

# Monosialoganglioside GM1 Deficiency Inhibits the Neurotrophic Effects of GDNF by Disrupting Lipid Raft Assembly

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## Research Article

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# Abstract

Recent studies have shown that monosialoganglioside GM1 deficiency can inhibit the signal transduction process of glial cell line-derived neurotrophic factor (GDNF), which plays an important role in the pathogenesis of Parkinson's disease (PD). However, its specific mechanism still needs to be explored. We inhibited the expression of GM1 by treating cells with D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP). CCK-8 assay, EdU cell proliferation assay and Western blot assay were used to evaluate the effect of GM1 deficiency on the proliferation and differentiation of SH-SY5Y cells induced by GDNF and on the GDNF-RET signaling pathway. Lipid rafts were isolated by Triton X-100 solubilization and OptiPrep™ density gradient centrifugation. The alterations of lipid raft assembly and the translocation of RET into lipid rafts were evaluated after PDMP treatment. We found that PDMP treatment inhibited the proliferation and differentiation of SH-SY5Y cells induced by GDNF and reduced the phosphorylation of RET and its downstream signaling molecules Erk and Akt. In addition, after PDMP treatment, caveolin-1 and flotillin-1, the prototypical markers of lipid rafts, diffused from lipid rafts to non-lipid raft microdomains, and GDNF-induced RET translocation into lipid rafts was also reduced. These alterations could be partially reversed by adding exogenous GM1. Our results suggest that ganglioside GM1 deficiency could compromise the neurotrophic effects and signals downstream of GDNF by altering the assembly of lipid raft membrane microdomains.

## Introduction

Parkinson's disease (PD) is a chronic degenerative disease of the central nervous system that seriously damages human health<sup>[1]</sup>. The main pathological change in PD is the progressive death of dopaminergic neurons in the substantia nigra pars compacta (SNpc), but the specific mechanism is unclear. Recent research suggests that changes in the relative abundance of monosialotetrahexosylganglioside (GM1) may play a crucial role in the development and progression of PD<sup>[2, 3]</sup>. Research data show that the GM1 content decreases with age in the brain. In people over 60, the total amount of gangliosides in the substantia nigra of the midbrain decreases, and this reduction is most pronounced in GM1 and GD1a<sup>[4]</sup>. The expression of the ganglioside synthesis-related genes B3gal4 and St3gal2 is conspicuously absent in PD patients, and the GM1 level is decreased in the substantia nigra, prefrontal cortex and various peripheral tissues<sup>[5, 6]</sup>. In B4galnt1 knockout mice, mice with ganglioside GM1 deficiency show the characteristic symptoms of PD, such as reduced dopamine levels in the striatum, loss of dopaminergic neurons in the SNpc, aggregation of  $\alpha$ -synuclein, and motor dysfunction. Supplementation with the exogenous GM1 derivative LIGA20 can significantly alleviate the changes described above<sup>[7]</sup>. GM1 deficiency can not only cause neuropathological features in the substantia nigra striatum and motor disorders of PD but also lead to gastrointestinal autonomic nervous dysfunction and the non-motor symptoms of the brain cognitive system of PD<sup>[8]</sup>. All these results suggest that deficiency of endogenous

ganglioside GM1 may be an initiator of the pathological process of PD and play an important role in it, but the specific mechanism is not clear.

Recent studies have shown that GM1 deficiency can inhibit the signal transduction process of glial cell line-derived neurotrophic factor (GDNF) and trigger neurodegeneration<sup>[7]</sup>. GDNF is a nutrient factor identified and purified by Lin et al. from the serum-free medium of the rat glial cell line B49, which can promote the survival and morphological differentiation of dopaminergic neurons in the embryonic midbrain<sup>[9]</sup>. The protective and restorative effects of GDNF on dopaminergic neurons have been confirmed by a large number of experiments, and GDNF is an important target of medical intervention for neuroprotection and regeneration in the treatment of PD<sup>[10, 11]</sup>. As an extracellular factor, GDNF needs to transmit signals into cells through membrane receptors. GDNF has a unique receptor system that consists of the transmembrane signal transduction receptor RET and the ligand binding receptor GFR $\alpha$ 1<sup>[12]</sup>. Lipid rafts play a vital role in the transmission of GDNF signals<sup>[13–15]</sup>.

Lipid rafts are highly dynamic membrane microdomains enriched in cholesterol and sphingolipids, and they are in liquid-ordered (Lo) phases with lower membrane fluidity than surrounding cell membranes<sup>[16, 17]</sup>. We and others showed that in the absence of GDNF, the signal transduction receptor RET is mainly located outside of lipid rafts<sup>[18]</sup>. After stimulation with GDNF, RET is translocated into lipid rafts, and multiple downstream effectors are recruited to activate RAS/Erk, PI3K/Akt and other signal transduction pathways involved in the regulation of cell survival, differentiation, migration, growth and other biological processes<sup>[13, 14]</sup>.

As lipid rafts are in highly dynamic Lo phases, the lipid and protein composition of lipid rafts is not fixed. Current data suggest that changes in lipid composition can regulate the assembly and characteristics of lipid rafts<sup>[19–21]</sup>. Recently, studies of various model membrane systems have indicated that GM1 is also involved in the regulation of lipid raft assembly<sup>[22–24]</sup>. However, it is not clear whether GM1 can influence the signal transduction process of GDNF-RET by altering lipid raft assembly in dopaminergic cells. To explore this problem, we used D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) to inhibit GM1 expression in SH-SY5Y cells<sup>[7, 25]</sup>. We then investigated the effect of GM1 deficiency on the role of GDNF in promoting proliferation and differentiation, and regulating the GDNF-RET signaling pathway. Lipid rafts were isolated by Triton X-100 solubilization and OptiPrep<sup>TM</sup> density gradient centrifugation and the alterations of lipid raft assembly and the localization of RET were determined after PDMP treatment. Our results showed that PDMP treatment inhibited the proliferation and differentiation of SH-SY5Y cells induced by GDNF and reduced the phosphorylation of RET as well as the activation of its downstream signaling molecules Erk and Akt. In addition, after PDMP treatment, caveolin-1 and flotillin-1, the representative markers of lipid rafts, diffused from lipid rafts to non-lipid raft microdomains, and GDNF-induced RET translocation into lipid rafts was also reduced. These alterations could be partially reversed by adding exogenous GM1. Our results suggest that ganglioside GM1 deficiency might compromise the neurotrophic effects of GDNF by altering the assembly of membrane microdomains and promote the progression of PD.

# Materials And Methods

## Cell culture

SH-SY5Y human neuroblastoma cells were seeded into 25 cm<sup>2</sup> culture flasks (NEST) with Dulbecco's minimum essential medium (DMEM)/F12 (KeyGen BioTech) containing 10% fetal bovine serum (Bovogen) and 1% penicillin/streptomycin (VICMED). All cells were cultured in a standard carbon dioxide incubator (5% CO<sub>2</sub>, 37°C; Thermo Fisher Scientific). One day after seeding, the medium was changed to mixed medium with 10 μM retinoic acid (RA; Aladdin), and the cells were incubated for 4 days before experiments. The medium was changed every 2 days.

## Cell proliferation assay

SH-SY5Y cells were inoculated into 96-well plates at a concentration of 3×10<sup>3</sup> cells/well. When cells reached 80% confluence, the culture medium was changed to DMEM/F12 containing 3% fetal bovine serum (Bovogen) for incubation with PDMP (20 μM; Abcam) with or without GM1 (100 μM; J&K Scientific) for 24 h. Then, the culture medium was removed, cells were washed with PBS and incubated with fresh serum-free DMEM/F12 containing recombinant human GDNF (50 ng/mL; R&D Systems) for 12, 24, 36, or 48 h after incubation in serum-free medium for 4 h. The same volumes of fresh serum-free DMEM/F12 were added to control wells. Proliferation was measured using a cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's protocol. The absorbance at 450 nm was measured using a microplate reader (Thermo Labsystems).

For the EdU staining assay, SH-SY5Y cells were seeded in a 48-well plate at a concentration of 1×10<sup>4</sup> cells/well. When cells reached 70% confluence, cells were treated with PDMP and GM1 as described above. Then, the culture medium was replaced with fresh serum-free DMEM/F12 containing GDNF (50 ng/mL, R&D Systems) and 10 μM 5-ethynyl-2'-deoxyuridine (EdU). After 24 h incubation in the presence of EdU, cells were fixed with 4% paraformaldehyde and permeabilized in 0.5% Triton X-100 in PBS for 10 min at room temperature. Then, the cells were incubated in 1×Apollo® staining reaction liquid for 30 min at room temperature in the dark, and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, KeyGen BioTech). The cells were examined and photographed with a fluorescence microscope.

## Immunofluorescence Immunocytochemistry

SH-SY5Y cells were cultured in DMEM/F12 containing 3% fetal bovine serum (Bovogen) in 24-well plates at 8,000 cells/1000 μL/well. Cells were either treated with PDMP (20 μM; Abcam) for 24 h immediately prior to seeding or left untreated. A fraction of the PDMP-treated SH-SY5Y cells were treated with GM1 (100 μM; J&K Scientific) for 24 h. All samples were cultured in DMEM/F12 without serum for 4 h before being stimulated with GDNF (50 ng/mL, R&D Systems) or left unstimulated at 37°C for 36 h.

For MAP2 staining, cells were fixed with 4% paraformaldehyde for 15 min at 4°C followed by permeabilization with 0.1% Triton-X 100 prepared in phosphate-buffered saline (PBS) for 15 min at room temperature (RT). Following 3 washes in PBS, cells were blocked with PBS containing 5% normal goat

serum (NGS) for 1 h at RT. Cells were then incubated with primary antibodies against MAP2 (1:300; Proteintech) in PBS (containing 5% NGS) overnight at 4°C. Following incubation, cells were washed with PBS and incubated with species-specific, CoraLite594-conjugated donkey anti-rabbit IgG (H+L) (Proteintech). To visualize nuclei, cells were incubated with 4',6-diamidino-2-phenylindole (DAPI, KeyGen BioTech) for 10 min at RT. The cells were then examined and photographed with a fluorescence microscope.

### **Preparation of detergent-resistant membranes.**

After indicated treatment, SH-SY5Y cells were placed on ice and washed twice with ice-cold PBS. The cells were then detergent extracted by adding detergent-resistant membrane (DRM) lysis buffer (TBS containing 1% Triton X-100 and 1% protease inhibitors) and rocking the plates gently at 4°C for 20 min. The crude lysates were then collected and spun at 16,200 g in a 4°C microfuge for 10 min. The supernatants were rapidly removed from the pellets and placed into new tubes. The pellets, which contained the DRMs, were washed quickly with ice-cold DRM lysis buffer and centrifuged again, and the supernatants were rapidly removed. Both the insoluble pellets and the supernatants were then treated with 2x sample buffer and boiled for 5 min.

### **Flotation gradients.**

A second method used to biochemically analyze lipid rafts was density gradient centrifugation with Optiprep™ reagent (Sigma). For these experiments, SH-SY5Y cells were maintained on 100 mm culture dishes precoated with collagen (NEST). After indicated treatment, cells were washed twice with ice-cold PBS. Subsequently, the cells were scraped of the dishes on ice, and this mixture was transferred to centrifuge tubes and homogenized by ultrasonication. Crude homogenates were mixed with 60% Optiprep™ containing 1% Triton X-100 to adjust the concentration of Optiprep™ to 45%, and the mixture was then placed at the bottom of an ultracentrifuge tube (Beckman). Next, 2 ml layers of 35%, 30%, 25%, 20% and 5% OptiPrep™ reagent mixed with isolation buffer were added to fill the tube. These gradients were centrifuged at 260,000 g, 4°C for 4.5 h, and each layer was carefully removed from the tube. Extracts were collected from each fraction, sample buffer was added and boiled for 5 min, and the extracts were analyzed by SDS–PAGE and immunoblotting.

### **Immunoblotting analysis.**

Denatured protein extracts were subjected to SDS–PAGE and blotted onto PVDF membranes (Immobilon P; Millipore). Membranes were incubated in 8% milk for 0.5 h before an overnight incubation with the primary antibody at 4°C. The immunoblots were washed three times with TBST and then incubated with a secondary antibody in TBST for 1 h. After washing three times in TBST, the blots were visualized using a chemiluminescent substrate (Tanon 5200 Multi). The antibodies used and their working dilutions were as follows: anti-Phospho-RET antibody (Tyr1062) (1:2000; Affinity); anti-RET antibody (1:5000; Abcam); anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (1:2000; Cell Signaling); anti-p44/42 MAPK (Erk1/2) antibody (1:1000; Cell Signaling); anti-Phospho-Akt (pan) antibody (1:2000; Cell

Signaling); anti-Akt (pan) antibody (1:1000; Cell Signaling); anti-Caveolin-1 antibody (1:10000; Proteintech); anti-Flotillin-1 antibody (1:10000; Proteintech); anti-CD71 antibody (1:1000; Proteintech); anti-TH antibody (1:2000; Proteintech); anti- $\alpha$ -synuclein antibody (1:2000; R&D Systems); anti- $\beta$ -Actin antibody (1:10000; Sigma). ImageJ software (Media Cybernetics, Rockville, MD) was employed for quantitative analysis of band density.

## Statistical Analysis

The results are presented as the mean  $\pm$  SD of a minimum of three independent sets of experiments. All statistical analyses were performed in Graph Prism 5.0. One-way analysis of variance (ANOVA) or Student's t test was used as indicated.  $P < 0.05$  was considered statistically significant.

## Results

### Exogenous GM1 can partially restore GM1 content reduced by PDMP treatment

To reduce the content of GM1 in the plasma membrane, SH-SY5Y cells were treated with 20  $\mu$ M PDMP, an inhibitor of glucosylceramide synthesis, which can partially reduce the level of GM1. To examine the effect of PDMP on cell viability, cells were treated with various concentrations of PDMP in 96-well plates (0 $\mu$ M, 10 $\mu$ M, 20 $\mu$ M, 30 $\mu$ M, 40 $\mu$ M, 50 $\mu$ M, 60 $\mu$ M, 70 $\mu$ M, 80 $\mu$ M, 90 $\mu$ M, 100 $\mu$ M, 120 $\mu$ M, 160 $\mu$ M). We found that the cell proliferation decreased with increasing PDMP doses, and that PDMP had no significant effect on cell viability at 20  $\mu$ M (Fig. 1A).

To confirm the inhibitory effect of PDMP on GM1 content, CtxB-Alexa 647 was used to label GM1. Treatment with 20  $\mu$ M PDMP significantly reduced the quantity of GM1. Notably, treatment with 100 $\mu$ M exogenous GM1 restored the GM1 content to almost to the basal level (Fig. 1B, C). This result is consistent with the report by Martino Calamai et al<sup>[26]</sup>.

### GM1 deficiency inhibits GDNF-induced cell proliferation

To investigate the effect of GM1 deficiency on the neurotrophic effects of GDNF, CCK-8 assays and EdU staining assays were performed to measure the proliferation of SH-SY5Y cells induced by GDNF after PDMP treatment. Cells gradually proliferated upon GDNF treatment, and the proliferation was significantly enhanced after 36 hours of treatment. In contrast, starting at 24 hours of treatment, cells treated with PDMP+GDNF proliferated significantly less than those treated with GDNF alone, and the change was reversed after the addition of exogenous GM1 (Fig. 2A). EdU staining results showed that after PDMP treatment, the proportion of EdU-positive cells induced by GDNF decreased significantly. The addition of 100  $\mu$ M GM1 significantly restored the effect of GDNF on the proliferation of SH-SY5Y cells, even to a degree comparable to those treated with GDNF alone (Fig. 2B, C).

In conclusion, reducing the content of GM1 can inhibit the effect of GDNF on proliferation, and exogenous GM1 can reverse this change.

## **GM1 deficiency inhibits GDNF-induced cell differentiation**

To examine the effects of GM1 on the pro-differentiation effect of GDNF, SH-SY5Y cells were treated under four conditions (Ctrl, GDNF, PDMP+GDNF and PDMP+GM1+GDNF), and microtubule-associated protein 2 (MAP2) immunofluorescent staining was performed. The axon length and the number of SH-SY5Y cells with axons were determined using ImageJ software. As shown in Fig. 3A, GDNF treatment increased the average axon length and the number of cells with axons, indicating the pro-differentiation effect of GDNF. Pretreatment with PDMP reduced the average axon length and the number of cells with axons induced by GDNF. Quantitative analysis revealed that exogenous GM1 increased the axon length at least by 0.1 inch compared to the PDMP treatment group (Fig. 3B). The number of cells with axons was also significantly increased by exogenous GM1 (Fig. 3C). In addition to the longer protrusions and increased number of cells with protrusions, the cells cultured with exogenous GM1 also maintained high tyrosine hydroxylase (TH) expression (Fig. 3D), which is specifically expressed by dopaminergic cells. In conclusion, reducing the content of GM1 can inhibit the effect of GDNF on differentiation induction, and exogenous GM1 can reverse this change.

## **GM1 deficiency impairs GDNF-RET signaling**

Stimulation with 50 ng/mL GDNF increased the phosphorylation of the primary GDNF signaling molecules RET, Erk and Akt. Pretreatment with PDMP reduced GDNF-RET signaling. Fig. 4 demonstrates that p-RET, p-Erk and p-Akt induced by GDNF were reduced to basal levels when GM1 was depleted. The crucial role of GM1 in these changes was further illustrated by the increase in the levels of p-RET, p-Erk and p-Akt when GM1 was exogenously supplied to the cells pretreated with PDMP.

## **GM1 deficiency disturbs the assembly of lipid rafts**

To investigate the alteration of lipid raft assembly, we performed Triton X-100 solubilization and OptiPrep™ density gradient centrifugation to isolate lipid rafts and examined the alterations in lipid raft markers in each sample by Western blotting. Immunoblotting of cell extracts from Triton X-100 solubilization samples revealed that treatment with 20 μM PDMP largely dispersed caveolin-1 and flotillin-1, the markers of lipid rafts, from the DRMs, also known as lipid rafts, to the detergent-soluble, non-raft membrane domains, and that this could be partially reversed by the addition of exogenous GM1. However, the total protein levels of CD71, a non-lipid raft marker, were not different between the GM1-deficient cells and the GM1-supplemented cells (Fig. 5A, B).

Fractions from OptiPrep™ density gradient centrifugation were also analyzed by Western immunoblotting. Not surprisingly, the results were consistent with those described above (Fig. 5C). Caveolin-1 and flotillin-1 in SH-SY5Y cells were mainly localized in lipid raft fractions, while CD71 was

distributed in non-lipid rafts. After pretreatment with PDMP, caveolin-1 and flotillin-1 tended to disperse from lipid raft to non-raft fractions, which could be partially reversed by the addition of GM1. At the same time, the localization of CD71 in non-lipid rafts was not changed by PDMP treatment. These results suggested that GM1 deficiency could result in changes in lipid raft compositions.

## **RET translocation into lipid rafts is blocked in GM1-reduced SH-SY5Y cells**

Many studies have shown that lipid rafts play a vital role in the transmission of GDNF-RET signals. Upon GDNF treatment, RET translocates into lipid rafts and activates RAS/Erk, PI3K/Akt and various other signal transduction pathways. To investigate the effect of lipid raft alterations induced by GM1 deficiency on GDNF-RET signals, we isolated lipid rafts and determined the translocation of RET into lipid rafts. First, we performed Triton X-100 solubilization to isolate lipid rafts. The results showed that, without GDNF treatment, RET was primarily located in detergent-soluble, non-raft membrane domains, and very little RET was detected in lipid raft fractions. After 20 minutes of treatment with 50 ng/mL GDNF, RET in lipid raft membrane domains increased. Pretreatment with PDMP inhibited the translocation of RET into lipid rafts induced by GDNF. In addition, GDNF-induced RET distribution in lipid rafts was partially restored following the restoration of GM1 content in PDMP pretreated cells (Fig. 6A).

To confirm these results, we used another biochemical method, OptiPrep™ density gradient centrifugation, to isolate lipid rafts. Consistent with the observations from Triton X-100 solubilization experiments, the majority of RET was located outside of DRMs without GDNF treatment, and after GDNF treatment, RET was translocated into lipid rafts. When GM1 was reduced, GDNF-induced RET translocation into lipid rafts was inhibited, which could be restored by GM1 addition (Fig. 6B).

## **GM1 is decreased in MPP<sup>+</sup>- induced PD cell model**

MPP<sup>+</sup> treatment is frequently used to establish a cell model of PD<sup>[27, 28]</sup>. In this study, SH-SY5Y cells were treated with 2.5 mM, 5 mM, or 7.5 mM MPP<sup>+</sup> for 24 h, and cell viability was determined by CCK-8 assay. The results showed that at 5 mM, MPP<sup>+</sup> significantly reduced cell viability to approximately 70% of that of the control group (Fig. 7A). Therefore, we used 5 mM MPP<sup>+</sup> to treat SH-SY5Y cells. To investigate the effect of 5 mM MPP<sup>+</sup> on GM1 content, we labeled GM1 with CtxB-Alexa 647 and quantified it by ImageJ software. We found that GM1 was significantly decreased after MPP<sup>+</sup> treatment (Fig. 7B, C). This change could be partially reversed after adding exogenous GM1.

## **Synergistic protective effect of GM1 and GDNF in an MPP<sup>+</sup>-induced PD cell model**

To determine the synergistic protective effect of GM1 and GDNF on the MPP<sup>+</sup>-induced PD cell model, we first selected the appropriate concentration of GDNF. MPP<sup>+</sup>-injured SH-SY5Y cells were treated with

various concentrations of GDNF (10 ng/mL, 20 ng/mL, 30 ng/mL, 40 ng/mL, 50 ng/mL, 60 ng/mL) for 36 h. Subsequently, cell viability was determined by CCK-8 assay. The results showed that 10 ng/mL GDNF had no significant protective effect on MPP<sup>+</sup>-injured SH-SY5Y cells (Fig. 8A). We selected this concentration of GDNF to investigate the synergistic protective effect of GM1 and GDNF.

SH-SY5Y cells were treated with 20  $\mu$ M PDMP with or without GM1 for 24 h followed by incubation in serum-free medium for 4 h. Subsequently, cells were stimulated with GDNF for 36 h. Cell viability was determined by CCK-8 assay and the expression of TH protein was evaluated by Western blot assay. The results showed that treatment with GM1 alone at 40  $\mu$ M or 100  $\mu$ M had no protective effect on injured cells, while 10 ng/mL GDNF together with 40  $\mu$ M or 100  $\mu$ M GM1 increased cell viability and the expression of TH protein in MPP<sup>+</sup>-injured SH-SY5Y cells (Fig. 8B, C).

## Discussion

Gangliosides are highly expressed in the nervous system of vertebrates<sup>[29]</sup>. In PD, the level of GM1 in dopaminergic neurons of the occipital cortex and SNpc is significantly lower than that of the control group at the same age, suggesting that GM1 deficiency may be a risk factor for PD<sup>[7]</sup>. Lack of dopaminergic neurons in the striatum and the SNpc and aggregation of  $\alpha$ -synuclein, which are typical characteristics of PD, were observed in mice with GM1 deficiency<sup>[30]</sup>. However, the specific mechanism of ganglioside GM1 deficiency leading to neurodegenerative changes is still unclear. Dopaminergic neurons require specific neurotrophic factors for proper differentiation and maintenance in vivo<sup>[31, 32]</sup>. As the most important neurotrophic factor in the midbrain dopamine system, GDNF can promote the survival and morphologic differentiation of embryonic midbrain dopaminergic neurons and enhance their uptake of dopamine<sup>[33]</sup>. Recent studies have shown that GDNF signal transduction is blocked in GM1-deficient mice. In our study, PDMP treatment inhibited GDNF-RET signal transduction and GDNF-induced proliferation and differentiation of SH-SY5Y cells, while the addition of exogenous GM1 partially reversed these negative effects. These observations are consistent with those of Ohmi et al<sup>[34]</sup>.

Lipid rafts are highly dynamic microdomains within cell membranes enriched in cholesterol and sphingolipids<sup>[16, 35]</sup>. It has been shown that, in a variety of neurodegenerative diseases, such as PD, Alzheimer's disease and familial amyotrophic lateral sclerosis, lipid rafts appear to have abnormal lipid composition and functional deficiencies in the brain, which can promote the pathogenesis and development of these diseases<sup>[36–38]</sup>. Our previous study also showed that the lipid and protein components of lipid rafts were significantly altered in MPP<sup>+</sup>-induced PD cell models<sup>[39]</sup>. Furthermore, studies of various unit membrane models have shown that GM1 can affect the phase separation of lipid membranes and the assembly of microdomains<sup>[22, 24, 40, 41]</sup>. However, it is not clear whether GM1 can affect the signal transduction process of GDNF by affecting lipid raft assembly in SH-SY5Y cells.

Our results showed that the lipid raft markers caveolin-1 and flotillin-1 were redistributed from lipid rafts to non-lipid raft regions when GM1 was reduced. In addition, we also found that GDNF induced less RET

translocation into lipid rafts and decreased RET phosphorylation in GM1-deficient cells. After the addition of exogenous GM1, GDNF-induced RET translocation to lipid rafts can be partially restored. These results indicate that lipid raft dysfunction caused by ganglioside GM1 deficiency affects GDNF-RET signaling.

Furthermore, our study also found that 5 mM MPP<sup>+</sup> could significantly decrease GM1 content, and that the addition of exogenous GM1 reversed the reduction. Treatment with 100 μM GM1 or 10 ng/mL GDNF alone had no significant effect on cell viability or TH protein expression in the MPP<sup>+</sup>-induced cell model of PD, while treatment with 100 μM GM1 combined with 10 ng/mL GDNF significantly increased cell viability and TH protein expression, suggesting that GM1 and GDNF have a synergistic protective effect on MPP<sup>+</sup>-induced cell injury.

Our results suggest that ganglioside GM1 deficiency might compromise the neurotrophic effects of GDNF by altering the assembly of lipid raft membrane microdomains and promote the progression of PD.

## Declarations

### Ethics approval

Not applicable for that section.

### Consent to participate

Not applicable for that section.

### Consent for publication

Not applicable for that section.

### Availability of data and materials:

SH-SY5Y cells were purchased from Cell Bank of Chinese Academy of Sciences([SCSP-5014](#)).

### Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

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**Authors' contributions**  Li Li conceived and designed the experiments; Jiang Shimin and Zhou Tai performed the experiments; Zhou Yao, Wang Zhongcheng and Zhang Kejia analyzed the data. Zhou Yao contributed reagents/materials/analysis tools; Li Li and Jiang Shimin wrote the paper. All authors read and approved the final manuscript.

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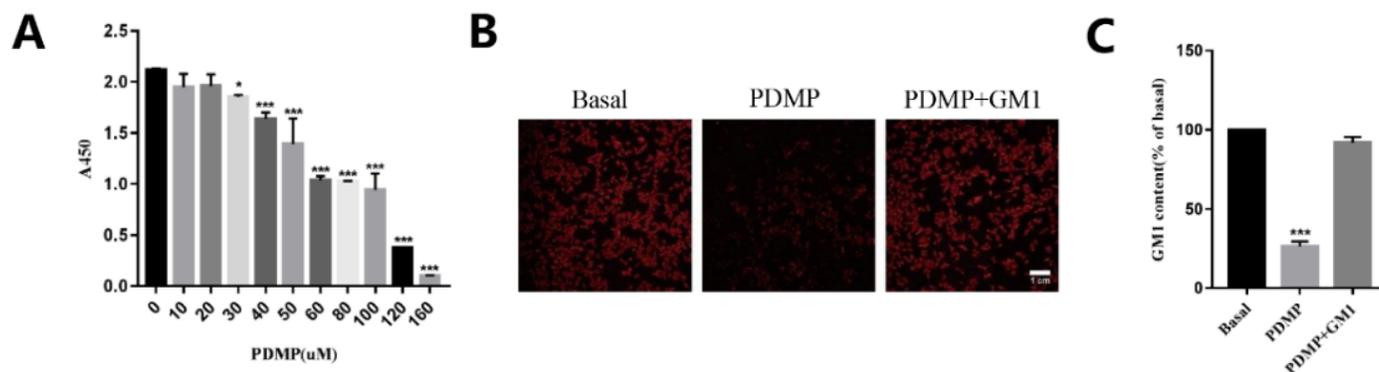
## References

1. Niu Y, Zhang J, Dong M (2021) Nrf2 as a potential target for Parkinson's disease therapy. *J Mol Med (Berl)* 99:917–931
2. Sipione S, Monyor J, Galleguillos D, Steinberg N, Kadam V (2020) Gangliosides in the Brain: Physiology, Pathophysiology and Therapeutic Applications. *Front Neurosci* 14:572965
3. Chiricozzi E et al (2020) GM1 Ganglioside Is A Key Factor in Maintaining the Mammalian Neuronal Functions Avoiding Neurodegeneration. *Int J Mol Sci* 21:E868
4. Huebecker M et al (2019) Reduced sphingolipid hydrolase activities, substrate accumulation and ganglioside decline in Parkinson's disease. *Mol Neurodegener* 14:40
5. Verma M, Schneider JS (2019) siRNA-mediated knockdown of B3GALT4 decreases GM1 ganglioside expression and enhances vulnerability for neurodegeneration. *Mol Cell Neurosci* 95:25–30
6. Schneider JS (2018) Altered expression of genes involved in ganglioside biosynthesis in substantia nigra neurons in Parkinson's disease. *PLoS ONE* 13:e0199189
7. Hadaczek P et al (2015) GDNF signaling implemented by GM1 ganglioside; failure in Parkinson's disease and GM1-deficient murine model. *Exp Neurol* 263:177–189
8. Wu G et al (2020) Mice deficient in GM1 manifest both motor and non-motor symptoms of Parkinson's disease; successful treatment with synthetic GM1 ganglioside. *Exp Neurol* 329:113284
9. Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 260:1130–1132
10. Kordower JH, Bjorklund A (2013) Trophic factor gene therapy for Parkinson's disease. *Mov Disord* 28:96–109
11. de d'Anglemont X, Pascual A, López-Barneo J (2015) GDNF-based therapies, GDNF-producing interneurons, and trophic support of the dopaminergic nigrostriatal pathway. Implications for Parkinson's disease. *Front Neuroanat* 9:10
12. Jmaeff S, Sidorova Y, Nedev H, Saarma M, Saragovi HU (2020) Small-molecule agonists of the RET receptor tyrosine kinase activate biased trophic signals that are influenced by the presence of GFRα1 co-receptors. *J Biol Chem* 295:6532–6542
13. Morales D (2016) Lipid Raft-Excluded GFRα1/Ret Fails to Transmit GDNF Signaling In Vivo. *J Neurosci* 36:1442–1444
14. Li L et al (2017) The Actin Cytoskeleton Is Involved in Glial Cell Line-Derived Neurotrophic Factor (GDNF)-Induced Ret Translocation into Lipid Rafts in Dopaminergic Neuronal Cells. *Int J Mol Sci* 18:E1922

15. Li L et al (2017) NCAM-140 Translocation into Lipid Rafts Mediates the Neuroprotective Effects of GDNF. *Mol Neurobiol* 54:2739–2751
16. Zacharias DA, Violin JD, Newton AC, Tsien RY (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* 296:913–916
17. Sonnino S, Aureli M, Mauri L, Ciampa MG, Prinetti A (2015) Membrane lipid domains in the nervous system. *Front Biosci (Landmark Ed)* 20:280–302
18. Pierchala BA, Milbrandt J, Johnson EM (2006) Glial cell line-derived neurotrophic factor-dependent recruitment of Ret into lipid rafts enhances signaling by partitioning Ret from proteasome-dependent degradation. *J Neurosci* 26:2777–2787
19. Staneva G, Osipenko DS, Galimzyanov TR, Pavlov KV, Akimov SA (2016) Metabolic Precursor of Cholesterol Causes Formation of Chained Aggregates of Liquid-Ordered Domains. *Langmuir* 32:1591–1600
20. Khadka NK, Ho CS, Pan J (2015) Macroscopic and Nanoscopic Heterogeneous Structures in a Three-Component Lipid Bilayer Mixtures Determined by Atomic Force Microscopy. *Langmuir* 31:12417–12425
21. Heftberger P, Kollmitzer B, Rieder AA, Amenitsch H, Pabst G (2015) In situ determination of structure and fluctuations of coexisting fluid membrane domains. *Biophys J* 108:854–862
22. Puff N, Watanabe C, Seigneuret M, Angelova MI, Staneva G (2014) Lo/Ld phase coexistence modulation induced by GM1. *Biochim Biophys Acta* 1838:2105–2114
23. Patel DS et al (2016) Influence of Ganglioside GM1 Concentration on Lipid Clustering and Membrane Properties and Curvature. *Biophys J* 111:1987–1999
24. Galimzyanov TR et al (2017) Line Activity of Ganglioside GM1 Regulates the Raft Size Distribution in a Cholesterol-Dependent Manner. *Langmuir* 33:3517–3524
25. Jiang L et al (2014) Effects of gangliosides on the activity of the plasma membrane Ca<sup>2+</sup>-ATPase. *Biochim Biophys Acta* 1838:1255–1265
26. Calamai M et al (2016) Single molecule experiments emphasize GM1 as a key player of the different cytotoxicity of structurally distinct Aβ<sub>1-42</sub> oligomers. *Biochim Biophys Acta* 1858:386–392
27. Ramalingam M, Kim S-J (2016) Insulin on activation of autophagy with integrins and syndecans against MPP<sup>+</sup>-induced α-synuclein neurotoxicity. *Neurosci Lett* 633:94–100
28. Nakaso K, Ito S, Nakashima K (2008) Caffeine activates the PI3K/Akt pathway and prevents apoptotic cell death in a Parkinson's disease model of SH-SY5Y cells. *Neurosci Lett* 432:146–150
29. McGonigal R et al (2021) Neuronally expressed a-series gangliosides are sufficient to prevent the lethal age-dependent phenotype in GM3-only expressing mice. *J Neurochem* 158:217–232
30. Wu G, Lu Z-H, Kulkarni N, Amin R, Ledeen RW (2011) Mice lacking major brain gangliosides develop parkinsonism. *Neurochem Res* 36:1706–1714
31. Airaksinen MS, Saarma M (2002) The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci* 3:383–394

32. Andressoo J-O, Saarma M (2008) Signalling mechanisms underlying development and maintenance of dopamine neurons. *Curr Opin Neurobiol* 18:297–306
33. Choi-Lundberg DL, Bohn MC (1995) Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in rat. *Brain Res Dev Brain Res* 85:80–88
34. Ohmi Y et al (2009) Gangliosides play pivotal roles in the regulation of complement systems and in the maintenance of integrity in nerve tissues. *Proc Natl Acad Sci U S A* 106:22405–22410
35. Pike LJ (2004) Lipid rafts: heterogeneity on the high seas. *Biochem J* 378:281–292
36. Fabelo N et al (2011) Severe alterations in lipid composition of frontal cortex lipid rafts from Parkinson's disease and incidental Parkinson's disease. *Mol Med* 17:1107–1118
37. Martín V et al (2010) Lipid alterations in lipid rafts from Alzheimer's disease human brain cortex. *J Alzheimers Dis* 19:489–502
38. Zhai J et al (2009) Proteomic characterization of lipid raft proteins in amyotrophic lateral sclerosis mouse spinal cord. *FEBS J* 276:3308–3323
39. Mu P et al (2020) Glial cell line-derived neurotrophic factor alters lipid composition and protein distribution in MPP+-injured differentiated SH-SY5Y cells. *J Cell Physiol* 235:9347–9360
40. Yuan C, Johnston LJ (2000) Distribution of ganglioside GM1 in L-alpha-dipalmitoylphosphatidylcholine/cholesterol monolayers: a model for lipid rafts. *Biophys J* 79:2768–2781
41. Bao R, Li L, Qiu F, Yang Y (2011) Atomic force microscopy study of ganglioside GM1 concentration effect on lateral phase separation of sphingomyelin/dioleoylphosphatidylcholine/cholesterol bilayers. *J Phys Chem B* 115:5923–5929

## Figures



**Figure 1**

PDMP reduces GM1 content in SH-SY5Y cells. (A) SH-SY5Y cells were treated with PDMP (10 $\mu$ M, 20 $\mu$ M, 30 $\mu$ M, 40 $\mu$ M, 50 $\mu$ M, 60 $\mu$ M, 70 $\mu$ M, 80 $\mu$ M, 90 $\mu$ M, 100 $\mu$ M, 120 $\mu$ M, 160 $\mu$ M). After 24 h of treatment, cell

viability was determined by a CCK-8 kit (\* represents the comparison between the experimental group and the control group: \*P < 0.5, \*\*\*P < 0.001). (B, C) SH-SY5Y cells were treated with 20  $\mu$ M PDMP with or without 100  $\mu$ M GM1 for 24 h followed by washing and staining with CtxB-Alexa 647 (red fluorescence). GM1 content under indicated conditions was quantified by ImageJ software. Scale bar = 50 $\mu$ m (\* represents the comparison between the experimental group and the control group: \*\*\*P < 0.001).

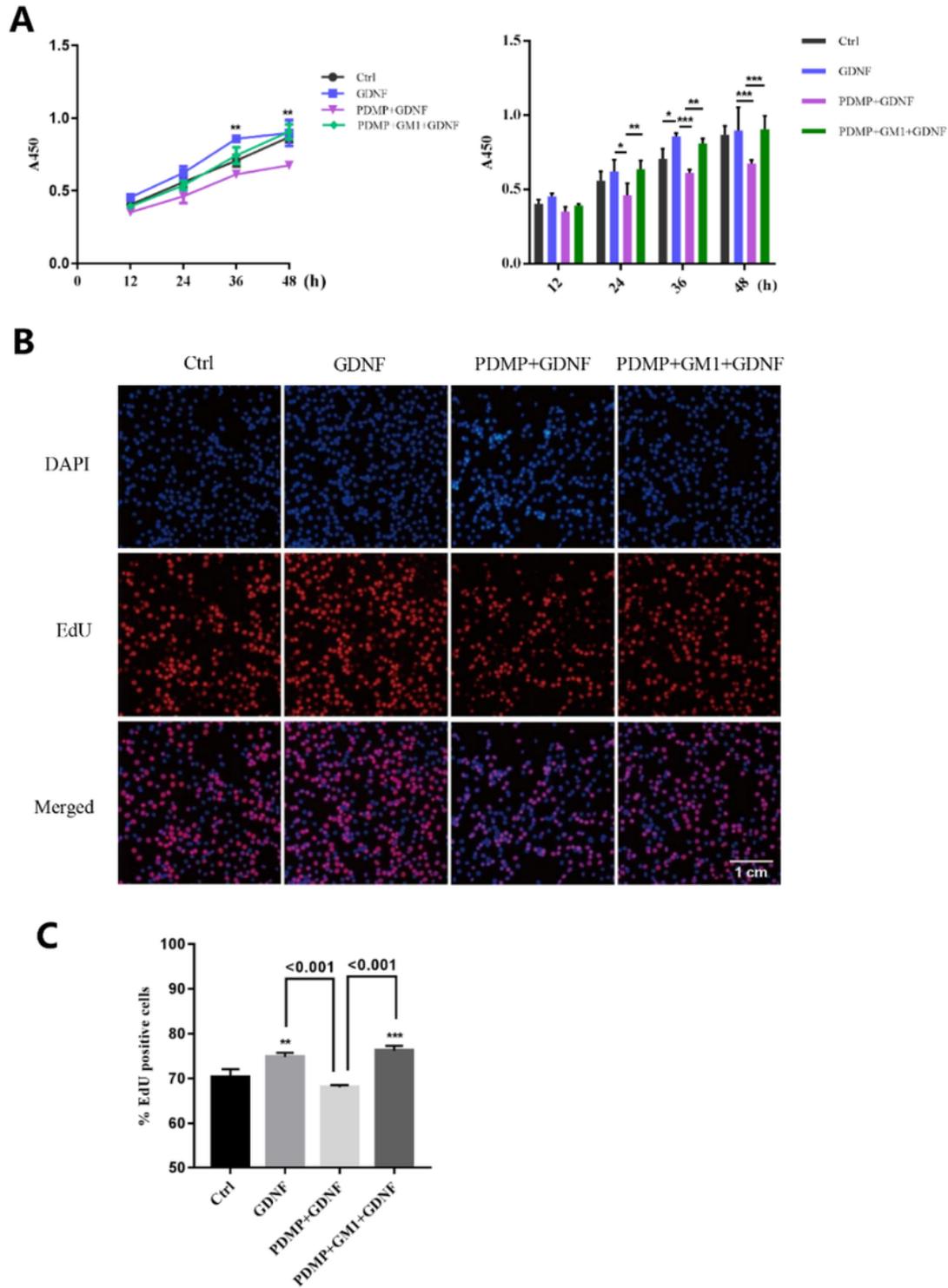
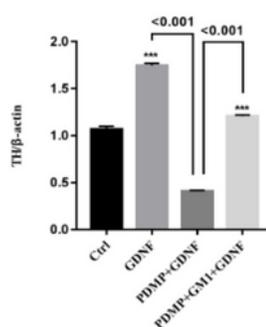
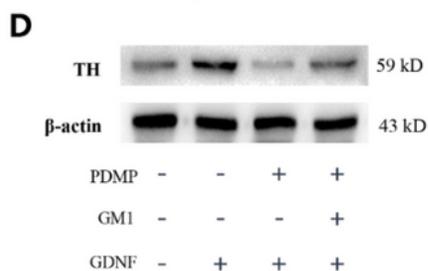
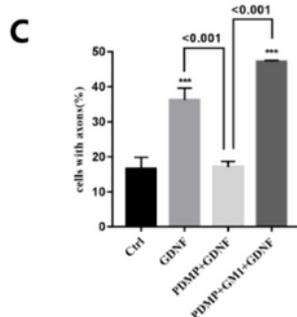
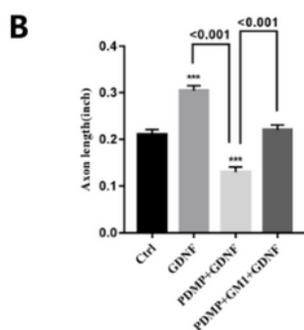
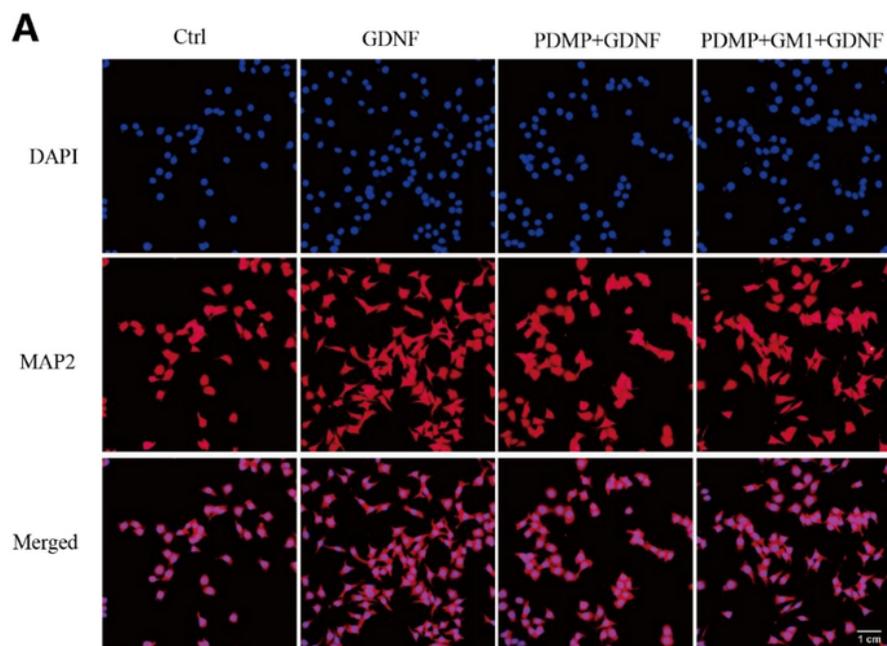


Figure 2

GM1 deficiency inhibits the proliferation of SH-SY5Y cells induced by GDNF. (A) SH-SY5Y cells were treated with 20  $\mu$ M PDMP with or without 100  $\mu$ M GM1 for 24 h followed by incubation in serum-free medium for 4 h. CCK-8 assays were used to detect the difference in cell proliferation among the 4 groups at 12, 24, 36 and 48 h after GDNF treatment. (B, C) SH-SY5Y cells were treated with 20  $\mu$ M PDMP with or without 100  $\mu$ M GM1 for 24 h followed by incubation in serum-free medium for 4 h. Subsequently, cells were stimulated with 50 ng/mL GDNF for 36 h. SH-SY5Y cell proliferation was evaluated by an EdU staining assay. The percentage of EdU-positive cells was quantified by ImageJ software. Percentage of EdU-positive cells = number of red nuclei / number of blue nuclei. Scale bar = 50  $\mu$ m (\* represents the comparison between the experimental group and the control group: \*\*P < 0.01, \*\*\*P < 0.001; GDNF group versus PDMP+GDNF group: P < 0.001; PDMP+GDNF group versus PDMP+GM1+GDNF group: P < 0.001).



**Figure 3**

GM1 deficiency inhibits the pro-differentiation effect of GDNF on SH-SY5Y cells. SH-SY5Y cells were treated with 20  $\mu$ M PDMP with or without 100  $\mu$ M GM1 for 24 h followed by incubation in serum-free medium for 4 h. Subsequently, cells were stimulated with 50 ng/mL GDNF for 36 h. (A) MAP2 (red fluorescence) and DAPI (blue fluorescence) immunofluorescent staining were performed to observe the neurites and nuclei. (B, C) The axon length and the number of cells with axons were quantified by ImageJ

software. Bar = 50  $\mu$ m (\* represents the comparison between the experimental group and the control group: \*\*\* $P < 0.001$ ; GDNF group versus PDMP+GDNF group:  $P < 0.001$ ; PDMP+GDNF group versus PDMP+GM1+GDNF group:  $P < 0.001$ ). (D) The expression of TH protein was determined by Western blot (\* represents the comparison between the experimental group and the control group: \*\*\* $P < 0.001$ ; GDNF group versus PDMP+GDNF group:  $P < 0.001$ ; PDMP+GDNF group versus PDMP+GM1+GDNF group:  $P < 0.001$ ).

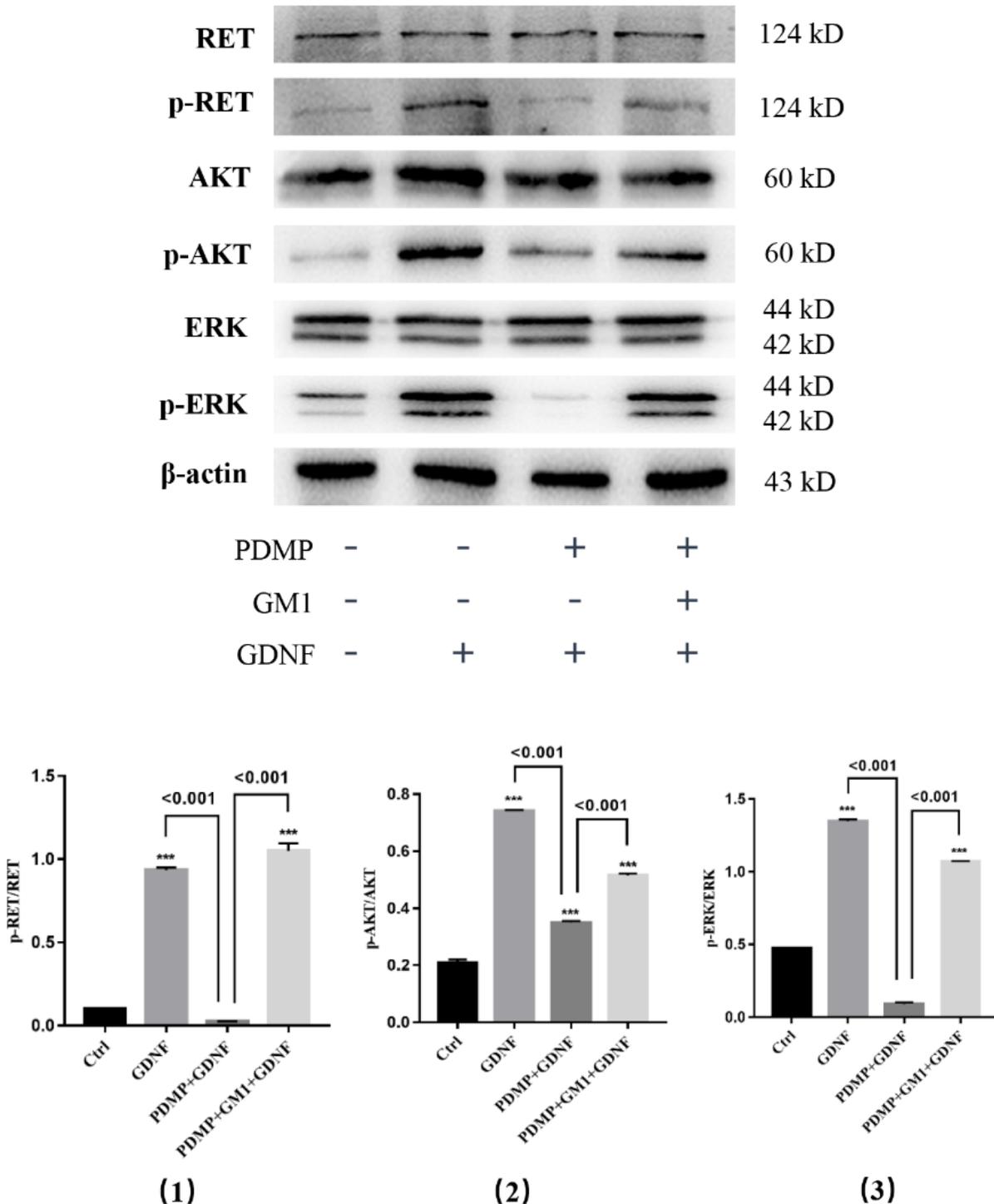
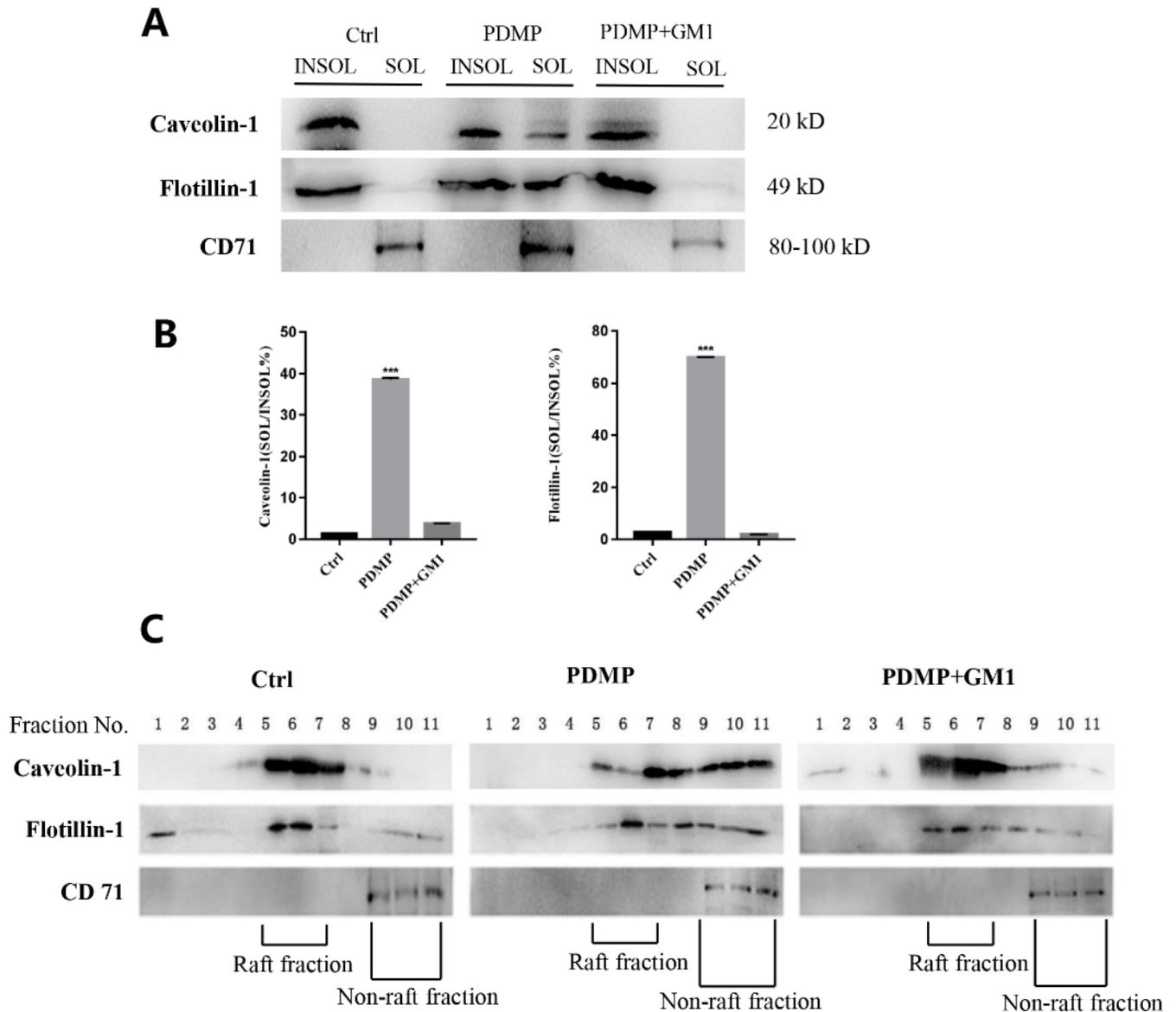


Figure 4

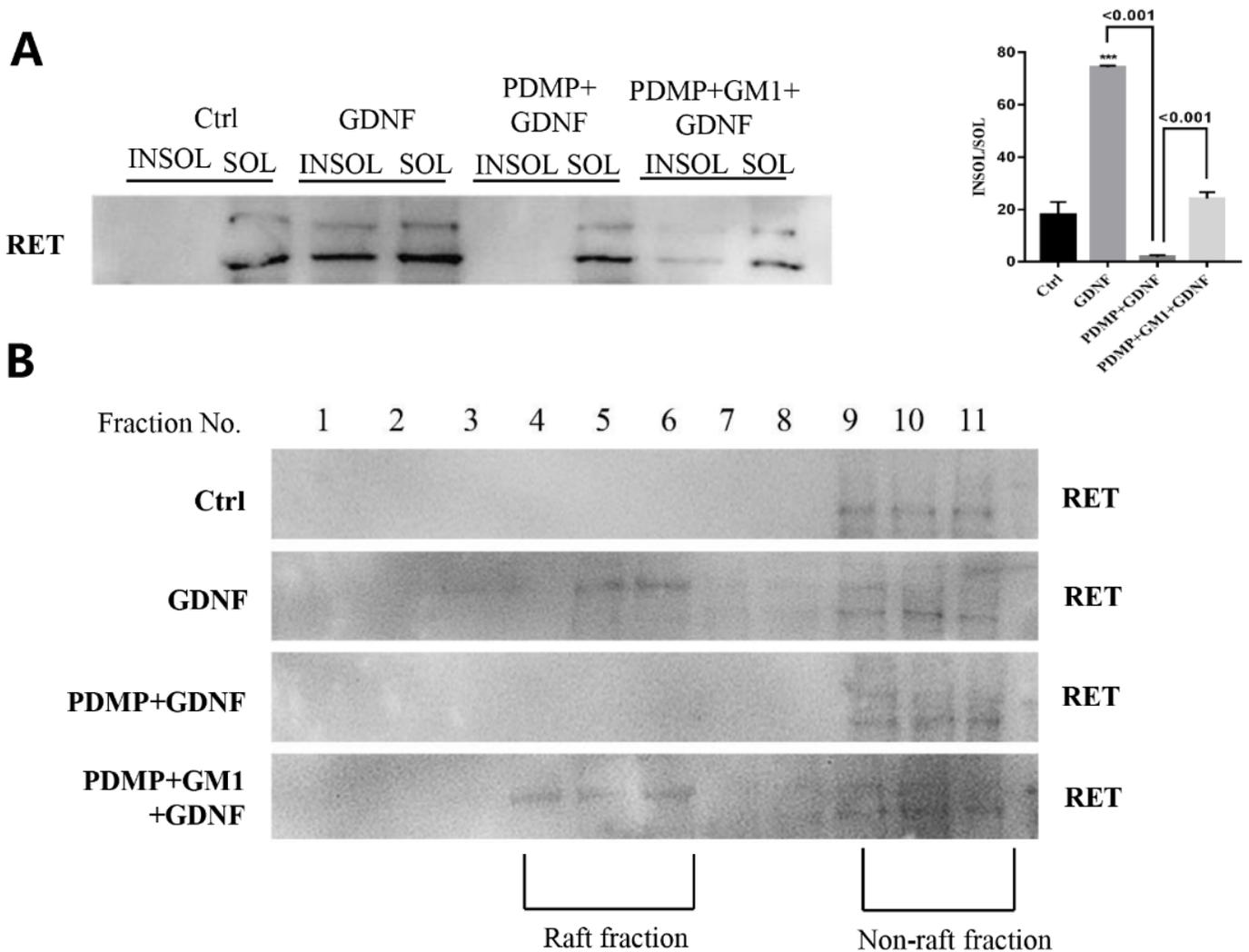
GM1 deficiency inhibits the GDNF signaling pathway. SH-SY5Y cells were treated with 20  $\mu$ M PDMP with or without 100  $\mu$ M GM1 for 24 h followed by incubation in serum-free medium for 4 h. Subsequently, cells were stimulated with 50 ng/mL GDNF for 20 min. The levels of relevant proteins were assessed by Western blot, and densitometric analysis was performed by ImageJ software (\* represents the comparison between the experimental group and the control group: \*\*\*P < 0.001; GDNF group versus PDMP+GDNF group: P < 0.001; PDMP+GDNF group versus PDMP+GM1+GDNF group: P < 0.001).



**Figure 5**

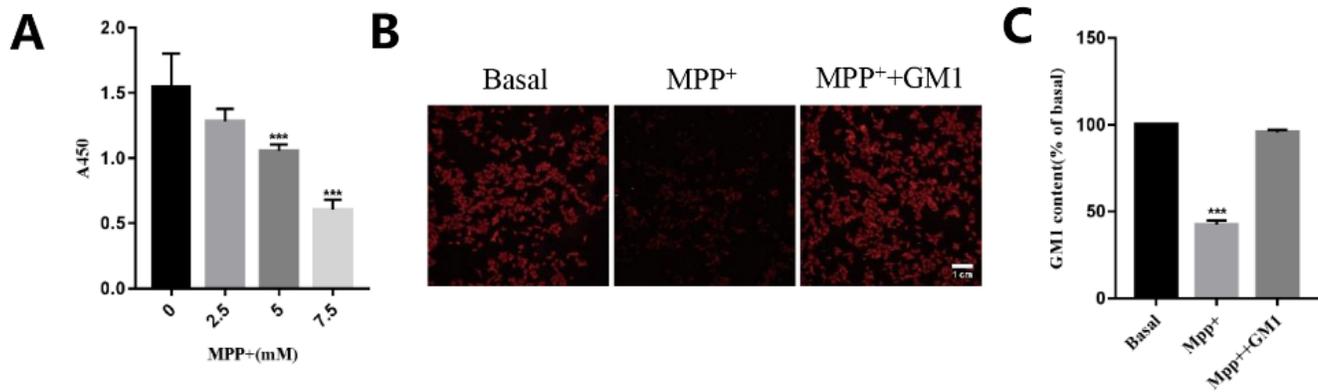
GM1 deficiency alters the assembly of lipid rafts. SH-SY5Y cells were treated with 20  $\mu$ M PDMP with or without 100  $\mu$ M GM1 for 24 h. (A, B) Lipid rafts were isolated by Triton X-100 solubilization. The expression of caveolin-1, flotillin-1 and CD71 was assessed by Western blot analysis, and densitometric analysis was performed by ImageJ software (\* represents the comparison between the experimental group and the control group: \*\*\*P < 0.001). (C) Lipid rafts were isolated by OptiPrep™ density gradient

centrifugation. Distribution of caveolin-1, flotillin-1 and CD71 were analyzed by Western blot using fractions separated by OptiPrep™ density gradient centrifugation. Lipid rafts were located in fractions 5–7.



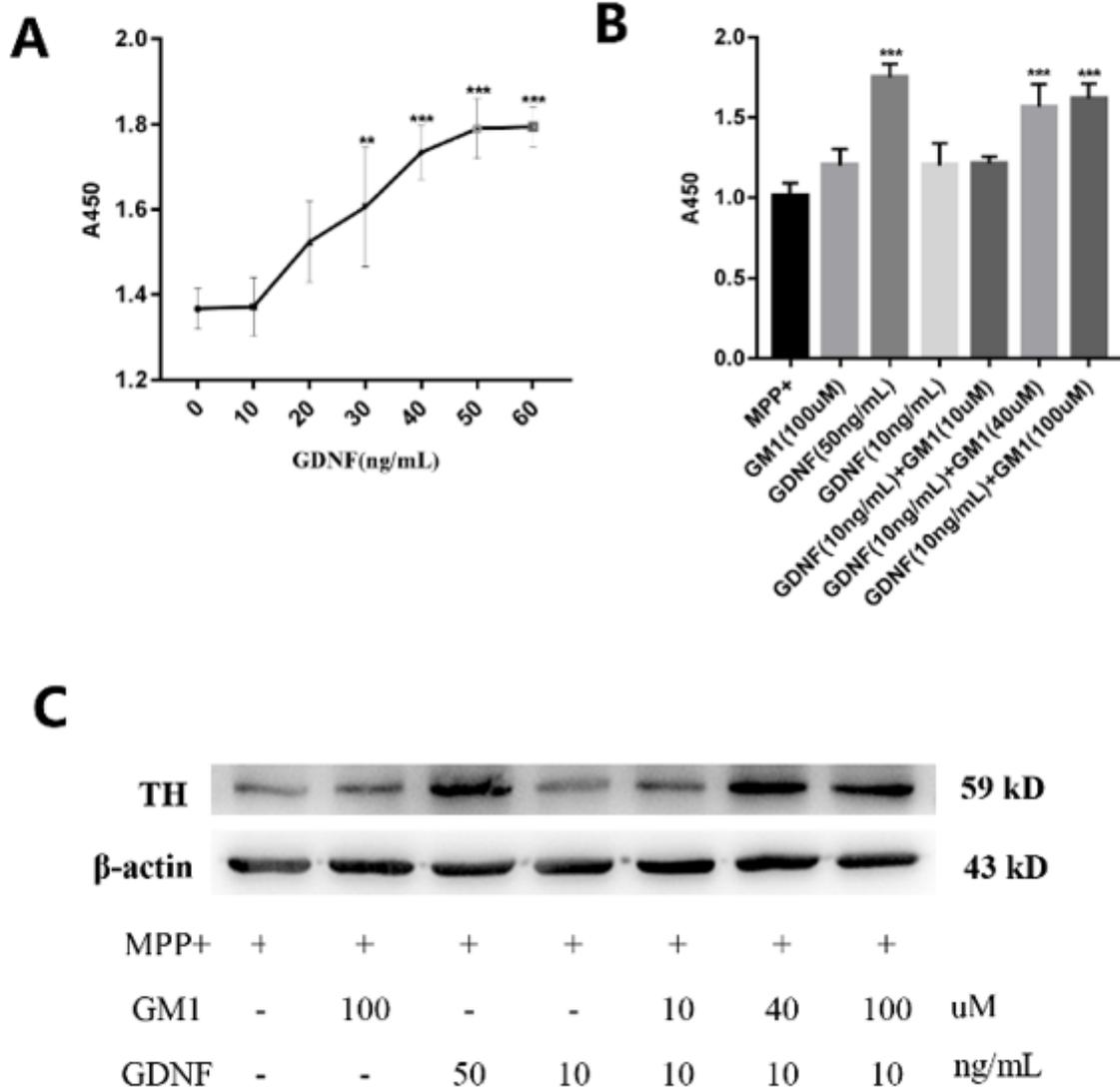
**Figure 6**

GM1 deficiency inhibits the translocation of RET to lipid rafts. SH-SY5Y cells were treated with 20 μM PDMP with or without 100 μM GM1 for 24 h followed by incubation in serum-free medium for 4 h. Subsequently, cells were stimulated with 50 ng/mL GDNF for 20 min. (A) Lipid rafts were isolated by Triton X-100 solubilization. The expression of RET was assessed by Western blot analysis, and the band intensities of the Western blots were quantified using ImageJ software (\* represents the comparison between the experimental group and the control group: \*\*\*P < 0.001; GDNF group versus PDMP+GDNF group: P < 0.001; PDMP+GDNF group versus PDMP+GM1+GDNF group: P < 0.001). (B) Lipid rafts were isolated by OptiPrep™ density gradient centrifugation. Localization of RET in the density gradient was analyzed by Western blot analysis using fractions separated by OptiPrep™ density gradient centrifugation.



**Figure 7**

GM1 content is decreased in SH-SY5Y cells treated with MPP+. (A) SH-SY5Y cells were treated with MPP+ (2.5 mM, 5 mM, 7.5 mM) for 24 h. CCK-8 assay was used to assess cell viability (\* represents the comparison between the experimental group and the control group: \*\*\*P < 0.001). (B, C) SH-SY5Y cells were treated with 5 mM MPP+ with or without 100  $\mu$ M GM1 for 24 h and then labeled with CtxB-Alexa 647 (red fluorescence). GM1 content was quantified by ImageJ software. Scale bar = 50 $\mu$ m (\* represents the comparison between the experimental group and the control group: \*\*\*P < 0.001).



**Figure 8**

GM1 potentiates the protective effect of GDNF in an MPP<sup>+</sup>-induced PD cell model. (A) Dose-dependent increase in cell viability assessed by CCK-8 assay. (\* represents the comparison between the experimental group and the control group: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001). (B, C) MPP<sup>+</sup>-injured SH-SY5Y cells were treated with various concentrations of GM1 for 24 h followed by incubation in serum-free medium for 4 h. Subsequently, cells were stimulated with different concentrations of GDNF for 36 h. CCK-8 assay and Western blot assay were used to assess cell viability and the expression of TH protein, respectively. (\* represents the comparison between the experimental group and the control group: \*\*\*P < 0.001).