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Ginkgo biloba Extract Improves Dendritic Spine Injury in Cerebellar Purkinje Cells Induced by MPTP in Mice by Regulating the PLK2-SPAR Pathway

Yilin Lyu[1,2] | Yumei Zhang[1]

[1]College of Basic Medical Science, Shenyang Medical College, Shenyang, Liaoning, China | [2]Faku County Central Hospital, Shenyang, Liaoning, China

Correspondence: Yumei Zhang (junmeiyijia2003@symc.edu.cn)

Received: 3 July 2024 | **Revised:** 7 February 2025 | **Accepted:** 17 February 2025

Funding: This work was supported by the Basic scientific research project of colleges and universities of Liaoning Provincial Department of Education (Grant/Award LJKZ1147).

Keywords: dendritic spine | *Ginkgo biloba* extract | Parkinson's disease | polo-like kinase 2 | Purkinje cells | spine-associated Rap guanosine triphosphatase activating protein

ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disease, and, currently, there is no cure for patients with PD. Studies have shown that *Ginkgo biloba* extract (EGb) has good neuroprotective effects against PD. The cerebellum is widely involved in cognitive function and may be related to the regulation of static tremors in PD. However, research on the corresponding microstructures is limited. Purkinje cells (PCs) are the only efferent neurons present in the cerebellum, and dendritic spines in PCs are considered the key structures for transmitting neuronal excitatory signals. When neurons are activated, polo-like kinase 2 (PLK2) is expressed, leading to the degradation of spine-associated Rap guanosine triphosphatase activating protein (SPAR) and, ultimately, the loss of postsynaptic density protein 95 (PSD-95), causing changes in the morphology or quantity of dendritic spines. This raises the question of whether the neuroprotective effect of EGb involves the PLK2-SPAR pathway. In this study, we used 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to establish a mouse model of dopamine neuronal injury. Golgi staining was performed to observe the dendritic spine changes. Immunohistochemistry was used to detect the expression of PLK2, SPAR, and PSD-95. The results showed that EGb improves MPTP-induced behavioral changes, dopamine neuronal injury, and dendritic spine damage in mice. In addition, EGb reversed the changes in PLK2, SPAR, and PSD-95 expressions caused by MPTP, revealing the potential mechanism by which EGb improves the condition of patients with PD.

1 | Introduction

Parkinson's disease (PD) is a common neurodegenerative disease characterized by the loss of dopaminergic cells in the substantia nigra (SN) (Tizabi et al. 2021). Currently, there are no effective drugs for the treatment of PD in clinical practice. Therefore, research on the pathology and treatment of PD is particularly important and urgent. Owing to the loss of dopamine in the striatum, the pathophysiological mechanisms and treatment of PD are primarily focused on the basal ganglia–thalamus–cortex pathway, whereas the cerebellum is often overlooked. However, the degeneration of basal ganglia neurons cannot fully explain the widespread motor and non-motor symptoms, suggesting that other brain regions may be involved. The cerebellum plays a crucial role in motor control and learning as well as in many non-motor processes such as cognition (Schmahmann 1991; Kim et al. 1994). Multiple studies have confirmed that the cerebellum and basal ganglia are involved in the development of a wide

range of motor and non-motor symptoms in PD (Bostan and Strick 2010; Wu and Hallett 2013). Overall, increasing evidence supports the crucial role of the cerebellum in PD (Solstrand Dahlberg et al. 2020; Burciu et al. 2017). Although cerebellar volume accounts for only 10% of the entire brain, it contains 80% of the neurons (Sereno et al. 2020). Purkinje cells (PCs) are unique efferent neurons in the cerebellar cortex and have numerous spines on their dendrites (Zecevic and Rakic 1976). PCs play an important role in daily motor activities, and the morphological dysfunction of PCs directly leads to cerebellar problems (Huang and Verbeek 2019; Tsai et al. 2012). However, research on changes in the dendritic spines of PCs in patients with PD is limited. Revealing these changes and their mechanisms may provide new targets for PD treatment. Spine-associated Rap guanosine triphosphatase activating protein (SPAR) is located in the postsynaptic density (PSD) of dendritic spines and forms a complex with molecules such as PSD protein 95 (PSD-95). It is closely associated with the maturation and maintenance of dendritic spines. Recently, Pak and Sheng (2003) found that when neurons are activated, the induced production of polo-like kinase 2 (PLK2) can phosphorylate and degrade SPAR, ultimately leading to the instability of dendritic spine structures and forming the PLK2-SPAR signaling pathway. However, it is not yet known whether this pathway plays a role in dendritic spine changes in cerebellar PCs. *Ginkgo biloba* extract (EGb) is a mixture of various effective medicinal components extracted from the dried leaves of *G. biloba* (Zang et al. 2023). EGb has a long history of medicinal use in China, and its medicinal value is increasingly appreciated. Previous studies have shown that EGb can improve the cognitive, learning, and mnemonic abilities of humans and animals (Hoffman et al. 2004), as well as affect synaptic remodeling in the hippocampus of the central nervous system (Kondratskaya et al. 2004). Increasing evidence has confirmed that EGb imparts significant neuroprotective effects against cerebral stroke, PD, and senile dementia by attenuating structural brain damage and neurological deficiencies (Christen 2004; Ji et al. 2020; Nishimon et al. 2020), but its mechanism of action is complex and still not fully understood. To date, no animal model has fully recreated all pathological hallmarks of PD. Currently, the model that most closely mimics PD is the pharmacological administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to nonhuman primates, with the MPTP mouse model being the most frequently used. Several mouse strains exhibit varying sensitivities to MPTP. For example, C57BL/6 mice are the most sensitive, whereas BALB/c mice are less vulnerable to MPTP neurotoxicity (Filipov et al. 2009). Among the MPTP administration regimens, the acute MPTP model appears to generate the most reliable nigrostriatal damage (Santoro et al. 2023; Smeyne and Jackson-Lewis 2005). Therefore, the acute MPTP model is considered a suitable platform for screening neuroprotective compounds (Santoro et al. 2023). In this study, we used an acute MPTP mouse model to observe the protective effects of EGb on behavioral tests, the number of tyrosine hydroxylase (TH)–positive neurons in the SN, dendritic spine density of PCs, and expression levels of PLK2, SPAR, and PSD-95 in the cerebellum. This study aimed to explore the mechanism of EGb in treating PD by protecting the dendritic spines of PCs in the cerebellum and to provide a scientific basis for the development of natural drugs with anti-PD effects.

2 | Materials and Methods

2.1 | Experimental Design

A total of 18 male C57BL/6 mice aged 8 weeks and weighing approximately 20 g were sourced from Liaoning Changsheng Biotechnology Co. Ltd. (License: SCXK [Liaoning] 2020-0001). The mice were randomly divided into three groups as follows: normal control group (NC; $n = 6$), MPTP treatment group (MPTP; $n = 6$), and MPTP + EGb treatment group (EGb; $n = 6$).

2.2 | Ethical Clearance

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Shenyang Medical College (approval no.: SYYXY 2021092902).

2.3 | Animal Experiments

MPTP (GlpBio, Shanghai, China) was dissolved in saline and injected intraperitoneally at a dose of 25 mg/kg every 2 h over a period of 1 day for a total of four injections (Santoro et al. 2023; Weerasinghe-Mudiyanselage et al. 2021). All mice in the NC group were injected with the same dose of saline, and those in the EGb group were intraperitoneally injected with 40 mg/kg of EGb (Solarbio, Beijing, China) within 30 min after the first administration of MPTP and then once a day for a total of eight doses. EGb was also dissolved in physiological saline, and the pH was adjusted to 7.4. The doses of EGb were determined from previous studies (Pan et al. 2020; Guan et al. 2018; Zhu et al. 2016; Rojas et al. 2008).

2.4 | Behavioral Experiments

Animal behavioral tests were conducted 1, 3, and 8 days after the last injection of MPTP or saline. The swim test was performed as previously described by Donnan et al. (1987). The scoring criteria were as follows: Mice who could swim continuously for 1 min scored 3.0 points; those who could swim for the most of the duration of the test and floated occasionally scored 2.5 points; those with a floating time accounting for more than 50% of the entire test time scored 2.0 points; occasional swimmers scored 1.5 points; and those who occasionally swam with their hind legs and remained afloat on the side of the water tank scored 1.0 point. The interval between each of the three tests was 1 min, and we considered the average value of the three tests for further analysis. Traction behavior tests were performed according to the approach described by Kuribara et al. (1977). The scores were assigned according to the following criteria: within 10 s, those who used two hind limbs to grab a metal pole scored 3 points, those who used one hind limb to grab a metal pole scored 2 points, those who used two limbs that did not grab a metal pole but did not fall scored 1 point, and those who fell scored 0 points. The interval between each test was 1 min, and the average value of three tests was considered.

2.5 | Tissue Sampling

After anesthetization via an intraperitoneal injection of chloral hydrate (350 mg/kg), the brain tissues of the mouse were removed and separated from the midline. One side was fixed with 4% paraformaldehyde and subjected to immunohistochemistry (IHC) testing. The other side was prepared for Golgi staining. Sampling was performed after the last behavioral experiment.

2.6 | Golgi Staining

Brain slices were obtained by cutting 100 μm thick sections using a microtome (LEICA CM 1950). Golgi staining was performed according to the manufacturer's instructions (Hito Golgi-Cox OptimStain Kit). Briefly, the brain tissue was rinsed using double-distilled water for 2–3 s to remove blood from the surface. The tissue was transferred into a mixed impregnation solution and stored at room temperature for 2 weeks in a dark room. The tissue was then transferred into Solution-3 and stored at 4°C for 2–3 days in a dark room. The tissues were embedded in low-gelling temperature agarose. The sections were cut and mounted onto gelatin-coated slides, and a few drops of Solution-3 were added to the mounted sections. The slides were air-dried overnight at room temperature in the dark and rinsed twice with distilled water two times for 3 min each. Next, 2 mL of Solution-4, 2 mL of Solution-5, and 6 mL of double-distilled water were mixed in a 12 mL staining jar, and the slides were placed in the solution mixture. The staining jar was closed tightly, and the slides were stored for 10 min. The slides were rinsed in distilled water twice for 4 min each and dehydrated in 50%, 75%, and 95% ethanol for 5 min each. The slides were again dehydrated in 100% ethanol three times for 5 min each. The slides were placed in xylene twice for 5 min each, and a coverslip was placed over the sections using an undiluted xylene-based resinous mounting medium. The slides were viewed after drying using bright-field microscopy.

2.7 | Dendritic Spine Analysis

The sections were observed under a 1000× objective (LEICA DM4 B) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Six impregnated and clearly visible cerebellar PCs were studied from each hemisphere, and three distal dendritic branchlets in each of the six cells studied per mouse were analyzed. Visually identified dendritic spines along the distal dendrites over 10 μm in length were counted. Spine density was calculated by dividing the number of spines on a segment by the length of the segment (Verslegers et al. 2015; González-Tapia et al. 2015).

2.8 | Immunohistochemistry

The brain hemispheres were fixed in 4% paraformaldehyde for more than 1 week and then dehydrated with 20% and 30% sucrose until the tissue sank to the bottom. Next, the specimens were cut into 25 μm thick brain slices and stained according to conventional methods. The main steps were as follows: (1) After antigen thermal repair, 0.3% of Triton-X 100 solution was added to increase cell membrane permeability; 3% of H_2O_2 was added dropwise to block endogenous peroxidase, and a normal goat serum working solution was gradually added dropwise to prevent nonspecific staining. (2) Preparation of the rabbit primary antibody: TH ([1:200]; Abcepta company, AP60061), PLK2 ([1:300], Abcepta Corporation, AP7252a), SPAR ([1:300], Proteintech company), 25086-1-AP, PSD-95 ([1:300], Abmart company), and T55407M were added to the brain slices (300 μL) at the prescribed dilution ratio and stored overnight at 4°C. The anti-rabbit secondary antibody (Elabscience, E-IR-R215) was added dropwise, and the slices were incubated at room temperature for 1 h. Subsequently, 3,3′-diaminobenzidine staining was performed. The brain slices were transferred onto gelatin-coated glass slides and dehydrated with a series of alcohol solutions. Neutral gum was added to the brain slice, and the slice was covered with a glass slide. Statistical analysis was performed by cell counting or calculating the average optical density (AOD). For each animal, three sections were used to calculate the AOD. The AOD was calculated after transforming the mean gray level using ImageJ software. This calculation was performed as previously described by Peyvandi Karizbodagh et al. (2021).

2.9 | Stereology

TH-positive SN neurons were counted from a mouse in either group by an optical fractionator as described previously (West 1999). Double-blinded counting was performed manually.

2.10 | Statistical Analysis

Data analysis was performed using GraphPad Prism, version 9.0.0 (GraphPad Software Inc., La Jolla, CA, USA). Student's *t*-tests were performed for intergroup comparisons. Data are presented as means ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

3 | Results

3.1 | EGb Alleviated Dopamine Neuronal Loss in MPTP-Injected Mice

We used IHC to evaluate dopaminergic neurons in the SN of the midbrain (Figure 1A), calculate the number of TH-positive neurons (relative to the NC group; MPTP: 0.5190 ± 0.0087 and EGb: 0.9948 ± 0.0209), and conduct intergroup comparative analyses. This analysis showed that the number of TH-positive neurons in the MPTP group was significantly lower than that in the NC group ($p < 0.0001$). However, this value was significantly higher in the EGb group than in the MPTP group ($p < 0.0001$; Figure 1B). This indicates that EGb ameliorates acute MPTP-induced damage to dopaminergic neurons in the SN of mice.

3.2 | EGb Improved Behavioral Deficits in MPTP-Injected Mice

Traction behavior and swimming tests were conducted to determine the behavioral impairments associated with PD in mice (Figure 1C,D). MPTP treatment significantly affected the scores

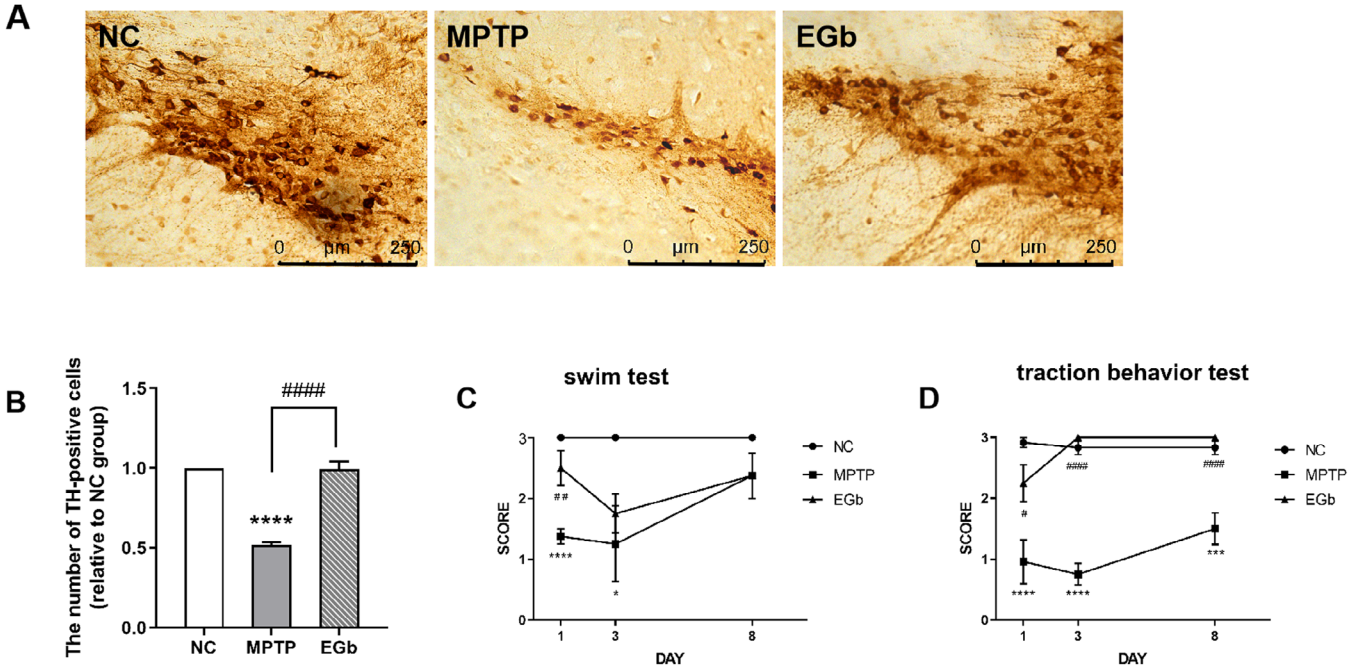

FIGURE 1 | EGb improves dopaminergic neuronal loss in the substantia nigra and behavioral deficits in MPTP-injected mice. (A and B) Brain sections immunostained for TH immunoreactivity in the substantia nigra, and the number of TH-positive cells is quantified. Scale bar = 250 μm. (C) Swimming test results of each group. (D) Tractional behavior test results of each group. Data are expressed as means ± the standard error of mean, $n = 6$; ****$p < 0.0001$, ***$p < 0.001$, and *$p < 0.05$ versus the NC group; ####$p < 0.0001$, ##$p < 0.01$, and #$p < 0.05$ versus the MPTP group. EGb, *Ginkgo biloba* extract; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NC, normal control; TH, tyrosine hydroxylase.

of the experimental animals, and EGb reversed this behavioral damage. Although the behavior of mice in the MPTP group improved over time, the differences between the groups were still statistically significant. Specifically, in the traction behavior test, EGB significantly improved the limb activity in mice.

3.3 | EGb Reversed the Decrease in Dendritic Spine Density Caused by MPTP in PCs

Golgi staining was performed to evaluate the density of dendritic spines (Figure 2A). The statistical results showed that the density of dendritic spines in the MPTP-treated group was lower than that in the NC group ($p < 0.0001$). However, EGb ameliorated the decrease in dendritic spine density caused by MPTP. The differences between MPTP and EGb groups are statistically significant ($p < 0.0001$; NC: 1.4270 ± 0.0351/μm, MPTP: 0.8101 ± 0.0551/μm, and EGb: 1.4250 ± 0.0276/μm; Figure 2B).

3.4 | EGb Reduced MPTP-Induced Increase in PLK2 Expression in Cerebellar PCs

The expression of PLK2 in cerebellar PCs was analyzed using AOD values (NC: 0.3508 ± 0.0115, MPTP: 0.5560 ± 0.0056, and EGb: 0.3247 ± 0.0028). The MPTP group showed increased PLK2 expression compared with that in the NC group ($p < 0.0001$), and EGb reversed MPTP-induced changes in PLK2 expression ($p < 0.0001$; Figure 3A,B).

3.5 | EGb Increased MPTP-Induced Decrease in SPAR Expression in Cerebellar PCs

As shown by IHC (Figure 3C,D), MPTP treatment markedly reduced SPAR expression in cerebellar PCs compared with that in the NC group ($p = 0.0011$). However, EGb increased MPTP-induced decrease in SPAR expression ($p = 0.0042$; NC: 0.5332 ± 0.0257, MPTP: 0.4107 ± 0.0079, and EGb: 0.5395 ± 0.0339).

3.6 | EGb Increased MPTP-Induced Decrease in PSD-95 Expression in Cerebellar PCs

The MPTP group exhibited reduced PSD-95 expression in cerebellar PCs compared with the NC group (Figure 3E,F; $p = 0.0003$). Consistent with the impact on SPAR, EGb increased the MPTP-induced decrease in PSD-95 expression ($p = 0.0003$; NC: 0.6233 ± 0.0179, MPTP: 0.4947 ± 0.00147, and EGb: 0.6243 ± 0.0194).

4 | Discussion

In this study, we used an acute MPTP mouse model to observe the protective effects of EGb on behavioral tests, dopaminergic neurons in the SN, and dendritic spines of cerebellar PCs. The results revealed that EGb improved dopaminergic cell loss in the SN and reduced behavioral impairment. TH-positive cells, as determined by IHC, can indicate damage to or repair of dopaminergic neurons. MPTP decreased the number of TH-positive neurons,

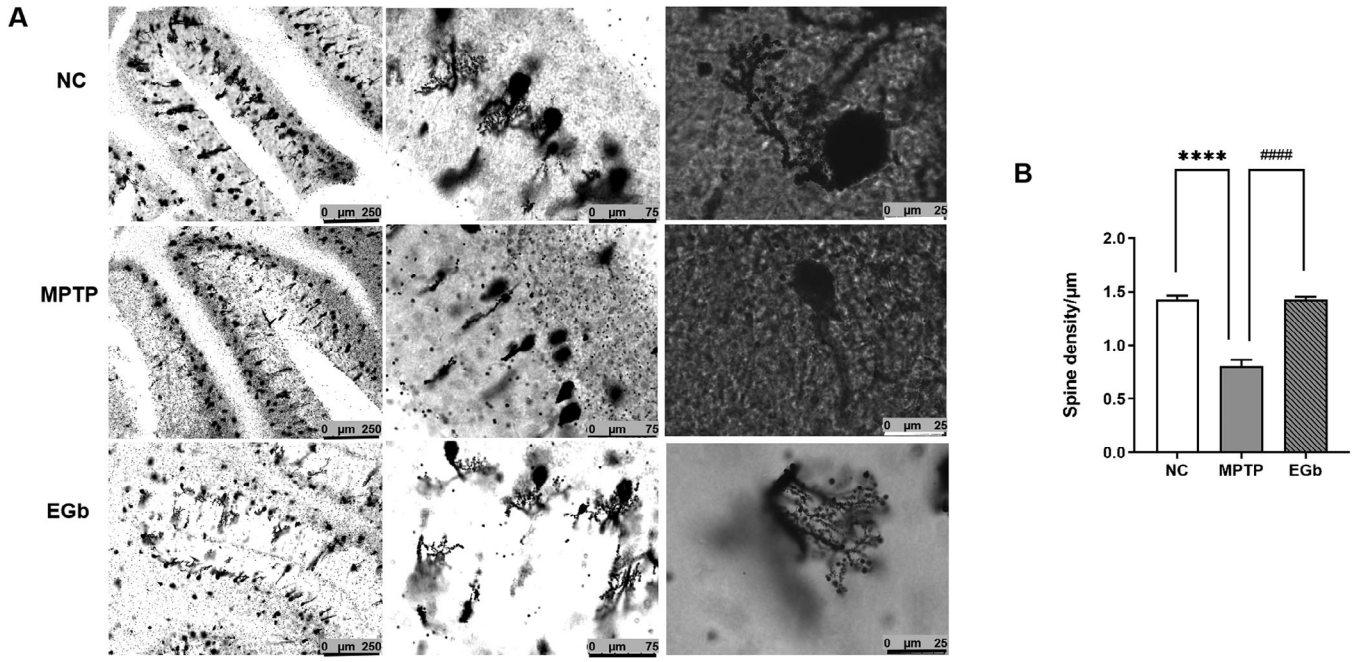

FIGURE 2 | EGb increases the number of dendritic spines in MPTP-treated cerebellar Purkinje cells. (A) Representative Golgi staining images of dendritic spines in each group. The scale bar represents 250, 75, and 25 μm, respectively. (B) Quantitative analysis of the dendritic spine density in Purkinje cells. Data are expressed as means ± the standard error of mean, $n = 6$; ****$p < 0.0001$ versus the NC group; ####$p < 0.0001$ versus the MPTP group. EGb, *Ginkgo biloba* extract; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NC, normal control; TH, tyrosine hydroxylase.

whereas EGb increased their number. The scores of both the traction behavior and swimming tests significantly decreased 1 day after MPTP injection. EGb reversed behavioral impairments compared with those observed in the MPTP group. Although the behavior of mice in the MPTP group improved over time, EGb still showed good protective effect against MPTP-induced impairments. Takada et al. (1993) confirmed that MPTP is also toxic to cerebellar PCs in mice. We found that MPTP decreased dendritic spine density in cerebellar PCs, whereas EGb reversed this MPTP-induced decline.

Collectively, our findings correspond to previous studies and anticipation (Takada et al. 1993; Weerasinghe-Mudiyanselage et al. 2021). We have not only successfully established the PD model in mice but also confirmed that EGb has a protective effect on animal behavior, dopaminergic neurons in the SN, and dendritic spines of cerebellar PCs.

Although PD pathology predominantly involves the SN–striatum circuit. Accumulated evidence suggests that pathological changes extend beyond the basal ganglia into other parts of the brain, including the cerebellum (Li et al. 2023). Over the past decade, studies have shown that the cerebellum is widely involved in cognitive function, may be related to the regulation of resting tremors, and plays an important compensatory role in motor dysfunction in patients with PD (Bugalho et al. 2006; Buckner 2013; Zhong et al. 2022). PCs, as one of the main types of neurons in the cerebellum, play a dominant role in the cerebellar circuitry (Jiang et al. 2013). Heman et al. (2012) confirmed a persistent activation of PCs in the cerebellum correlated with dopaminergic degeneration. However, current research on the corresponding microstructure of the PCs is limited. A better understanding of the functional and morphological changes in the cerebellum associated with PD will be of great significance for understanding the pathophysiology of PD and may help develop new treatment strategies and targets. The findings of the present study indicated that acute MPTP treatment could alter dendritic spine density in cerebellar PCs. Herein, our findings provided evidence for the association between cerebellum and PD. Moreover, the protective effect of EGb on dendritic spines of cerebellar PCs suggested that EGb had the potential to become one of the drugs for the treatment of PD.

Dendritic spines are the main sites of excitatory synaptic transmission in neurons and are specialized structures for synaptic transmission. Abnormal changes in the number and morphology of dendritic spines are considered fundamental pathological features of various brain diseases (Blanpied and Ehlers 2004; Fiala et al. 2002). Owing to the complexity of dendritic spines and the specificity of their functions, they have received increasing attention in the field of neuroscience. Understanding the molecular mechanisms underlying the maintenance of spinal morphology will contribute to advancing research on the prevention and treatment of central nervous system diseases. The PLK2-SPAR pathway, a recently discovered signaling pathway that exists within dendritic spines, may participate in the central nervous system's damage through excitotoxicity and repair processes to a certain or greater extent by regulating the stability of dendritic spines (Pak and Sheng 2003). Our research on the dendritic spines of striatal medium spiny neurons supports this finding (Su et al. 2022). PSD-95 is an important excitatory postsynaptic scaffold protein that is involved in dendritic spine growth and synaptic function (Kim and Sheng 2004). With the degradation of SPAR, the expression of PSD-95 also decreases, which, to some extent, leads to a reduction in the number of mature synapses and dendritic spines. Clarifying the mechanism of

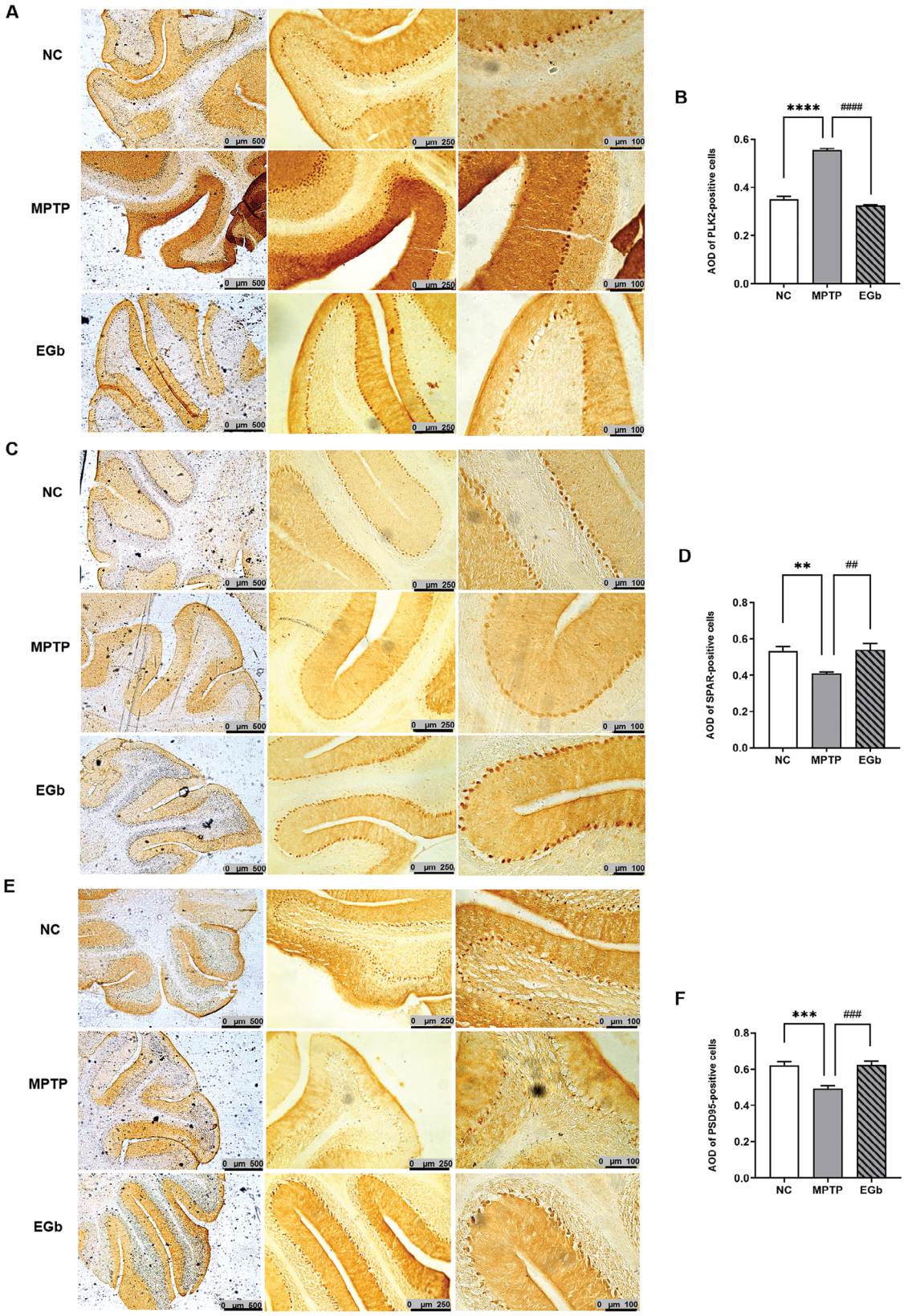

FIGURE 3 | EGb lowers the expression levels of PLK2 but increases the expression of SPAR and PSD-95 in cerebellar Purkinje cells. (A, C, and E) Representative immunohistochemical staining for PLK2, SPAR, and PSD-95 in each group. The scale bar represents 500, 250, and 100 μm, respectively. (B, D, and F) Quantification of the AOD of PLK2-, SPAR-, and PSD-95-positive cells in Purkinje cells. Data are expressed as means ± the standard error of mean, $n = 6$; ****$p < 0.0001$, ***$p < 0.001$, and **$p < 0.01$ versus the NC group; ####$p < 0.0001$, ###$p < 0.001$, and ##$p < 0.01$ versus the MPTP group PLK2, polo-like kinase 2. EGb, *Ginkgo biloba* extract; NC, normal control; TH, tyrosine hydroxylase; AOD, average optical density; PSD-95, postsynaptic density protein 95; SPAR, spine-associated Rap guanosine triphosphatase activating protein; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

changes in the dendritic spines of cerebellar PCs may provide new research directions for PD treatment. The discoveries of this study are consistent with those of previous studies (Pak and Sheng 2003). The AOD analysis results showed that MPTP upregulated the expression of SNK, and SPAR expression was downregulated in cerebellar PCs. EGb reversed this effect by decreasing and increasing the expressions of PLK2 and SPAR, respectively. Furthermore, EGb increased the content of PSD-95 in cerebellar PCs. These results indicate that EGb can have a significant protective effect on cytoskeletal proteins in dendritic spines by intervening in the PLK2-SPAR pathway after synaptic activation, thereby maintaining the stability of dendritic spines in PCs and exerting neuroprotective effects. In conclusion, the natural drug extract EGb showed some degree of protection against damage to behavior and dopaminergic neurons in the SN in MPTP model mice. In addition, we observed the changes in the dendritic spines of cerebellar PCs destroyed by MPTP using Golgi staining and found that EGb can improve this injury by regulating the PLK2-SPAR pathway. However, owing to the complexity of the components of EGb, the exact components and sites of intervention require further in-depth research to confirm this hypothesis.

Author Contributions

Yilin Lyu: acquisition of data, analysis of data, drafting the manuscript. **Yumei Zhang**: conception and design of the study, revising the manuscript critically for important intellectual content.

Acknowledgments

This work was supported by the Basic scientific research project of colleges and universities of Liaoning Provincial Department of Education, Grant/Award Number LJKZ1147.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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