

Gastrodin ameliorated features of MPTP-induced Parkinson's disease via activating VMAT2 maintained dopamine homeostasis

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Research Article

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Abstract

The vesicular monoamine transporter (VMAT2) plays a crucial role in maintaining dopamine (DA) homeostasis through packaged DA into vesicles, which has been suggested to being an excellent marker for presynaptic dopaminergic nerve terminals in the nigrostriatal of Parkinson's disease. Gastrodin (GTD), the major bioactive compound of *Gastrodia elata*, has shown neuroprotective in animal models for many neurological disorders. However, it is unclear whether GTD confers neuroprotection via activating VMAT2 to maintain DA homeostasis in an animal model of Parkinson's disease (PD) using the DA neuron-specific toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Here, we first identified that GTD could ameliorate cell damage and the dysfunction of DA homeostasis in PC12 cells induced by MPTP via up-regulating VMAT2 expression. Moreover, GTD could enhance VMAT2 protein expression, increase striatal vesicle volume, and ameliorate DA dysregulation in MPTP-induced PD mice. Furthermore, we found that the DA homeostasis and therapeutic effect of GTD could be reversed by the VMAT2 inhibitor in vitro and in vivo. Finally, we confirmed that GTD could increase VMAT2 expression by activating MEK/ERK pathway. In summary, our data showed that GTD attenuated MPTP neurotoxicity through activating of the MEK/ERK/VMAT2 signaling pathway maintained DA homeostasis, suggesting that the manipulation of VMAT2 by GTD may provide a potential therapeutic strategy for PD.

Introduction

Parkinson's disease (PD) is the second common progressive neurodegenerative disease characterized by the classical motor features of parkinsonism associated with loss of dopaminergic neurons and Lewy bodies in the nigrostriatal pathway[1]. Most currently available drugs for PD relieve symptoms, but their effects are limited and they do not address disease progression. Patients with early PD characteristically experience a remarkable benefit of levodopa manifesting a well-characterized role in incentivizing physically effortful behavior, unfortunately, this situation is not everlasting for the pathological process of PD is not delayed[2]. Hence, novel therapies with higher efficacy and fewer adverse effects are urgently needed.

It is widely known that the dysfunction of DA homeostasis plays an important role in the pathogenesis of PD[3]. DA, a neurotransmitter regulating motor function, can accumulate in the cytoplasm when the synaptic vesicle recycling of DA is disturbed, generating toxicity to dopaminergic neurons through metabolism and oxidation[4]. DA vesicle storage is an important physiological process of dopaminergic neurons, which is closely related to the loss of dopaminergic neurons in nigrostriatal dopaminergic[5]. Dysfunction of DA vesicle storage can result in abnormal DA metabolism, which is involved in many pathogenesis mechanisms of PD, such as abnormal protein accumulation and phosphorylation, mitochondrial dysfunction, and oxidative damage[6]. The vesicular monoamine transporter 2 (VMAT2), a H⁺-ATPase reverses transport protein, maintains DA homeostasis through accumulating and compartmentalizing DA with the DA transporter. VMAT2 can package DA into small synapses and dense core vesicles subsequent release from neurons, thus isolating the toxicity of DA to neurons[7]. The study has detected a loss in striatal VMAT2 binding in the PD brain with in vivo imaging and postmortem

binding[8]. Aging-induced reductions in locomotion are more pronounced in heterozygous VMAT2 knockout (KO) mice, which indicates VMAT2 is closely related to age-related changes in dopaminergic function[9]. Many environmental toxins, such as MPTP, can also cause the dysfunction of DA homeostasis and the loss of VMAT2[10]. Interestingly, VMAT2 overexpressed mice can increase vesicle capacity, improve the prognosis of anxiety-like and depression-like behavior, and reduce MPTP toxic damage to mice[11, 12], which indicated VMAT2 is a potentially effective target for the treatment of PD.

Gastrodin (GTD) is the key bioactive compound of Tianma (*Gastrodia elata* Blume) with many bioactivities such as anti-oxidative, anti-inflammatory, modulating neurotransmitters, and suppressing microglial activation, etc[13]. GTD confers neuroprotection on dopaminergic neurons in PD animal models or clinical trials and serves as a multifaceted player in several signaling pathways[13–15]. However, it is still unclear whether GTD can relieve dysregulation of VMAT2 transporter to maintain DA homeostasis, thus helping the symptoms and pathology of PD. This study investigated whether and how GTD regulates the dysregulation of the VMAT2 transporter to maintain DA homeostasis for ameliorating the features of MPTP-induced PD in vitro and in vivo (Fig. 1).

Materials And Methods

Chemicals and Reagents

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, S0620A) was purchased from Sigma-Aldrich (MO, USA). Gastrodin (GTD, HG201547198) and Reserpine (Res, HR2013W1) were purchased from Baoji Chenguang Biological Technology Co. Ltd (Xian, CHN). 3-Hydroxytyramine hydrochloride (AF9120701) and dihydroxy-phenyl acetic acid (DOPAC, AF20112402) were furnished by Chengdu Alfa biotechnology Co. Ltd (Chengdu, CHN). Anti-VMAT2 (D1219) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-TH (GR3219792-1), α -syn (GR3191908-8) antibodies were purchased from Abcam (Cambridge, UK). Anti-MEK (9126S), ERK (9107S), p-MEK (9154S), and p-ERK (14227S) antibodies were purchased from Cell Signaling Technology (Danvers, USA). Anti-bax (00073304) and bcl-2 (00075461) antibodies were purchased from Wuhan sanying biotechnology co. Ltd (Wuhan, CHN). Anti-GAPDH (LS191936) was purchased from Xavier Biotech Co., Ltd (Wuhan, CHN). GeneJET RNA purification Kit and RevertAid RT Reverse Transcription Kit were obtained from Thermo Fisher Scientific (Waltham, USA). Real Time PCR EasyTM-SYBR Green I was furnished by Chengdu Fuji Biotechnology Co. Ltd (Chengdu, CHN). The specific pairs of primers used for PCR were purchased from Sangon Biotechnology (Shanghai, CHN).

Cell culture

PC12 cells were obtained from the BLUEF Biotechnology Co., Ltd (Shanghai, China). The protocol of PC12 cell culture was according to the literature[16]. Briefly, PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) and incubated at 37°C in a humidified atmosphere containing 5% CO₂ incubator (Thermo Fisher Scientific Co., Ltd., USA).

Concentration selection of MPTP and GTD

PC12 cells were seeded into 96-well plates for 12 hours, and then treated with 100 μ L DMEM medium containing MPTP (500, 750, 1000, and 1250 μ M) for 12, 24, 36, and 48 hours, respectively. CCK-8 was used to detect cell viability, and the optimal MPTP modeling concentration and time were selected for subsequent experiments. In the same way, the different concentrations (0, 3.125, 6.25, 12.5, 25, and 50 μ M) of GTD were added in DMEM medium and monitoring the PC12 cells cell viability in the presence or absence of MPTP. The final decision was to treat with 1 mM MPTP for 24 hours, and the concentrations of Gastrodin were 6.25, 12.5, and 25 μ M.

Cell viability measurements

PC12 cells in the logarithmic growth phase are selected and digested with 0.25% trypsin (Gibco, USA). Then, PC12 cells were seeded into 96-well plates at a density of 6×10^4 cells/ml for 12 hours. The cells were incubated with different concentrations of GTD (25, 12.5, and 6.25 μ M) for 2 hours, and then exposed to 1mM MPTP or 500 nM Res. After 24 hours, 10 μ L of CCK-8 was added to each well, incubated for 4 hours and absorbance was measured at 450 nm.

Animals and Experimental protocol

All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication 80-23, revised 1996) and the Institute of Material Medica Integration and Transformation for Brain Disorders ethics committee approved the animal protocols (Chengdu, China). All efforts were made to minimize animal suffering. C57/BL6 mice (8 weeks, male, 20-22 g) were obtained from the Chongqing Enswell Biotechnology Co., Ltd. Mice were housed 6 per cage in a standard environment with controlled temperature and humidity conditions in the animal observation room (SPF) of Chengdu University of Traditional Chinese Medicine temperature of $22 \pm 2^\circ\text{C}$ and relative humidity of $50 \pm 10\%$. Food and water were available freely. All mice were allowed to acclimatize for 5 d prior to experiments. Doses of GTD and Res were prepared according to our previous study[17, 18]. Animals were randomly divided into the Control group (n=6), MPTP group (20mg/kg, n=6), Medopar group (20mg/kg MPTP + 50mg/kg Medopar, n=6), GTD-H group (20mg/kg MPTP + 30 mg/kg GTD, n=6), GTD-L group (20mg/kg MPTP + 15 mg/kg GTD, n=6), and GTD-H + Res (20mg/kg MPTP + 30 mg/kg GTD + 1mg/kg Res, n=6). All mice except for the control group were intraperitoneally injected by MPTP for 7 days. From day one of MPTP, the mice were orally administered by GTD or Medopar for 15 days, respectively[19]. Meanwhile, mice in Control and MPTP groups were given equal volume normal saline. MPTP and GTD were dissolved in saline and prepared just prior to dosing. All animals were humanely euthanized for further study after administration on the 15th day of GTD.

Behavioral evaluation

In our assay, the motor and non-motor dysfunction of PD model animals were assayed on the 14th day of GTD treatment by the open field test, autonomic behavior test, gait analysis test, pole and rotarod tests.

Open field test: The spontaneous motion of the mice was detected by the open field test. Mice movements were observed with an infrared light photo beam system. Mice were placed in the center of a square arena (47 cm× 47 cm × 40 cm) for 5 min in the open field. Then, the time, distance, and heat map of the movement of mice were analyzed.

Autonomic behavior test: Autonomic behavior test was used to measure the general behavior of animals and to assess behavioral changes in rodents exposed to new environments. The mice were placed in the activity box with a defined central square area of 30 cm × 30 cm. The number of autonomous activities and standing in 5 min was recorded.

Gait analysis test: Gait analysis was conducted as our previous research[20]. In short, the right hind and forelimbs of the mice were stained with different colors of pigment, respectively, they climbed from the opening end, and their gait characteristics were recorded. The stride length of the hindlimb and forelimb were measured separately.

Pole test: The pole test was a method used to detect the coordination ability of mice. The mice were placed head up on the top of a high rough-surfaced pole (55 cm height, 8 mm diameter). The time mice used to completely turn around and face the floor as T-turn and the time to climb down the pole as T-total were recorded. Trails were excluded if the mouse slid down or jumped out the pole. The test was performed three trials for each mouse with an interval time of at least 30 min[21].

Rotarod test: A Rotarod apparatus was used to assess motor coordination and balance. The mice were placed on the Rotarod and the rotating speed was set up as 5 to 40 rpm in 300 s. The latency time of each mouse to fall off the Rotarod was recorded. The mouse was tested three times with an interval of at least 30 min[21].

Detection of DA and DOPAC by HPLC

PC12 cells were seeded into 6-well plates at a density of 1.2×10^5 cells/ml for 12 hours, then incubated with GTD, MPTP and Res for 24 hours. The culture solution, PC12 cells, and striatum tissue homogenate were collected in each group respectively. Then 0.1M perchloric acid was added, centrifuged at $13000 \times g$ at 4 °C for 15 minutes, and filtered through a 0.22 µm micropore film to yield the sample solution for HPLC. The chromatographic separation was performed on a Poroshell 120 EC-C18 column (100 × 4.6 mm, 2.7 µm, Agilent Technologies, CA, USA). The eluent contained 69% 0.005 mol /L sodium lauryl sulfate, 30% acetonitrile-glacial, 0.9% acetic acid, and 0.1% 0.1 mol/L disodium ethylenediaminetetraacetate. The column temperature was maintained at 30 °C, a 20-µL of sample solution was injected for each run with the autosampler temperature at 4 °C and 1.0 ml/min elution, and excitation wavelength of UV detector is 280nm.

Immunocytochemistry

Immunohistochemistry of the paraffin blocks was performed as previously described[22]. The brain slices were dewaxed, rehydrated, blocked, incubated with primary antibodies (anti-VMAT2, 1:400, anti-TH, 1:500)

overnight for 4°C, incubated with secondary antibody, colored with a DAB kit, and analyzed by light microscopy.

Real-time PCR analysis

PC12 cells were seeded into 6-well plates at a density of 1.2×10^5 cells/ml for 12 hours, at the end of the administration, cells from each group were washed with cool PBS and collected. Then, RNA extraction, cDNA synthesis, and real-time PCR were performed according to the manufacturer's instructions. The primer pairs of MEK were 5'-TGCTGGACAACATGCTGCTCAC-3' and 5-GAAGATGCTTTTCGGAGGTGGAG-AC-3'. The primer pairs of ERK were 5-GCTGTCCGCAACCAGATCATCC-3' and 5-TCTCGCCGTCGCTGTAGAAGG-3'. The amplified products were confirmed by running 1.5% agarose gels.

Western blotting analysis

The striatum tissues of mice and collected cells were lysed with lysate, centrifuged, collected supernatant, added with sample buffer, and stored at -80°C. Approximately 50 µg of proteins were separated using either 8% or 15% SDS-PAGE and transferred to PVDF membrane using the wet transfer method. The membranes were blocked with 5% BSA in TBST buffer for 90 min at room temperature prior to exposure to primary antibodies. Membranes were incubated overnight at 4°C with the primary antibodies (anti-TH, 1:1000; anti-α-syn, 1:1000; anti-bcl-2, 1:1000; anti-bax, 1:1000; anti-VMAT2, 1:500; anti-p-MEK, 1:1000; anti-MEK, 1:1000; anti-p-ERK, 1:1000; anti-ERK, 1:1000; anti-GAPDH, 1:5000) and for approximately 4 h with the secondary antibodies at room. HRP-conjugated goat secondary antibodies at 1:5000 dilution were used for staining. Finally, the relative expression of the target proteins was analyzed by the Quantity One software.

Statistical analysis

All data are represented as the mean $\pm$ standard error of the mean (SEM). A comparison of the different groups was performed via one-way analysis of variance (ANOVA). Student's test was performed for comparisons between two groups. And Bonferroni post hoc analysis was conducted, and the statistical significance was tested at $P < 0.05$.

Results

GTD alleviated MPTP-induced neurotoxicity in PC12 cells.

Initially, the optimal concentration of MPTP injured PC12 cells was determined. Treatment with MPTP decreased the cell viability in a dose- and time-dependent manner (Fig. 2A). At 1mM and 24 hr, MPTP stably reduced the cell viability without excessive PC12 cell damaged (Fig. 2A). As such, subsequent experiments used 1mM MPTP for 24 hr as the optimal damage conditions. As showed in Fig. 2B, GTD at the concentration ranging from 3.125 to 50 µM had no obvious effects on the viability of PC12 cells and

the viability of all groups was approximate. Moreover, 1mM MPTP reduced cell viability to about 60% normalized to that in the control group, GTD (50 μ M, 25 μ M, 12.5 μ M, and 6.25 μ M) rescued the cell viability in a dose-dependent manner according to the CCK-8 assays (Fig. 2C and 2D). Furthermore, the expression level of TH, α -syn, bcl-2, and bax protein was detected by Western blot (Fig. 2E). Results showed that GTD markedly increased the expression of TH, decreased the expression of α -syn, and enhanced the ratio of bcl-2/bax protein expression in a dose-dependent manner compared with the MPTP group (Fig. 2F-2H). These results indicated that GTD played a neuroprotective role in MPTP-induced PC12 cells by inhibiting apoptosis.

GTD remodeled DA homeostasis and up-regulated VMAT2 in MPTP-induced PC12 cells and PD mice.

Growing evidence showed that MPTP could damage dopaminergic neurons by interrupting vesicular DA storage[23]. To assess whether GTD could relieve MPTP-induced PC12 cell damages through regulating the misregulation of DA storage. The DA releases and DA content in PC12 cells with different treatments were detected by HPLC. Our results showed that MPTP exposure observably decreased DA release, which is consistent with the previous study[24], while this decrease was reversed by GTD in a dose-dependent manner (Fig. 3A). The effects of GTD on the DA content were consistent with the DA release (Fig. 3B). Research had demonstrated that the cellular content of DA was considered to be mainly storage in synaptic vesicles to maintain DA homeostasis, thus protecting neurons from the detrimental effects of DA accumulation and breakdown in the cytosol[25]. The above results suggested that GTD could attenuate MPTP-induced PC12 cell damages through regulating DA homeostasis disorder.

It is well known that VMAT2 transporters located on vesicles play an important role in maintaining the balance of DA homeostasis via uptake of cytoplasmic DA into vesicles for storage[26]. Therefore, the expression levels of VMAT2 mRNA and protein were tested by qPCR and Western blot. As shown in Fig. 3C and 3D, MPTP treatment significantly decreased the expression levels of VMAT2 mRNA and protein, whereas that markedly increased in response to GTD. These results indicated that GTD could activate VMAT2 to promote DA uptake into vesicles for maintaining DA homeostasis.

To verify whether the results in vivo were consistent with those in PC12 cells, the expression of VMAT2 of the striatum tissue in MPTP-induced PD mice was measured by Western blot and Immunohistochemistry. Results revealed that the reduced expressions of VMAT2 transporters occurring in MPTP-induced mice, however, this phenomenon was reversed by GTD (Fig. 3E-3G). Moreover, the vesicles of striatal synapses were visualized by electron microscopy (Fig. 4H). The results showed that the volume and number of vesicles were decreased significantly in MPTP-induced mice compared with the control group, suggesting that they may prevent the readily releasable vesicle pool. After treatment of GTD, the volume and number of vesicles of striatal synapses were significantly increased in a dose-dependent manner. Furthermore, the decrease in striatal VMAT2 immunoreactivity would correspond with dysregulation of DA neurochemistry[11]. Next, the content of DA and DOPAC in striatal was detected by HPLC (Fig. 3I and 3J). Compared with the control group, the content of DA in striatal remarkably decreased while the content of DOPAC significantly increased in MPTP-induced mice. But GTD could reverse these changes in a dose-

dependent manner. These results are consistent with our previous hypothesis that GTD can up-regulate the expression of VMAT2 protein, increase striatal vesicle volume and ameliorate DA dysregulation.

GTD alleviated DA homeostasis disorder and protected PC12 cells from MPTP-induced neurotoxicity via regulating VMAT2.

Subsequently, we determined to explore whether GTD against MPTP toxicity depends on VMAT2 expression. To address this issue, reserpine (Res), a VMAT2 inhibitor, was used in vitro and in vivo. As showed in Fig. 4A and 4B, the content and release of DA were increased significantly by GTD in MPTP-treated PC12 cells, while Res abolished the effect of GTD. Meanwhile, cell viability was not affected by 500 nM Res, while Res reduced the neuroprotective effect of GTD (Fig. 4C). Hoechst staining also showed that Res inhibited the anti-apoptotic effect of GTD in MPTP-treated PC12 cells (Fig. 4D). In addition, our results also showed that Res counteracted the effects of GTD in the content of increased DA and decreased DOPAC turnover in MPTP-induced PD mice (Fig. 4E and 4F). These findings suggested that GTD against MPTP-induced neurotoxicity was dependent on the activation of VMAT2 remodeling DA homeostasis.

GTD attenuated the impaired neurobehaviors and pathological features of MPTP-induced PD mice via regulating VMAT2.

To investigate whether GTD exerted neuroprotective effects for impaired neurobehaviors of MPTP-induced PD mice was related to VMAT2, reserpine (Res) was used in MPTP-induced PD mice when GTD administration. First, the spontaneous motion of the mice was detected by the open field test and autonomic activity test. The spontaneous behavior of the mice treated with GTD significantly increased compared with the mice in the MPTP group (Fig. 5A- 5E). But compared with the mice treated with GTD, the movement times and distance in the open field test were markedly reduced and the number of standing and spontaneous activities in the autonomic activity test was also significantly decreased in the mice treated with Res (Fig. 5A- 5E). The gait analysis, the pole test, and the rotarod test are important methods used to detect the coordination ability of mice. The results showed that the coordination ability of the mice treated with GTD was significantly alleviated compared with the mice in the MPTP group (Fig. 5F- 5J). In the gait analysis, compared with the GTD group mice, the step lengths of forelimb and hindlimb were significantly shortened in the Res group mice (Fig. 5F and 5G). In the pole test, Res treated mice exhibited significantly longer T-turn and T-total than the GTD mice (Fig. 5H and 5I). In the rotarod test, the Res group mice maintained a shorter time on the rod than the GTD mice (Fig. 5J). These results demonstrated that GTD ameliorated the impaired behaviors of MPTP-induced PD mice via promoting VMAT2.

The pathological features of PD include the degeneration of dopaminergic neurons in the substantia nigra pars compacta of the midbrain. In addition, we also examined the effect of GTD on dopaminergic neurons in the presence of the inhibitor. Our results showed GTD treatment increased the number of TH-positive neurons, however, after Res treatment, the effect of GTD on the loss of dopaminergic neurons

was significantly reduced compared with the GTD group (Fig. 5K and 5L). The results suggested that GTD rescued the loss of dopaminergic neurons in the MPTP-induced PD mice via promoting VMAT2.

GTD activated the MEK/ERK signaling pathway in MPTP-induced PC12 cells and PD mice.

The research suggested that MEK/ERK signaling pathway was found to be involved in the regulation of the VMAT2 promoter[27]. To investigate whether GTD promoted VMAT2 through MEK/ERK signaling pathway, the expression levels of MEK and ERK were tested in this study. The mRNA and protein levels of MEK and ERK were first measured in MPTP-induced PC12 cells by qPCR and Western blot. As showed in Fig. 6B and 6C, the results displayed that the expression of the mRNA level of the MEK and ERK gene in the MPTP-induced PC12 cells was restrained. After treatment with GTD, the mRNA level of the MEK and ERK gene was improved compared with the MPTP group. The effects of GTD on the p-MEK/MEK and p-ERK/ERK were highly similar to that of the mRNA expression (Fig. 6A, 6D and 6E). These results were also confirmed in MPTP-induced PD mice. Compared with the control group, the protein levels of MEK phosphorylation and ERK phosphorylation were significantly reduced in the MPTP group (Fig. 6F-6H). After treatment of GTD, phosphorylation levels of MEK and ERK were significantly up-regulated. Taken together, these findings suggested that GTD ameliorated the neurotoxicity of MPTP was promoted VMAT2 through activating MEK/ERK pathway.

Discussion

Our present study demonstrated that GTD could mitigate the neurotoxicity of MPTP and ameliorate the features of MPTP-induced PD through remodeling DA homeostasis, which is by activating MEK/ERK pathway promoted VMAT2 transporter. The research indicated GTD was a potential natural product of treating PD via targeting VMAT2 promoted dopamine vesicular transport.

PD is a devastating neurological condition that affects millions of people worldwide[24]. A progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta and striatum are the characteristic pathological features of PD[28]. With the development of the disease, PD patients may lose normal postural reflexes, leading to falls, seriously affecting the quality of life[1]. Currently, dopamine-based therapies (eg, carbidopa-levodopa, dopamine agonists) typically help initial motor symptoms about 49%-53% of individuals with mild PD[29]. Although DA replete has significantly increased quality of life and life expectancy, no therapies halt or reverse disease progression[30]. Patients with PD not only have a deficiency of DA but also have the dysfunction of DA transporter[29]. It may be an effective therapeutic strategy that regulating DA transporter to maintain DA homeostasis for PD[31].

GTD possesses a broad range of beneficial effects on the central nervous system (CNS) diseases, such as PD[13]. Preliminary evidence suggested that GTD could relieve behavioral damage and increase the expression of TH-positive neurons in the ventral tegmental area in the 6-hydroxydopamine and MPTP induced PD animal model, showing a protective effect on TH-positive neuron[13, 32]. Consistent with previous research results, the protective effect of GTD on dopaminergic neurons in vivo and in vitro were also reproduced in our work. Besides, previous studies had shown that the mechanisms of GTD actions

in PD include suppressing apoptosis, antioxidative, inhibition of glial activation, etc[17, 32]. In addition, clinical data investigating the therapeutic effect of GTD on PD showed that GTD can improve mild cognitive impairment in patients with PD, suggesting GTD might be an effective adjuvant drug for this condition[13, 33]. But the molecular mechanism involved in GTD-induced neuroprotection was not cleared yet. In the present study, we explored the possible effects of GTD on MPTP-induced PD models in vitro and in vivo.

MPTP is converted into MPP⁺, which can damage dopaminergic neurons in many mammals with biochemical and cellular changes that are relatively similar to those observed in PD[34]. Therefore, MPTP treatment is widely used to induce neural degeneration for the PD mice model. Mechanism of its selectivity in targeting the dopaminergic neurons of the substantia nigra lies in several pathways[35]. There is a wealth of research demonstrating toxic consequences to dysregulated cytosolic DA and DA oxidation. The concentration of DA in the cytoplasm is at a low level under normal conditions, however, there is an increase in free DA in the cytoplasm, resulting in the metabolism and oxidation of DA produce poisons, which make them extremely susceptible to cell death in the pathological condition of PD[36]. One of the neurotoxic effects of MPTP is disrupted DA homeostasis[37], accompanied by the inhibition of VMAT2 and the alter of the cytosolic dopamine pool[24]. In our study, we found that dopamine neuron damages and DA homeostasis disorder were induced by MPTP in PC12 cells, but the effects were reversed by GTD. The number of synaptic vesicles and volume became less when dopamine storage dysfunction occurs[38]. Synaptic vesicle deficiencies and dopamine dysregulation were observed in the nigrostriatal tissues of mouse brains were induced by MPTP, but this phenomenon was restrained by GTD. These results were consistent with that the results in vitro, which showed that GTD could alleviate the defects in DA storage exposed to MPTP.

VMAT2, localized on vesicular membranes in dopaminergic neurons, is a strong candidate that functions to transport cytosolic DA and exogenous neuronal toxins into synaptic vesicles and is involved in neuroprotection against neurotoxin-induced cell death[39, 40]. The deficits of VMAT2 have been implicated in the dopaminergic neurons loss which is considered as a typical pathological feature of PD[41]. The accumulation of DA in the cytoplasm can be eliminated by VMAT2 to avoid the cytotoxic action of DA. In the research, we found that GTD can up-regulate the expression of the VMAT2 gene and protein, and its regulation of dopamine homeostasis can be eliminated by the VMAT2 inhibitor in MPTP-induced PC12 cell. Research shows that polymorphisms in the VMAT2 gene and in its regulatory regions might therefore serve as genetic risk factors for PD[42]. And the density of VMAT2 was reduced in the striatum and mid-brain of PD patients compared with healthy controls[43]. Therefore, we hypothesized that the neuroprotective effect of GTD on DA neurons may involve the regulation of VMAT2 protein function. Consistent with the results in vitro, we found that GTD could up-regulate the expression of VMAT2 protein in nigrostriatal tissues of MPTP-induced PD mice. Furthermore, the effects that GTD attenuated the loss of DA neurons and impaired neurobehaviors of MPTP-induced PD mice were restrained by VMAT2 inhibitor. These findings indicated that GTD increased VMAT2 expression and thereby attenuates MPTP-induced neurotoxicity.

MEK/ERK pathway plays an important role in various biological processes such as cell proliferation, differentiation, and apoptosis[44], which can regulate various cell signaling pathways by activating target genes including cAMP response element-binding protein (CREB) transcription factors[45]. In addition, the promoter of VMAT2 has the binding site of the CREB, and the inhibition of ERK will reduce the activity of the promoter of VMAT2[46]. The activation of ERK can increase the ability of CREB binding to the promoter region of VMAT2, leading to elevated VMAT2 expression, which contributes to DA release and cell survival[41]. Our results indicated that GTD significantly increased the mRNA and protein expression of the MEK/ERK signaling pathway in the MPTP-induced PD model, suggesting GTD stimulated VMAT2 expression through activating MEK/ERK pathway and leading to the protection of dopaminergic neurons.

In conclusion, the results of this study first demonstrate that GTD induced an increase in VMAT2 expression through the activation of the MEK/ERK pathway to ameliorate features of MPTP-induced PD. These experiments strongly support the future use of GTD for the treatment of PD and related complications.

Abbreviations

PD, Parkinson's disease; DA, dopamine; VMAT2, vesicular monoamine transporter 2; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; GTD, Gastrodin; CNS, central nervous system; Res, Reserpine; TH, tyrosine hydroxylase; α -syn, alpha-synuclein; MEK, mitogen-activated protein kinase kinase; ERK, extracellular regulated protein kinases; bcl-2, B-cell lymphoma-2; bax, Bcl-2 Associated X protein; DOPAC, dihydroxy-phenyl acetic acid; CREB, cAMP response element-binding protein.

Declarations

Authors' Contributions

Meihuan Zhao and Peng Xia performed the methodology, the practical work; Meihuan Zhao also did the writing of the original draft. Jie Wang completed animal modeling and drug administration; Ping Li and Qi Chen completed western blotting analyses; Tengyun Ma and Panwang Liu did the data curation and behavioral testing; Shijun Xu conceived, designed the study and critically reviewed, edited, and revised the paper contributed the conception and design of the study.

Availability of Data and Materials

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

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Ethics Approval and Consent to Participate

The study was conducted according to National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication 80-23, revised 1996), keeping, and use of experimental animals (approved by the Institute of Material Medica Integration and Transformation for Brain Disorders, ethical approval no. IBD2020006).

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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Figures

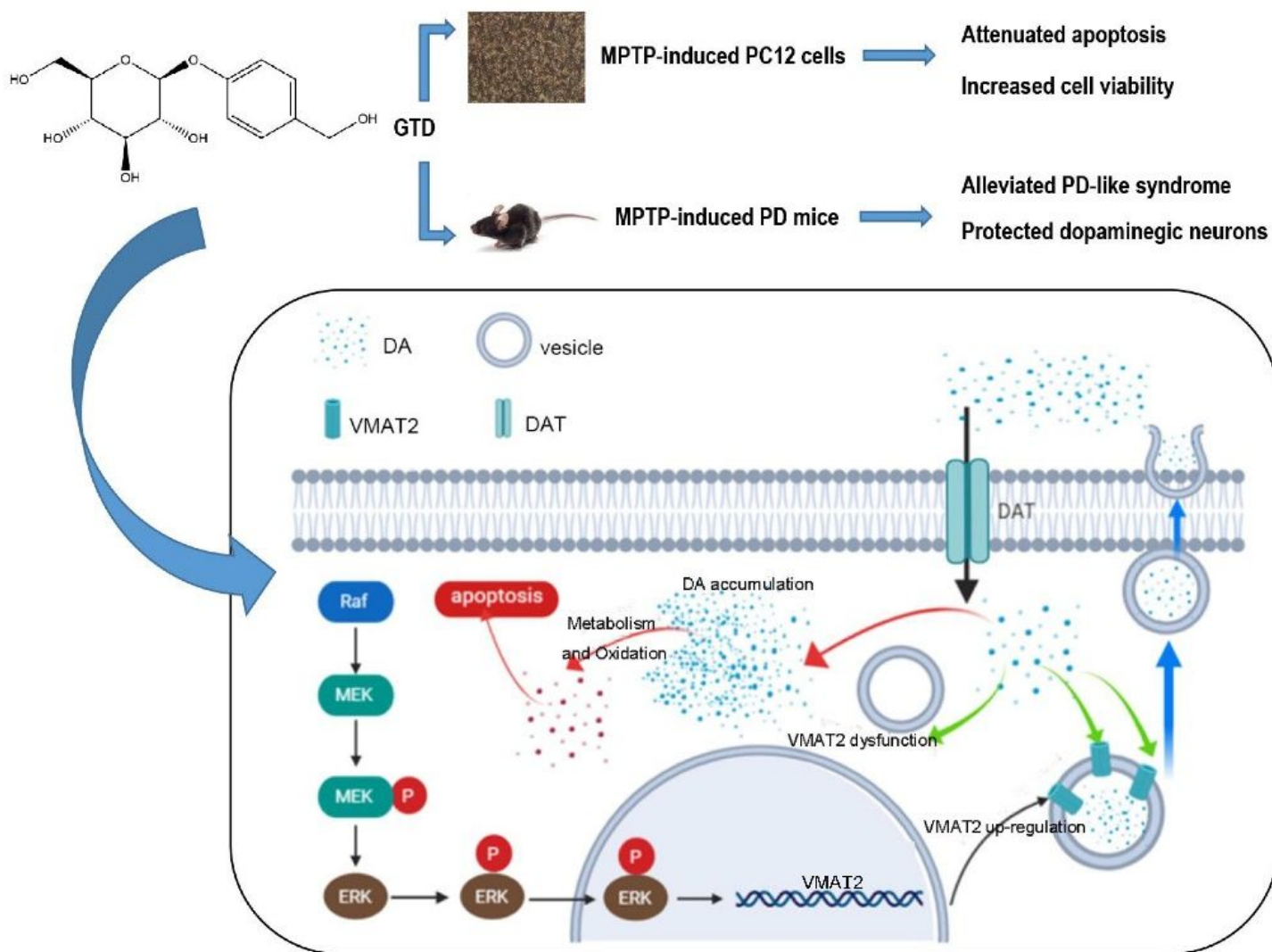


Figure 1

Graphical abstract. The in vitro data suggested that GTD protected PC12 cells from MPTP-induced neurotoxicity by upregulating the function of VMAT2 to promote vesicular dopamine transport. The in vivo data indicated that GTD protected dopaminergic neurons and improved behavior dysfunction by promoting VMAT2 protein expression to ameliorate dopamine dysregulation in MPTP-induced PD mice.

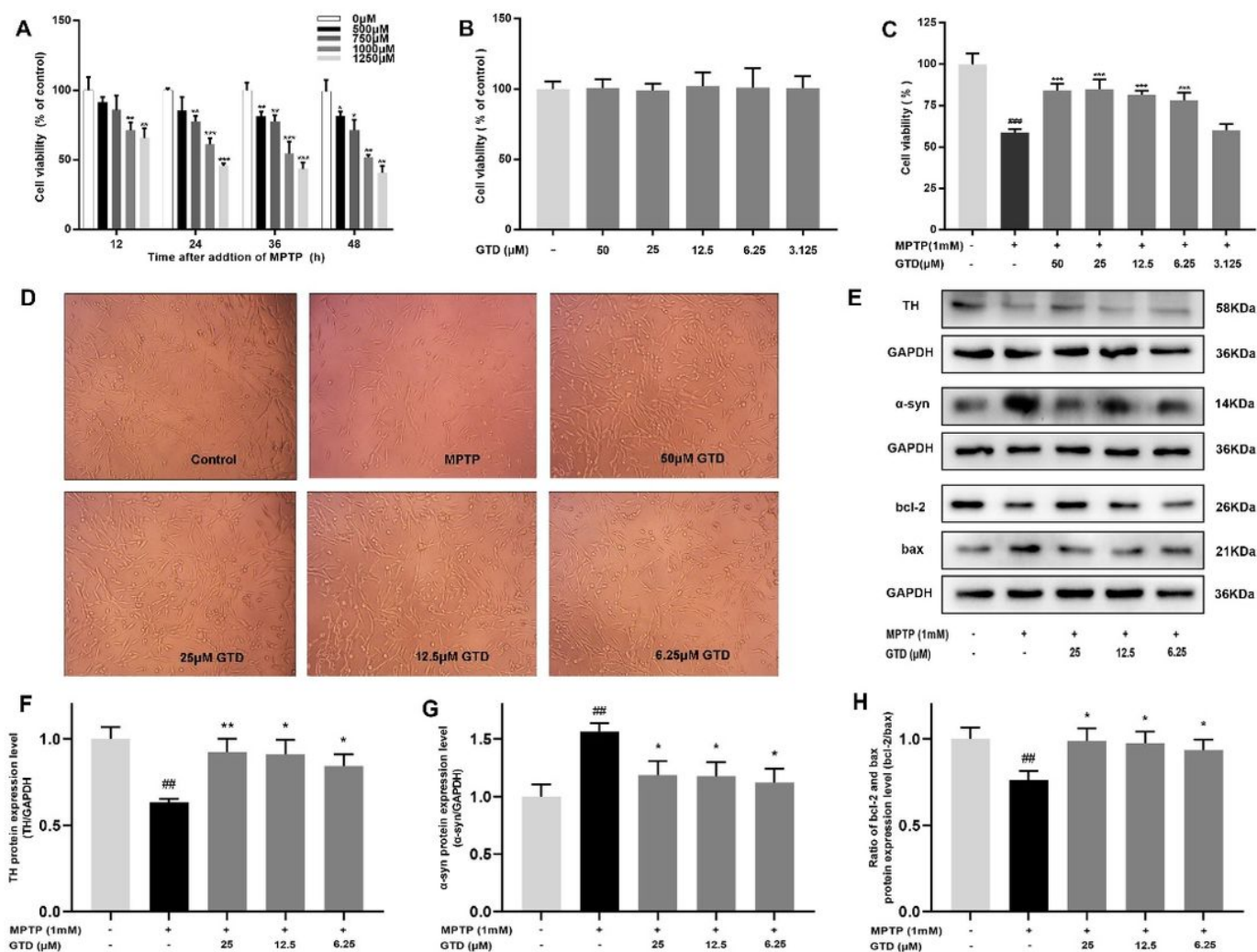


Figure 2

Screening right concentration of MPTP injured and effects of GTD on MPTP-induced cell damaged in PC12 cells. **(A)** PC12 cells viability damaged by different concentrations of MPTP at 12, 24, 36, and 48 hr. **(B)** PC12 cells viability after treatment for different concentrations of GTD for 24 hr. **(C)** The viability of PC12 cells induced by MPTP after treatment for different concentrations of GTD for 24 hr. **(D)** Phase-contrast micrographs (10×) of cells subjected to different treatments. Scale bar=100 μm. **(E)** Western blot analysis was performed to detect the levels of TH, α-syn, bcl-2 and bax in PC12 cells with indicated treatments. And effects of GTD on TH protein **(F)**, α-syn protein **(G)**, bcl-2/bax **(H)** expression were quantified in PC12 cells induced by MPTP. All the data in these figures are presented as mean ± SEM of three individual experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus MPTP group; ## $p < 0.01$ or ### $p < 0.001$ versus control group.

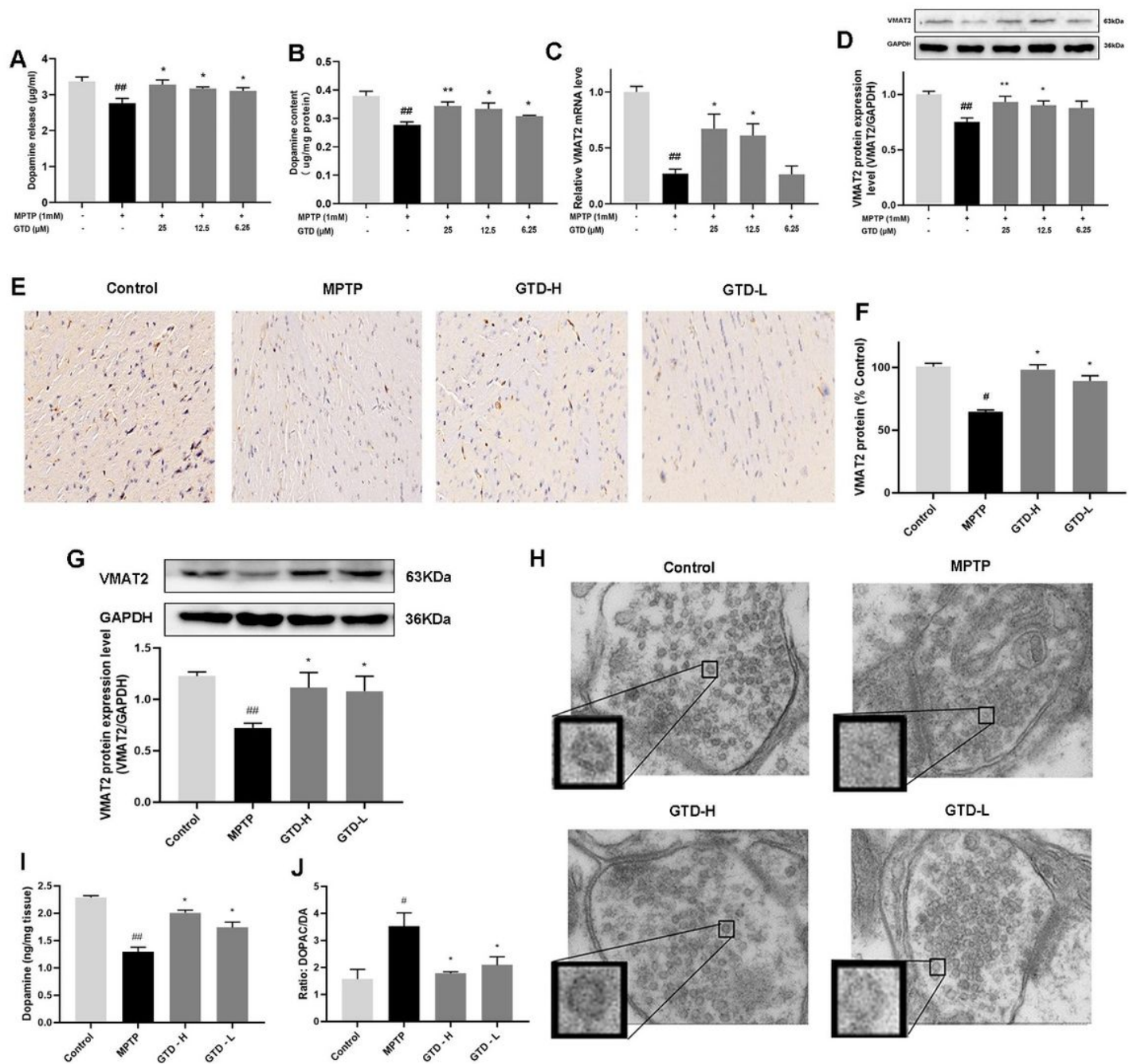


Figure 3

Effects of GTD on dopamine homeostasis imbalance, VMAT2 protein expression, and striatal vesicle in MPTP-induced PD model. (A and B) Dopamine release and content in MPTP treated PC12 cells were analyzed by HPLC. (C) Effect of GTD on VMAT2 mRNA levels in PC12 cells induced by MPTP. (D) Effect of GTD on VMAT2 protein expression in PC12 cells induced by MPTP. (E) Representative images from the immunohistochemical analysis of VMAT2 in the striatum of mice are shown, magnification: 200×. (F) Quantification of VMAT2 immunoreactivity in the striatum compared to the control mice. (G) Representative western blot images of VMAT2, and quantification of VMAT2/GAPDH in the striatum of mice. (H) Representative electron micrographs are included comparing the characteristics of neuronal

presynaptic terminals, scale bar=200nm. Levels of dopamine (I), dopamine turnover as measured by a ratio of DOPAC and dopamine (J). All the data in these figures are presented as mean $\pm$ SEM of three individual experiments. * p <0.05 or ** p <0.01 versus MPTP group; # p <0.05 or ## p <0.01 versus control group.

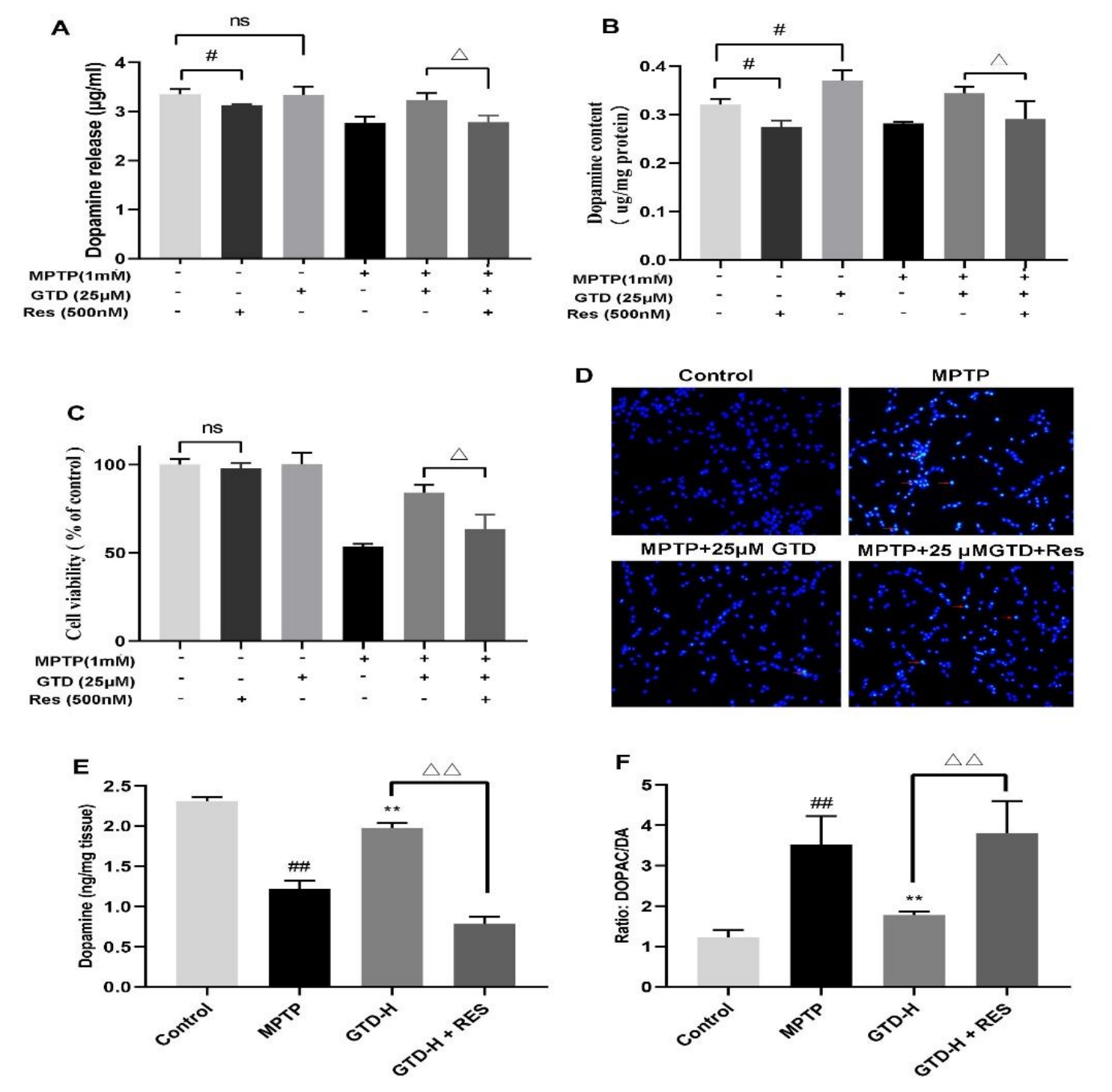


Figure 4

Effects of GTD on dopamine homeostasis imbalance and MPTP-induced PC12 cells damaged in the presence of the VMAT2 inhibitor. **(A and B)** Dopamine release and content in MPTP treated PC12 cells were analyzed by HPLC when VMAT2 inhibitor is present. **(C)** Cell viability was assessed by the CCK-8 assay. **(D)** PC12 cells were all stained by the Hoechst 33258, magnification: 200×. Levels of dopamine **(E)**, dopamine turnover as measured by a ratio of DOPAC and dopamine **(F)** in MPTP treated PD mice when VMAT2 inhibitor is present. # $p<0.05$ or ## $p<0.01$ versus control group; * $p<0.05$ versus MPTP group; $\Delta p<0.05$ or $\Delta\Delta p<0.01$ versus Res group; ns represents not statistically significant.

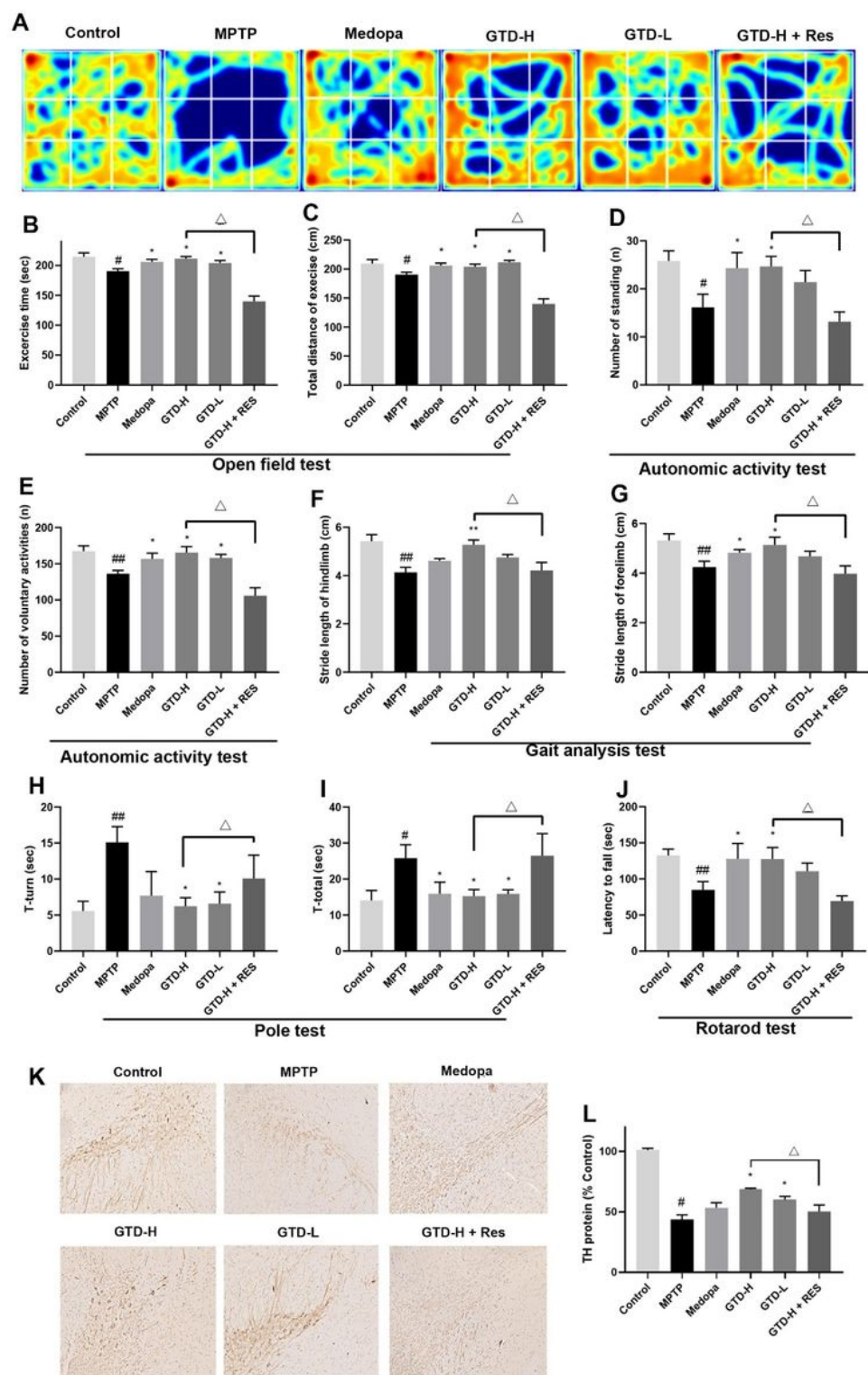


Figure 5

Effects of GTD on the impaired neurobehaviors and pathological features of MPTP-induced PD mice in the presence of the VMAT2 inhibitor. (A) Representative tracking heat map, (B) the exercise time, and (C) total distance of exercise were recorded in the open field test. (D and E) The number of standing and spontaneous activities was recorded in the autonomic activity. (Fand G) The step lengths of the forelimb and hindlimb were assessed in the gait analysis. (H) The time mice used to completely turn around and face the floor T-turn, and (I) the time to climb down the pole T-total were assessed in the pole test. (J) The latency time of mice to fall off the rod is shown for all groups in the Rotarod test. (K) Representative images from the immunohistochemical analysis of TH in the substantia nigra of mice are shown, magnification: 200×. (L) Quantification of TH immunoreactivity in the substantia nigra compared to the control mice. All the data in these figures are presented as mean $\pm$ SEM of three individual experiments. All the data in these figures are presented as mean $\pm$ SEM, n=6. # p <0.05, or ### p <0.01 versus control group; * p <0.05 versus MPTP group; Δ p <0.05 versus Res group.

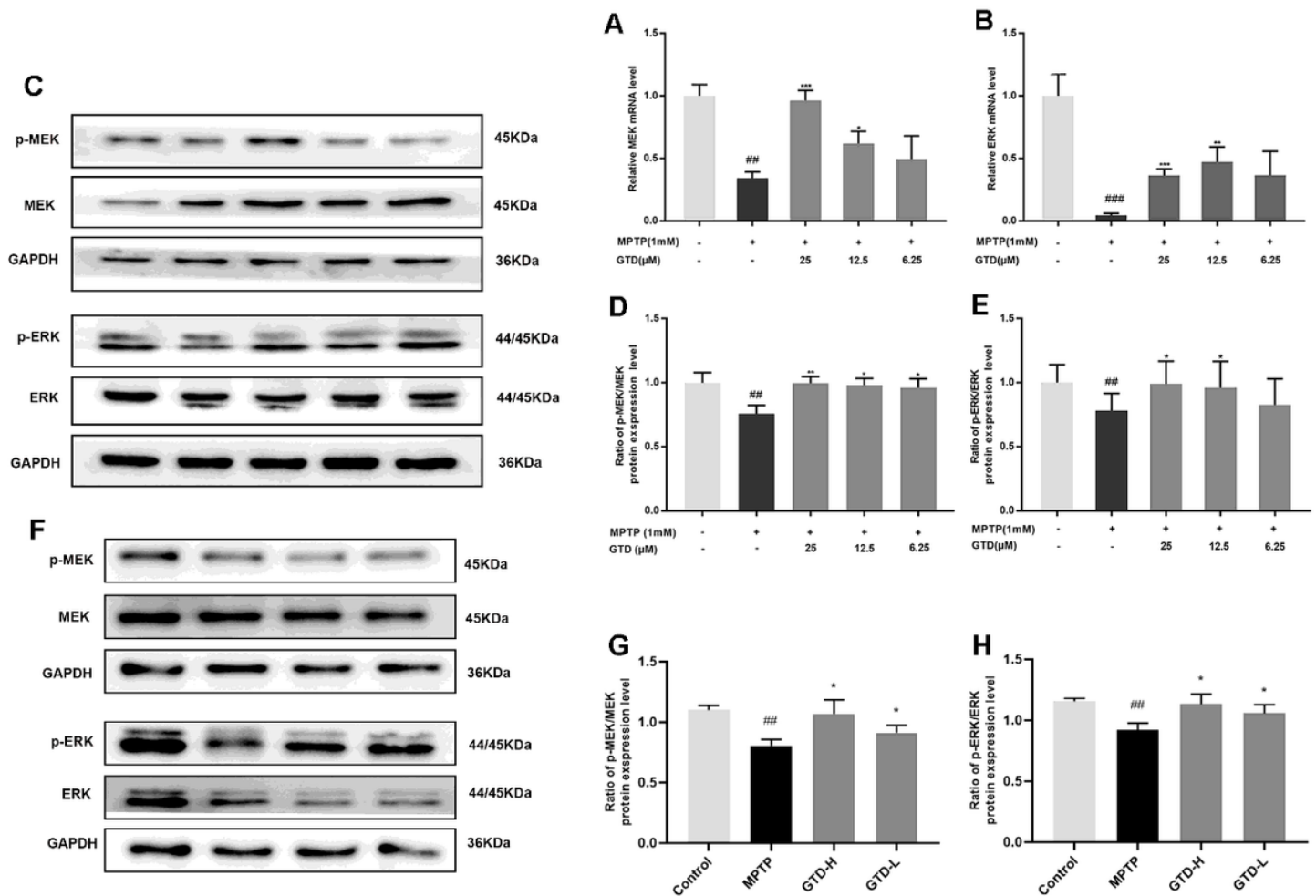


Figure 6

Effects of GTD on the MEK/ERK signaling pathway. (**A** and **B**) Effect of GTD on MEK and ERK mRNA levels in PC12 cells induced by MPTP. (**C**) Representative western blot of p-MEK, MEK, p-ERK, and ERK in PC12 cells subjected to different treatments. The effects of GTD on the expression ratio of p-MEK /MEK (**D**), p-ERK/ERK (**E**) in MPTP-induced PC12 cells. (**F**) Representative western blot of p-MEK, MEK, p-ERK, and ERK, and the effects of GTD on the expression ratio of p-MEK /MEK (**G**), p-ERK/ERK (**H**) in MPTP-induced PD mice. All the data in these figures are presented as mean $\pm$ SEM of three individual experiments. # $p<0.05$, ## $p<0.01$ or ### $p<0.001$ versus control group; * $p<0.05$, ** $p<0.01$, or *** $p<0.001$ versus MPTP group.