et al*. [15] observed that treatment with AG in patients with PD with depressive symptoms led to partial improvement in motor symptoms and sleep disturbances. This effect may be attributed to the high expression of 5-HT2C receptors in the basal ganglia and substantia nigra, which regulate the activity of dopaminergic neurons and thus influence motor control in the brain [15]. AG functions as an agonist of MLT receptors and as an antagonist of 5-HT2C receptors. Blocking 5-HT2C receptor overexpression enhances dopaminergic activity, thereby improving motor symptoms [16]. In this study, administration of the MLT receptor antagonist LU alone for 3 and 4 weeks led to approximately 10% and 14% reductions, respectively, in APO-induced rotations compared to untreated PD rats, suggesting that MLT receptor antagonists may also help control motor symptoms in PD. Ortiz-López *et al*. [17] reported that 2-week administration of LU (10 mg/kg) in adult female C57BL/6 mice effectively blocked MLT receptors but did not reduce despair-like behavior in the forced swim test (FST). Micale *et al*. [18] demonstrated that subcutaneous injection of LU (0.25 mg/kg) abolished the MLT-induced reduction in immobility time during the FST in rats, possibly due to the anticonvulsant and anxiolytic effects of MLT that enhance GABAergic transmission in the central nervous system [19]. Meng *et al*. [20] also observed that in PD model rats with 6-OHDA lesions, MLT levels in the lesioned striatum were positively correlated with the concentration of the excitatory amino acid glutamate and negatively correlated with that of the inhibitory amino acid gamma-aminobutyric acid (GABA). It is therefore hypothesized that LU exerts its effects by antagonizing the MLT receptor MT2, leading to a reduction in MLT levels, an increase in GABA concentration, and an indirect improvement in motor symptoms in PD rats [21].

MLT is a hormone primarily secreted by the pineal gland and is widely distributed throughout the mammalian body [22]. It exerts various physiological effects, including regulation of circadian rhythms, enhancement of free

radical scavenging and immune responses, and cytoprotective effects [5]. Under normal conditions, physiological MLT levels decline with age [23]. In this study, serum MLT levels in PD rats were approximately 39% and 41% higher than those in the control group after 2 and 4 weeks of AG treatment, respectively. Moreover, MLT levels in the PD group increased by an additional 22% in 4 weeks compared to 2 weeks. These findings suggest that MLT levels increase with Parkinson's disease progression, which is consistent with our previous results [20,24]. This compensatory increase in endogenous MLT may occur in response to dopaminergic neuronal loss, potentially serving as a neuroprotective mechanism to counteract neuronal degeneration. In this study, administration of the MLT receptor agonist AG for 2 weeks did not significantly alter MLT levels in either control or PD rats. However, after 4 weeks of treatment, serum MLT levels increased by approximately 28% in the control group.

Additionally, MLT levels in control and PD rats treated with the agonist increased by approximately 41% and 26%, respectively, at 4 weeks compared to 2 weeks. These findings suggest that the prolonged administration of AG can elevate MLT levels in normal and PD rats. This effect may result from the specific binding of AG to MT1 and MT2 receptors *in vivo*, thereby enhancing serum MLT levels, although the exact mechanism remains unclear. LU, a non-selective MT1/MT2 MLT receptor antagonist, reduces MLT levels. Following 2 and 4 weeks of LU administration, serum MLT levels in PD rats decreased by approximately 30% and 26%, respectively, compared to untreated PD rats. Moreover, MLT levels in LU-treated control rats also revealed a declining trend, further confirming that LU can reduce serum MLT levels in PD rats.

In this study, after 2 weeks of administration, neither the MLT receptor agonist nor antagonist had a significant effect on MEL-1A/B protein expression in PD rat striatum. After 4 weeks of treatment, administration of the agonist alone in the control group led to an increase in MEL-1A/B protein expression in both striata. In the PD group, MEL-1A/B expression in the intact striatum was approximately 35% higher than in the corresponding side of the control group. These findings suggest that the agonistic effect on MLT receptors is enhanced after 4 weeks of agonist administration, which is consistent with the observed elevation in serum MLT levels. Antagonist administration alone did not significantly alter MEL-1A/B protein expression in either the control or PD groups, indicating that MLT receptor antagonism in the striatum remained limited even after 4 weeks of treatment. These findings are consistent with those of Kang *et al*. [25], who reported that unilateral MFB injections of varying doses of 6-OHDA (8.75–20 µg) over 8 weeks did not significantly alter MT1 protein expression in the striatum or substantia nigra, suggesting that 6-OHDA has a limited effect on MEL-1A/B receptor protein levels in the striatum.

It is generally believed that 6-OHDA enters the neurons via dopamine transporters and induces oxidative stress by generating reactive oxygen species. This occurs primarily through the non-enzymatic auto-oxidation of 6-OHDA under physiological conditions [26–28], producing toxic byproducts, including quinones, hydrogen peroxide, superoxide radicals, and hydroxyl radicals [29,30]. In this study, the TH-positive cell number in the lesioned side of the SNc in PD rats decreased by approximately 89% and 91% at 2 and 4 weeks, respectively, compared to the lesioned side of the control group. Similarly, TH protein expression in the lesioned striatum of PD rats decreased by approximately 85% and 93% at 2 and 4 weeks, respectively. Notably, at 4 weeks, TH expression in the intact striatum of PD rats was reduced by approximately 19% compared to that in controls. These findings confirm that 6-OHDA-induced damage to midbrain dopaminergic neurons in the SNc intensifies over time, leading to a progressive decline in striatal dopamine levels, possibly affecting the contralateral dopaminergic system. These results are consistent with the findings of Sarre *et al*. [31], who observed that 6-OHDA injection into the MFB led to a complete loss of striatal dopamine within 3 weeks, with a gradual loss of dopaminergic neurons in the SNc. By week 5, approximately 90% of these neurons were lost, indicating that neurodegeneration continues even after the initial stabilization of striatal dopamine denervation [31]. Following MLT receptor agonist administration, the number of TH-positive cells in the lesioned SNc of PD rats increased by approximately 53% and 77% at 2 and 4 weeks, respectively, compared to untreated PD rats. Although TH protein expression in the lesioned striatum remained unchanged at 2 weeks, it increased by approximately 81% at 4 weeks post-treatment, suggesting that prolonged agonist administration is required to attenuate the decline in striatal dopamine levels. These findings align with those of Carriere *et al*. [32], who reported that chronic low-dose MLT administration preserved the structural integrity of the nigrostriatal pathway and exerted neuroprotective effects on dopaminergic neurons [33]. Brain-derived neurotrophic factor (BDNF) has been demonstrated to confer neuroprotective effects in PD animal models [34]. Lu *et al*. [35] also identified that AG increased the BDNF-positive neuron number in a depression model, suggesting that the neuroprotective effect of AG observed in this study may be mediated, at least in part, through BDNF expression upregulation. However, in agonist-treated PD rats, TH protein expression in the intact striatum was approximately 27% lower than that in the corresponding region in agonist-treated control rats. This may be due to compensatory increases in endogenous MLT levels on the unlesioned side following 6-OHDA-induced damage, which may be insufficient to confer protective effects on the dopaminergic neurons. Furthermore, Günaydın *et al*. [36] reported that daily administration of AG (40 mg/kg) for 18 days exacerbated rotenone-induced neu-

rotoxicity in PD rats by increasing caspase-3 expression, oxidative protein damage, and apoptosis. Therefore, it is speculated that TH protein loss in the intact striatum after 4 weeks of agonist treatment may be associated with compensatory changes in MLT homeostasis in the brain.

In PD rats treated with the MLT receptor antagonist, the number of TH-positive cells in the lesioned SNc increased by approximately 39% and 64% in 2 and 4 weeks, respectively, compared to untreated PD rats. TH protein expression in the striatum revealed a non-significant change after 2 weeks of treatment. However, it increased by approximately 76% in the lesioned striatum at 4 weeks, suggesting that prolonged antagonist administration may also help prevent the decline in striatal dopamine levels. LU, a non-selective MLT receptor antagonist, has been revealed in various studies to inhibit MLT-induced effects. For example, it reduces the stimulation of MLT of lipid metabolism in mouse fibroblasts [37]. It suppresses MLT-induced expression of Cu/Zn-superoxide dismutase and glutathione reductase in human corneal fibroblasts [10]. It also blocks the ability of MLT to downregulate nestin, phosphorylated c-Myc (S62), and c-Myc expression [38] and significantly inhibits MLT-induced enhancement of BKCa channel activity [12]. As previously mentioned, MLT levels tend to increase as Parkinson's disease progresses [39–41]. LU, by inhibiting MLT activity, may serve as an important modulator and could indirectly exert a protective effect on dopaminergic neurons.

This study observed that, at 2 weeks post-modeling, serum MLT levels in PD rats were positively correlated with the number of APO-induced rotations. After 2 weeks of treatment with the MLT receptor agonist, this positive correlation was further enhanced, whereas treatment with the antagonist for 2 weeks abolished the correlation. At 4 weeks post-modeling, a significant positive correlation was observed between serum MLT levels and APO-induced rotations in PD rats, suggesting that MLT levels continue to increase with disease progression, accompanied by worsening motor symptoms [24]. After 4 weeks of agonist administration, although serum MLT levels in PD rats increased, motor symptoms were markedly alleviated, and the correlation between the two variables disappeared. After 4 weeks of antagonist treatment, serum MLT levels decreased, motor symptoms were partially relieved, and the correlation between the two measures was eliminated. These findings indicate that prolonged administration of either an agonist or antagonist can disrupt the correlation between MLT levels and APO-induced rotational behavior.

This study identified that 2 weeks after modeling, serum MLT levels in PD rats were negatively correlated with the TH-positive cell number in the SNc. Treatment with the MLT receptor agonist for 2 weeks further enhanced this negative correlation, whereas treatment with the antagonist abolished this correlation. At 4 weeks post-modeling, serum MLT levels in PD rats were significantly negatively correlated with TH-positive cell counts in the SNc, indicating that MLT levels progressively increased over time as dopaminergic neurons further declined [42]. After 4 weeks of treatment with the agonist, MLT levels in PD rats increased; however, the dopaminergic neuron number also significantly increased, and the previously observed correlation disappeared. Similarly, 4 weeks of antagonist treatment resulted in decreased MLT levels along with an increase in dopaminergic neurons, suggesting that prolonged administration of either agonists or antagonists can disrupt the correlation between MLT levels and dopaminergic neuron counts.

5. Conclusions

The MLT receptor agonist AG ameliorates motor deficits in PD model rats and exerts neuroprotective effects on dopaminergic neurons in the SNc. The robust agonistic activity of MLT receptors elevates the circulating MLT levels. The putative mechanisms involve both direct activation of MT1/MT2 receptors (enhancing melatonergic signaling) and indirect modulation of dopaminergic tone via antagonism of the 5-HT2C receptors. Chronic administration may further augment endogenous MLT production and upregulate receptor expression, thereby strengthening antioxidant defenses, inhibiting apoptotic cascades, and potentially enhancing neurotrophic factor signaling (for example, BDNF). Experimental evidence, including behavioral recovery after 3–4 weeks of treatment, increased TH-positive neurons in the SNc, elevated striatal TH expression, and higher serum MLT concentrations, supports the notion that chronic receptor modulation and neurotrophic/antioxidant mechanisms are the basis for dopaminergic neuroprotection.

The MLT receptor antagonist LU also exerts indirect neuroprotective effects on dopaminergic neurons, albeit through a distinct and seemingly paradoxical mechanism. However, LU displays potent antagonism against MT1/MT2 receptors, thereby acutely reducing MLT levels, and prolonged administration in this study unexpectedly improved motor symptoms and enhanced TH expression. This paradox may be explained by the dual role of melatonergic signaling. Under physiological conditions, enhancing MLT activity promotes antioxidative and neurotrophic pathways, whereas under conditions of pathological overactivation, an excessive melatonergic drive may exacerbate excitatory imbalance and oxidative stress. In this context, LU-mediated antagonism may act as a compensatory mechanism to restore striatal glutamate/GABA homeostasis and mitigate excitotoxic stress. Previous reports, together with our data, support this interpretation, exhibiting a positive correlation between MLT and excitatory amino acid activity and a negative correlation with GABAergic tone. Moreover, LU is known to exert pleiotropic effects across tissues, including the regulation of antioxidant enzyme expression and ion channel activity, which may further contribute to its

neuroprotective action independent of MLT receptor signaling. Therefore, agonism and antagonism of MLT receptors may converge toward neuroprotection, albeit through fundamentally different regulatory mechanisms.

This study has certain limitations. The 6-OHDA lesion model does not fully recapitulate the pathological complexity of human PD, particularly with respect to non-motor manifestations and subtle histopathological alterations. Moreover, the 6-OHDA model primarily targets dopaminergic neurons in the nigrostriatal pathway; however, it fails to reproduce the hallmark features of PD, including α-synuclein aggregation and neuroinflammation. Therefore, future studies should consider complementary PD models, including the α-synuclein pre-formed fibril paradigm, to enhance translational relevance and capture broader aspects of disease pathology.

Availability of Data and Materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Author Contributions

LL and LH contributed to the study conception and design. Material preparation, data collection and analysis were performed by PL, RL, WL and JL. The first draft of the manuscript was written by RL and JL. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was conducted following the Guidelines for the Care and Use of Laboratory Animals in China and was approved by the Animal Ethics Committee of Fujian Medical University (Approval No. 2015-26).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN45522.

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