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Brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor inhibit ferrous iron influx via divalent metal transporter 1 and iron regulatory protein 1 regulation in ventral mesencephalic neurons

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ABSTRACT

Iron accumulation is observed in the substantia nigra of patients with Parkinson's disease. However, it is unknown whether neurotrophic factors, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) participate in the modulation of neuronal iron metabolism. Here, we investigated the effects and underlying mechanisms of BDNF and GDNF on the iron influx process in primary cultured ventral mesencephalic neurons. 6-hydroxydopamine-induced enhanced ferrous iron influx via improper up-regulation of divalent metal transporter 1 with iron responsive element (DMT1 + IRE) was consistently relieved by BDNF and GDNF. Both the mRNA and protein levels of DMT1 + IRE were down-regulated by BDNF or GDNF treatment alone. We further demonstrated the involvement of iron regulatory protein 1 (IRP1) in BDNF- and GDNF-induced DMT1 + IRE expression. Extracellular-regulated kinase 1/2 (ERK1/2) and Akt were activated and participated in these processes. Inhibition of ERK1/2 and Akt phosphorylation abolished the down-regulation of IRP1 and DMT1 + IRE induced by BDNF and GDNF. Taken together, these results show that BDNF and GDNF ameliorate iron accumulation via the ERK/Akt pathway, followed by inhibition of IRP1 and DMT1 + IRE expression, which may provide new targets for the neuroprotective effects of these neurotrophic factors.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized in its late phase by the sustained loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) [1–3]. Extensive evidence has shown that selective high levels of iron and oxidative stress in the SNpc play a key role in PD pathogenesis [4–8]. Excess iron can generate high levels of reactive oxygen species (ROS) through the Fenton reaction by acting on the dopamine metabolite H_2O_2 [9–11]. Iron homeostasis is achieved by several iron trafficking proteins [12,13]. We previously reported that the aberrant regulation of divalent metal transporter 1 (DMT1/Nramp2/SLC11A2) by the iron responsive element (IRE, DMT1 + IRE) may account for the abnormal iron accumulation in the SN of PD animal models [14,15], which agrees with a study showing that increases in DMT1 were associated with degeneration of DAergic neurons in PD patients and were a common phenomenon in animal models of PD [16]. Thus, DMT1 may serve as a promising molecular target for therapeutic interventions that would slow PD progression [16].

Autopsy results of PD patients showed marked reductions of brain-derived neurotrophic factor (BDNF) mRNA and protein levels in the striatum and SN compared with other brain regions [17–19]. The replenishment of neurotrophic factors (NTFs), such as BDNF and glial cell line-derived neurotrophic factor (GDNF), to an appropriate site of action may provide important neuronal support in PD [20,21]. BDNF and GDNF, the most often studied neurotrophins related to neurodegeneration, belong to two different families of NTFs. By binding to their high-affinity receptors tyrosine kinase receptor B (TrkB) and GDNF family receptor α (GFRα), respectively, BDNF and GDNF could promote the survival and morphological differentiation of midbrain

Abbreviations: 6-OHDA, 6-hydroxydopamine; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; DMT1 + IRE, divalent metal transporter 1 with iron responsive element; ERK1/2, extracellular-regulated kinase1/2; GDNF, glial cell line-derived neurotrophic factor; IRP 1, iron regulatory protein 1; MEK, mitogen-activated protein kinase; NTFs, neurotrophic factors; PD, Parkinson's disease; PI3K, phosphatidylinositol 3-kinase; SNpc, substantia nigra pars compacta; TfR1, transferrin receptor 1; TH, tyrosine hydroxylase; VM, ventral mesencephalic

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DAergic neurons and increase dopamine up-take [22–27]. Using organotypic culture, BDNF treatment after 6-hydroxydopamine (6-OHDA) lesion has been reported to not only improve cell survival but also transcriptionally up-regulate tyrosine hydroxylase (TH) mRNA expression [25]. Other investigations have shown that GDNF can prevent the death of dopamine neurons and promote functional recovery in 1-methyl-4-phenyl-l 1, 2, 3, 6- tetrahydropyridine (MPTP)-treated rodent and nonhuman primate PD models *in vivo* [28–30]. However, little is known about the ability of these NTFs to regulate trace metal ion metabolism, such as iron, in the central nervous system (CNS).

In this report, we tested the hypothesis that BDNF and GDNF could protect primary cultured ventral mesencephalic (VM) neurons from 6-OHDA induced iron accumulation by regulating the iron importer DMT1 + IRE and possibly its post-transcriptional activator, iron regulatory protein 1 (IRP1). Utilizing pharmacological approaches, we also aimed to elucidate the involvement of intracellular pathways in this process.

2. Materials and methods

2.1. Materials

All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Qingdao University. Dulbecco′s modified Eagle′s medium Nutrient Mixture-F12 (DMEM/F12) and B27 were obtained from Gibco (Grand Island, NY, USA). Recombinant BDNF, GDNF and 6-OHDA were obtained from Sigma (St Louis, MO, USA). The specific phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and MEK inhibitor PD98059 were purchased from Beyotime (Jiangsu, China). The primary antibodies rabbit anti-DMT1 + IRE, rabbit anti-transferrin receptor 1 (TfR1), and rabbit anti-IRP1 were obtained from Alpha Diagnostic (San Antonio, TX, USA); rabbit anti-ERK1/2, rabbit anti-phospho-ERK1/2 (T202/Y204), rabbit anti-Akt and rabbit anti-phospho-Akt (Ser473) were obtained from Cell Signaling Technology (Beverly, MA, USA); and mouse anti-TH was obtained from Sigma (St Louis, MO, USA). All other chemicals and regents were of the highest grade available from local commercial sources.

2.2. VM neuron culture and pharmacological treatments

Primary rat VM neuron cultures were obtained from embryonic day 14–15 Wistar rats as described previously by our laboratory [31,32]. Briefly, VM tissues were dissected from embryonic day 14–15 rat brains and dissociated mechanically. After centrifugation, cells were suspended in DMEM/F12 supplemented with 2% B27, 100 U/ml penicillin, and 100 μg/ml streptomycin and seeded on poly-D-lysine-coated cover slips or 12-well culture plates at a density of 6×10^5 cells/ml or 1.5×10^6 cells/ml, respectively. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 18 h, and then, the medium was changed. Cultures were replenished with fresh medium 3 days later and used at 6 days *in vitro* (DIV). Neuron purity was approximately 96% based on immunofluorescence staining with the specific neuron marker MAP2. Approximately 2% of the neurons exhibited TH positivity, indicating DAergic neurons (data not shown).

Neuronal treatments with pharmacological agents were performed without B27 supplement. For both BDNF and GDNF, 10 ng/ml is a commonly used concentration that exerts neuroprotective effects *in vitro* [33–37]. A concentration of 10 μM 6-OHDA was used based on our previous study, which does not cause significant cell loss [31].

2.3. Calcein loading of cells and ferrous iron influx assay

The ferrous iron influx into neurons was determined by measuring the quenching of calcein fluorescence as previously described by our laboratory [14,38]. Cells seeded onto coverslips were incubated with calcein-AM (0.5 μM final concentrations) in Hepes-buffered saline (HBS, 10 mM Hepes, 150 mM NaCl, pH 7.4) for 30 min at 37 °C. After three washes with HBS, the cells were perfused with 0.5 mM ferrous iron (ferrous sulfate in ascorbic acid solution, 1:44 molar ratio, pH 6.0) to maintain extracellular stabilization of the iron concentration. Then, calcein fluorescence was recorded using an Olympus FV500 confocal microscope at 488 nm excitation and 525 nm emission wavelengths, and fluorescence intensity was measured every 3 min for 10 repetitions.

The fluorescence intensity representing the mean value of 35–40 separate cells from four separate fields was monitored at × 20 magnification at each time point and processed with Fluoview 5.0 Software.

2.4. Western blots

After three washes with cold PBS, the cells were lysed with lysis buffer. Insoluble material was removed by centrifugation. A total of 20–40 μg of protein was separated using 10% SDS-polyacrylamide gels and then transferred onto PVDF membranes. After 2 h of blocking with 10% non-fat milk at room temperature, the membranes were incubated with primary antibodies, including a DMT1 + IRE antibody (1:800), IRP1 (1:1000), TfR1 (1:800), Akt, phospho-Akt, ERK1/2 and phospho-ERK1/2 (1:1000) overnight at 4 °C. β-actin was detected by an anti-β-actin monoclonal antibody (1:8000) according to a similar procedure to ensure equal samples of protein. Membranes were then incubated in peroxidase-conjugated secondary anti-rabbit secondary antibody (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 h, and excess IgG was removed with TBST washes. Cross-reactivity was visualized using ECL western blotting detection reagents and then analyzed with scanning densitometry by a UVP Image System.

2.5. Total RNA extraction and quantitative real-time PCR

Total RNA was isolated from neurons treated as described above using the Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Then, 2 μg of total RNA was reversed transcribed in a 20 μl reaction with oligo-dT primers using a reverse-transcription system (Promega). Quantitative real-time RCR was used to detect the changes in DMT1 + IRE. A TaqMan probe and primers were designed with respect to the sequences using the default settings of Primer Express 2.0 (PE Applied Biosystems). Each set of primers was used with a TaqMan probe labeled at the 5'-end with the 6-carboxyfluorescein (FAM) reporter dye and at the 3'-end with the 6-carboxy-tetramethylrhodamine (TAMRA) quencher dye. The following primers and probes were employed. DMT1 + IRE: sense: 5′-TGG CTG TCA CGA GTG CTT ACA-3′, antisense: 5′-CCA TGG CCT TGG ACA GCT ATT-3′, probe: 5′-TTA CCC TGT AGC ATT AGG CAG CAC C-3′; GAPDH: sense: 5′-CCC CCA ATG TAT CCG TTG TG-3′, antisense: 5′-GTA GCC CAG GAT GCC CTT TAG T-3′, probe: 5′-TCT GAC ATG CCG CCT GGA GAA ACC-3′.

Amplification and detection were performed with the following conditions: an initial hold at 95 °C for 10 s, followed by 35 cycles at 95 °C for 5 s and 60 °C for 45 s.

2.6. Double immunofluorescence labeling

Primary cultured VM neurons were detected with an anti-MAP2 antibody. Briefly, cells were fixed with 4% paraformaldehyde followed by blocking with PBS containing 0.3% Triton X-100 and 10% normal goat serum. Then, cells were incubated with primary antibodies, including mouse anti-MAP2 (1:500), rabbit anti-phospho-ERK1/2 (1:200) or rabbit anti-phospho-Akt (1:200) overnight at 4 °C. The cells were then washed with PBS and incubated for 1 h with the secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG (H + L) (1:500) and Alexa Fluor® 555 goat anti-rabbit IgG (H + L) (1:500) at room temperature. After rinsing with PBS, the cells were examined using a Fluoview FV500 laser confocal scanning microscope. Control coverslips incubated

in a solution without primary antibodies showed no staining (data not shown).

2.7. Statistical analysis

The results are presented as the mean ± S.E.M. An unpaired Student's t test was performed to analyze the differences between two groups. One-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test, was used to compare differences between three or more groups. Iron influx studies were analyzed using two-way ANOVA. A probability of $P < 0.05$ indicated statistical significance.

3. Results

3.1. BDNF and GDNF attenuated 6-OHDA-induced ferrous iron influx in primary cultured VM neurons

The fluorescent dye calcein was used to monitor the ferrous iron influx of VM neurons with 0.5 mM ferrous iron perfusion. The fluorescence intensity declined gradually inside cells, indicating a transmembrane ferrous iron influx. In agreement with our data in MES23.5 cells [15], in 10 μM 6-OHDA-treated cells, more rapid fluorescence quenching and a significant decrease in the fluorescence intensity occurred compared with controls. However, pretreatment with 10 ng/ml BDNF or 10 ng/ml GDNF for 4 h fully blocked this process and produced a fluorescence intensity similar to the control levels (Fig. 1A,B), indicating that BDNF and GDNF suppress the increased ferrous iron influx caused by 6-OHDA.

3.2. BDNF and GDNF down-regulated DMT1 + IRE expression in primary cultured VM neurons

DMT1 is the importer of ferrous iron, and previous research has shown that the brain appears to express a higher amount of DMT1 with IRE compared with DMT1 without IRE [39]. In the present study, VM neurons were incubated with a vehicle, BDNF (10 ng/ml), GDNF (10 ng/ml), and 6-OHDA (10 μM) for 24 h or incubated with BDNF (10 ng/ml) or GDNF (10 ng/ml) for 4 h prior to 6-OHDA (10 μM) treatment for another 24 h, and the protein and mRNA levels of DMT1 + IRE were determined. As shown in Fig. 2A and B, singular BDNF or GDNF treatment suppressed DMT1 + IRE protein levels to nearly 26% or 21% below the control level, respectively. DMT1 + IRE protein levels in 6-OHDA-treated VM neurons were up-regulated 1.5-fold compared with the control level. However, pretreatment with BDNF fully blocked 6-OHDA-induced DMT1 + IRE up-regulation. Similar effects were observed in the cells pretreated with GDNF. In addition, no significant differences were observed in the protein expression of DMT1 + IRE between the BDNF and GDNF groups (Fig. 2C).

To further investigate whether this altered expression of DMT1 + IRE protein was due to the change in DMT1 + IRE mRNA transcription, quantitative real-time PCR was conducted. GAPDH mRNA showed stable expression in the different groups (data not shown). BDNF and GDNF incubation down-regulated DMT1 + IRE mRNA levels to 50% and 40% of the basal levels, respectively, and thus fully abolished the 6-OHDA-induced up-regulation of DMT1 + IRE (Fig. 2D,E). These results indicated that both BDNF and GDNF could exert suppressive effects on DMT1 + IRE expression at both the protein and mRNA levels.

3.3. BDNF- and GDNF-induced down-regulation of DMT1 + IRE was IRE/IRP dependent in primary cultured VM neurons

We next investigated whether BDNF and GDNF modulate DMT1 + IRE directly or produce an indirect outcome by targeting other molecules. IRPs register cytosolic iron concentrations and post-transcriptionally regulate the expression of iron metabolism genes, such as DMT1 + IRE and TfR1, by binding to the IRE in the 3' untranslated region (UTR). IRP1 is much more abundant than IRP2 in most cells and tissues, and IRP1 commonly makes a greater contribution to IRE-binding activity than IRP2 [40]. Therefore, we tested the effects of BDNF and GDNF on IRP1 levels. We observed approximately 19% and 17% down-regulation of IRP1 protein levels in BDNF (10 ng/ml)- and GDNF (10 ng/ml)-treated VM neurons, respectively. As demonstrated previously in 6-OHDA-treated MES23.5 cells [15], 10 μM 6-OHDA treatment up-regulated IRP1 protein levels by 20% in primary cultured VM neurons. As expected, BDNF and GDNF (10 ng/ml) fully abolished the 6-OHDA-induced up-regulation of IRP1 (Fig. 3A,B). Additionally, no changes were observed between the BDNF and GDNF groups (Fig. 3C).

To further confirm that BDNF and GDNF participated in IRP1 regulation, we detected the expression of TfR1 after BDNF and GDNF treatment. Similar to the regulation of DMT1 + IRE induced by BDNF and GDNF, the results showed that BDNF or GDNF treatment suppressed the basal levels of TfR1 expression, and pretreatment with these NTFs fully blocked the 6-OHDA-induced TfR1 up-regulation (Fig. 4).

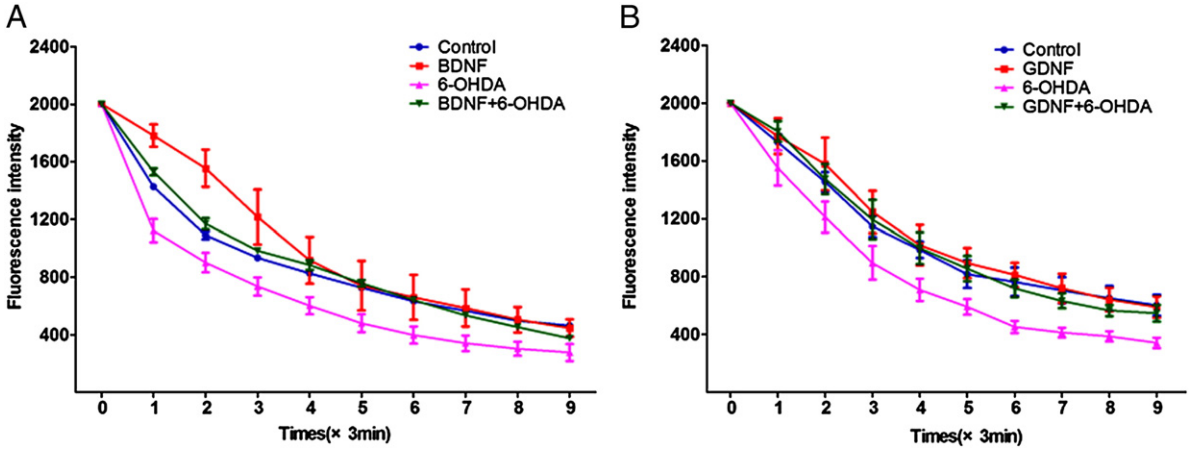

Fig. 1. BDNF, GDNF attenuated iron influx in 6-OHDA-treated VM neurons. Ferrous iron influx in VM neurons was determined by quenching calcein fluorescence, an indicator of intracellular iron levels. When 0.5 mM ferrous iron was used to perfuse the cells, a more rapid decrease in fluorescence intensity occurred in cells treated with 6-OHDA compared with controls, indicating increased ferrous iron influx in 6-OHDA-treated cells. The fluorescence intensity was restored to control levels when cells were pretreated with 10 ng/ml BDNF (A, two-way ANOVA, $F = 43.620$, $P < 0.05$, 6-OHDA group compared with control; $P < 0.05$, BDNF + 6-OHDA group compared with 6-OHDA group) and 10 ng/ml GDNF for 4 h (B, two-way ANOVA, $F = 34.744$, $P < 0.05$, 6-OHDA group compared with control; $P < 0.05$, GDNF + 6-OHDA group compared with 6-OHDA group). The fluorescence intensity represents the mean value of 35–40 separate cells monitored at × 20 magnification at each time point. Data are presented as the mean ± S.E.M. of four independent experiments.

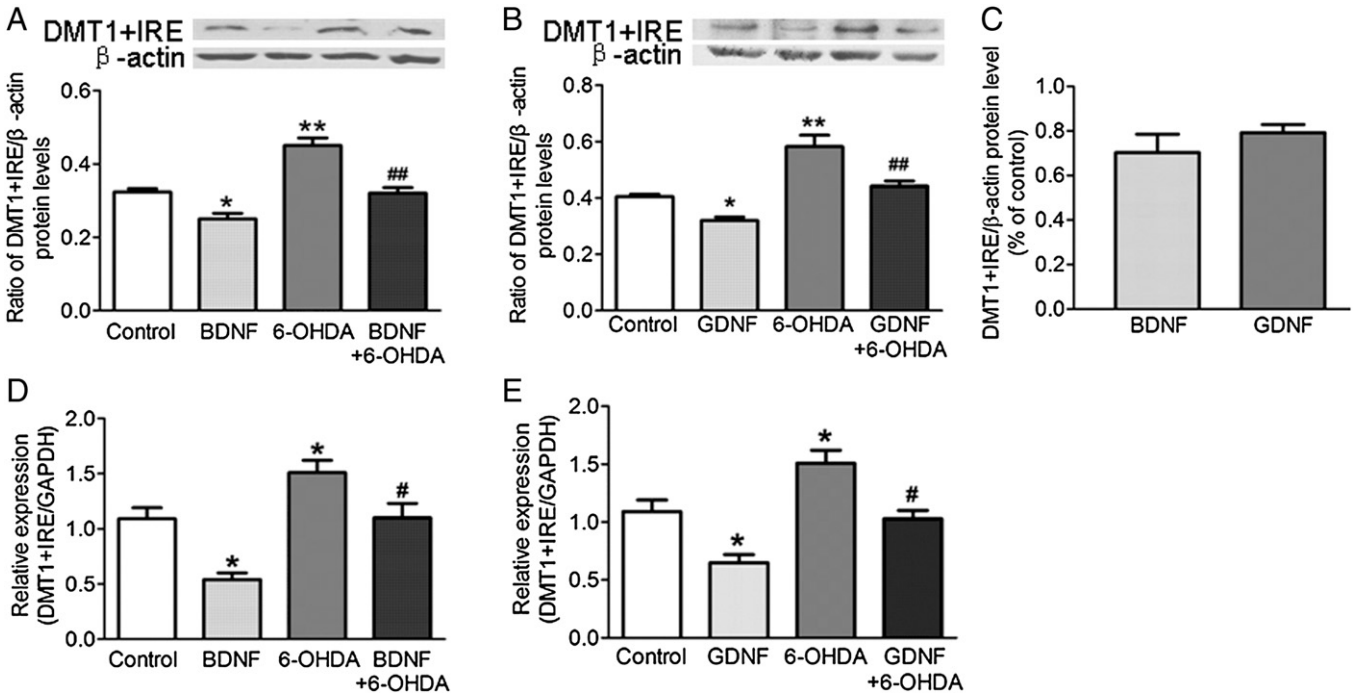

Fig. 2. BDNF and GDNF down-regulated DMT1 + IRE expression and abolished 6-OHDA-induced DMT1 + IRE upregulation in VM neurons. A and B: DMT1 + IRE protein levels were up-regulated in 6-OHDA-treated primary cultured DIV6 VM neurons, which could be fully abolished by pretreatment with BDNF or GDNF for 4 h prior to 6-OHDA incubation. Singular BDNF or GDNF treatment down-regulated DMT1 + IRE levels. β-actin was used as a loading control. Data are presented as the ratio of DMT1 + IRE to β-actin. C: No significant differences were observed in the expression of DMT1 + IRE between the BDNF and GDNF groups. D and E: DMT1 mRNA levels were up-regulated in the 6-OHDA group and restored to control levels with 10 ng/ml BDNF or 10 ng/ml GDNF pretreatment. Down-regulation of DMT1 + IRE mRNA was observed when VM cultures were subjected to BDNF or GDNF. Data are presented as fold changes in mRNA expression of treatment vs. control. Each bar represents the mean ± S.E.M. of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the 6-OHDA group.

These results confirmed our hypothesis that DMT1 + IRE serves as a target of BDNF and GDNF through the regulation of the IRE/IRP system.

3.4. BDNF- and GDNF- induced activation of the MEK/ERK and PI3K/Akt signaling pathways in primary cultured VM neurons

BDNF binds to its high affinity receptor TrkB, and GDNF specifically binds to GFRα, both of which can induce the activation of the MEK/ERK and PI3K/Akt signaling pathways, which are important for neuronal survival [41,42]. The activation of ERK1/2 and Akt depends on their phosphorylation; hence, phospho-specific antibodies can be used to assess their relative activities. VM neurons were incubated with BDNF and GDNF for the indicated periods (0, 0.5, 1, 2 and 4 h) and then collected for western blot analysis of phospho-ERK1/2 and phospho-Akt. As shown in Fig. 5A and E, we determined that ERK1/2 phosphorylation occurred in a time-dependent manner, with maximum activation observed within 0.5 h in both the BDNF- and GDNF-treated groups. However, the activation was more dramatic (up to 7-fold greater) in BDNF-treated cells compared with GDNF-treated cells. Phospho-ERK1/2 levels remained above basal levels after 4 h of treatment in the BDNF group; however, phospho-ERK1/2 had returned to basal levels in the GDNF group. Akt also underwent time-dependent phosphorylation in response to BDNF and GDNF treatment in VM neurons (Fig. 5C,G). Maximum activation was achieved within 30 min of treatment in both the

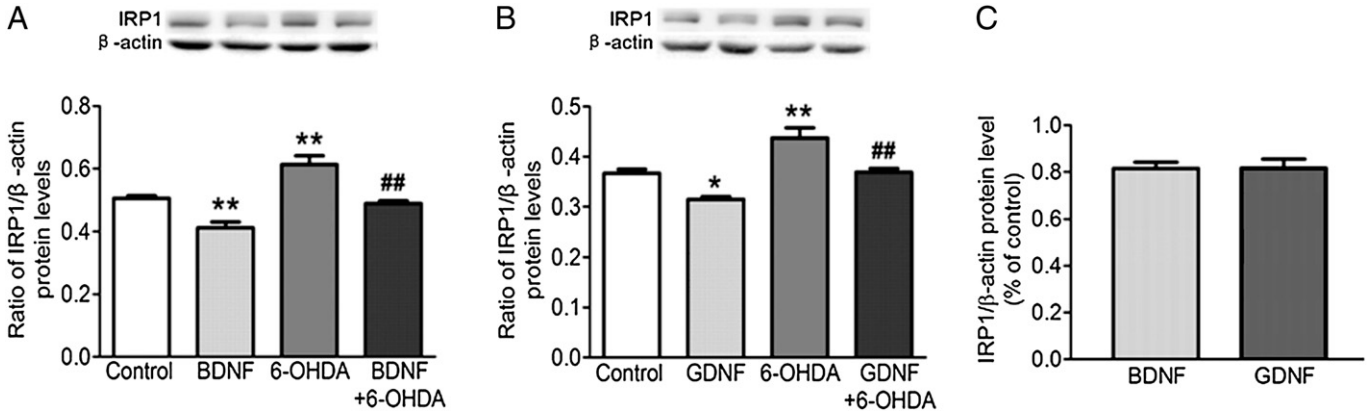

Fig. 3. BDNF and GDNF abolished 6-OHDA-induced IRP1 upregulation in VM neurons. A, B: Pretreatment with BDNF (A) or GDNF (B) for 4 h prior to 6-OHDA incubation effectively prevented the 6-OHDA-induced up-regulation of IRP1. A decline in IRP1 expression was observed in BDNF and GDNF (10 ng/ml) alone treatment groups. C: No significant differences were observed between BDNF and GDNF treatment. β-actin was used as a loading control. Each bar represents the mean ± S.E.M. of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control, $^{\#\#}P < 0.01$, compared with the 6-OHDA group.

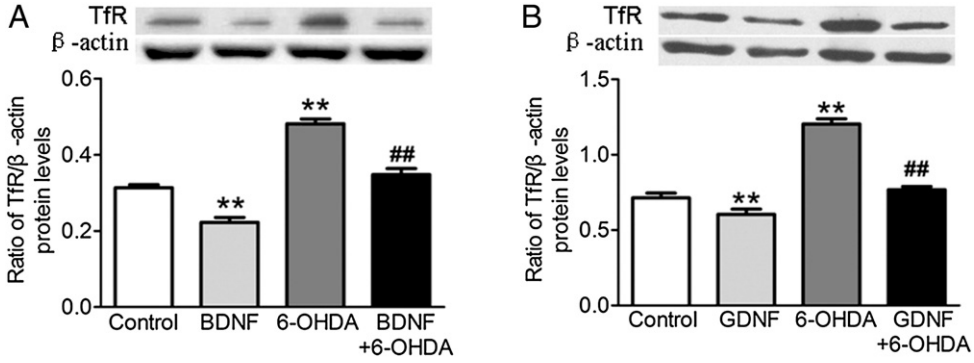

Fig. 4. BDNF and GDNF abolished 6-OHDA-induced TfR1 upregulation in VM neurons. TfR1 protein levels were up-regulated in 6-OHDA-treated primary cultured VM neurons, and this up-regulation was fully abolished by pretreatment with BDNF and GDNF for 4 h prior to 6-OHDA incubation. BDNF or GDNF treatment alone caused the down-regulation of TfR1 levels. β-actin was used as a loading control. Each bar represents the mean ± S.E.M. of four independent experiments. $^{**}P < 0.05$, $^{\#\#}P < 0.01$, compared with control.

BDNF- and GDNF-treated cells. However, as observed for ERK, the magnitude of Akt activation was significantly lower in the GDNF-treated groups compared with the BDNF-treated groups. No changes in the total ERK1/2 and total Akt levels were observed at any time point.

We also examined the expression of the phosphorylated forms of ERK1/2 and Akt at the cellular level. BDNF and GDNF treatment for 30 min increased phospho-ERK1/2 and phospho-Akt immunoreactivity in MAP2-positive neurons. PD98059 (5 μM), an inhibitor of MEK, and LY294002 (2.5 μM), an inhibitor of PI3K, were able to block these effects (Fig. 5B,D,F,G).

3.5. MEK/ERK and PI3K/Akt pathways participate in the BDNF- and GDNF-induced down-regulation of IRP1 and DMT1 + IRE

To further explore the possibility of the participation of the MEK/ERK and PI3K/Akt pathways in the regulation of IRP1 and DMT1 + IRE expression, we measured the effects of the specific inhibitors PD98059 (5 μM) and LY294002 (2.5 μM) on the protein and mRNA levels of IRP1 and DMT1 + IRE. Primary cultured VM neurons were pretreated with specific inhibitors for 0.5 h, and then, 10 ng/ml BDNF or GDNF were added for another 24 h. As shown in Fig. 6A and D, pretreatment with the inhibitors fully blocked BDNF- and GDNF-mediated down-regulation of IRP1, suggesting that the ERK1/2 and Akt pathways mediated the IRP1 down-regulation induced by BDNF and GDNF. In the presence of these signaling inhibitors, BDNF- and GDNF-induced down-regulation of the DMT1 + IRE protein (Fig. 6B,E), and mRNA (Fig. 6C,F) was completely abrogated. Remarkably, although IRP1 levels were at basal levels in the PD98059 + BDNF and PD98059 + GDNF groups, the mRNA expression of DMT1 + IRE was even higher compared with the control condition (Fig. 6C,F). We propose that there may be additional transcriptional mechanism participating in the regulation of DMT1 + IRE rather than IRP1 alone. (See Fig. 7.)

4. Discussion

In the present study, we demonstrated the potential of two established NTFs, BDNF and GDNF, to act directly on primary cultured VM neurons to protect these neurons against 6-OHDA-induced iron accumulation. In 1954, Cohen and colleagues [43] discovered the first NTF nerve growth factor (NGF) acting on nerve cells; afterwards a large number of NTFs and their receptors, which play multiple roles in the developing and mature CNS, were identified. BDNF and GDNF are types of nutritional factors that are widely distributed in the brain and known to be important for DAergic neuron survival during development and in adulthood. Recently, GDNF has been suggested as the most potent trophic factor for DAergic neurons both *in vitro* and *in vivo* [44]. Current strategies for exploring alternative or supplementary therapeutic strategies for PD include the evaluation of neurotrophic factors for DAergic neurons.

One study showed that treatment with NGF, another member of neurotrophin family, induced a significant decrease in DMT1 and TfR1 expression in PC12 cells [45]. However, controversial data have been reported in PC12 cell cultures subjected to NGF [46,47]. The PC12 cell line belongs to an adrenergic neural tumor pheochromocytoma cell line and has the ability to proliferate and differentiate, implying that this cell line is different from mature neurons in some aspects of cell metabolism, such as iron needs. NTFs have been shown to protect neurons against oxidative stress by inducing an increase in the activities of antioxidant enzymes and modulate the expression of apoptosis-related proteins in a pro-survival manner [37]. However, knowledge of the relationship between NTFs and iron homeostasis crosstalk is unclear at best. 6-OHDA is a classical neurotoxin for PD models, and we previously observed that 6-OHDA administration increased cellular iron up-take *in vitro*. In the present study, we used primary cultured VM neurons to test whether BDNF and GDNF could modulate intracellular iron metabolism. We found that pre-incubation with BDNF (10 ng/ml) or GDNF (10 ng/ml) for 4 h prior to 6-OHDA (10 μM) treatment blocked 6-OHDA enhanced ferrous iron influx. In this manner, these two NTFs could protect VM neurons against 6-OHDA-induced iron accumulation. These data suggest that modulation of iron metabolism may provide a novel mechanism by which BDNF and GDNF participate in the neuroprotective effect.

Iron accumulates with aging and has been linked to several neurodegenerative diseases, especially PD [48,49]. An approximate 25% to 100% increase in SN iron levels in patients with PD compared with normal controls has been observed [50]. Previous studies have shown that in the SNpc of PD patients and the MPTP-lesioned mouse model, increased DMT1 + IRE expression is associated with local iron accumulation and DAergic neuron degeneration. In Belgrade rats and mk/mk mice, studies have shown less iron accumulation and a lower susceptibility in DMT1 mutant DAergic neurons after a Parkinsonian toxin injection [16,51]. These data support the importance of DMT1 in iron-mediated neurodegeneration in PD. As we expected, the alleviation of 6-OHDA-induced iron accumulation by BDNF and GDNF was mediated by DMT1, as indicated by the result that BDNF or GDNF treatment alone down-regulated DMT1 + IRE protein and mRNA levels, although 10 ng/ml is higher than the physiological concentration of these neurotrophic factors. In other words, these trophic factors were able to decrease iron uptake in the absence of the toxin. These results indicate that under normal conditions, these trophic factors may be valuable regulators of iron metabolism (e.g., they could down-regulate DMT1 and inhibit excess iron uptake). In the present study, we focus on the healing properties of BDNF and GDNF. Although low BDNF and GDNF concentrations under physiological conditions may mediate a relatively minor regulation of iron metabolism, these brain neurotrophic factors may be used as a therapeutic strategy to block aberrant iron uptake in the diseased state. Our previous publication revealed that neuroprotective agents, such as ginsenoside-Rg1, could protect MPP^+-treated MES23.5 cells via attenuating DMT1

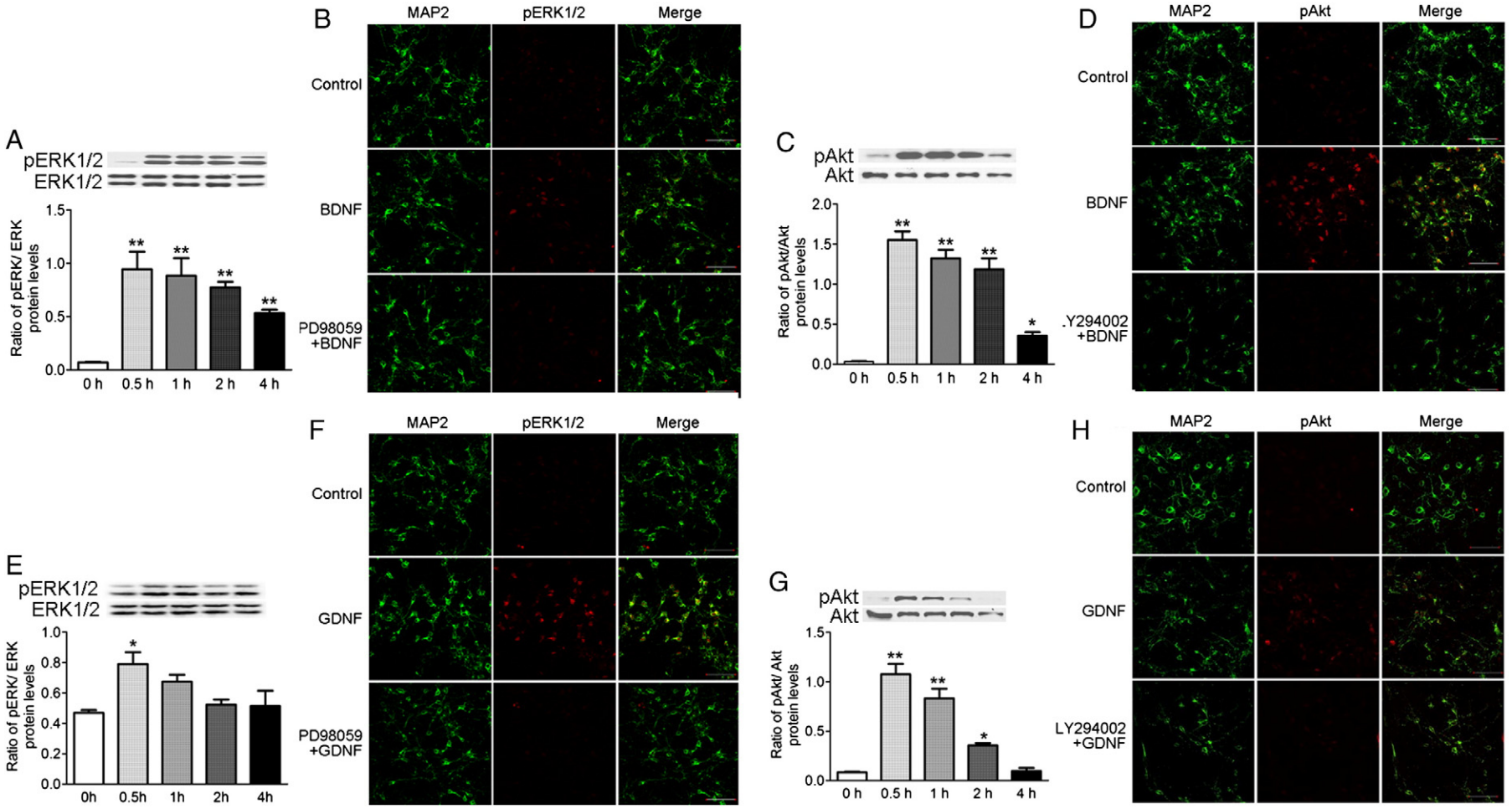

Fig. 5. BDNF and GDNF induced the phosphorylation of ERK1/2 and Akt in VM neurons. A, C, E, G: Western blots analysis of ERK1/2 and Akt phosphorylation in cultured DIV6 VM neurons exposed to 10 ng/ml BDNF or GDNF for the indicated times. Significantly increased levels of phosphorylated ERK1/2 within 0.5 h after BDNF administration were observed; the phospho-ERK1/2 level was gradually restored but remained above basal levels at 4 h. Phosphorylated Akt expression was similar to that of phosphorylated ERK1/2. Maximum activation of ERK1/2 was also achieved within 0.5 h after GDNF treatment; however, the response was weaker compared with that in cells with BDNF exposure. GDNF also induced Akt phosphorylation at 0.5 h; however, the levels had returned to basal levels at 4 h. Each bar represents the mean ± S.E.M of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with 0 h. B, D, F, H: Double immunofluorescence labeling also showed phospho-ERK1/2 and phospho-Akt in MAP2-positive neurons with BDNF and GDNF treatment for 0.5 h. Pre-incubation with the MEK inhibitor PD98059 (5 μM) or the PI3K inhibitor LY294002 (2.5 μM) for 0.5 h fully abolished BDNF- and GDNF-induced ERK1/2 or Akt phosphorylation, respectively. Scale bar = 50 μm.

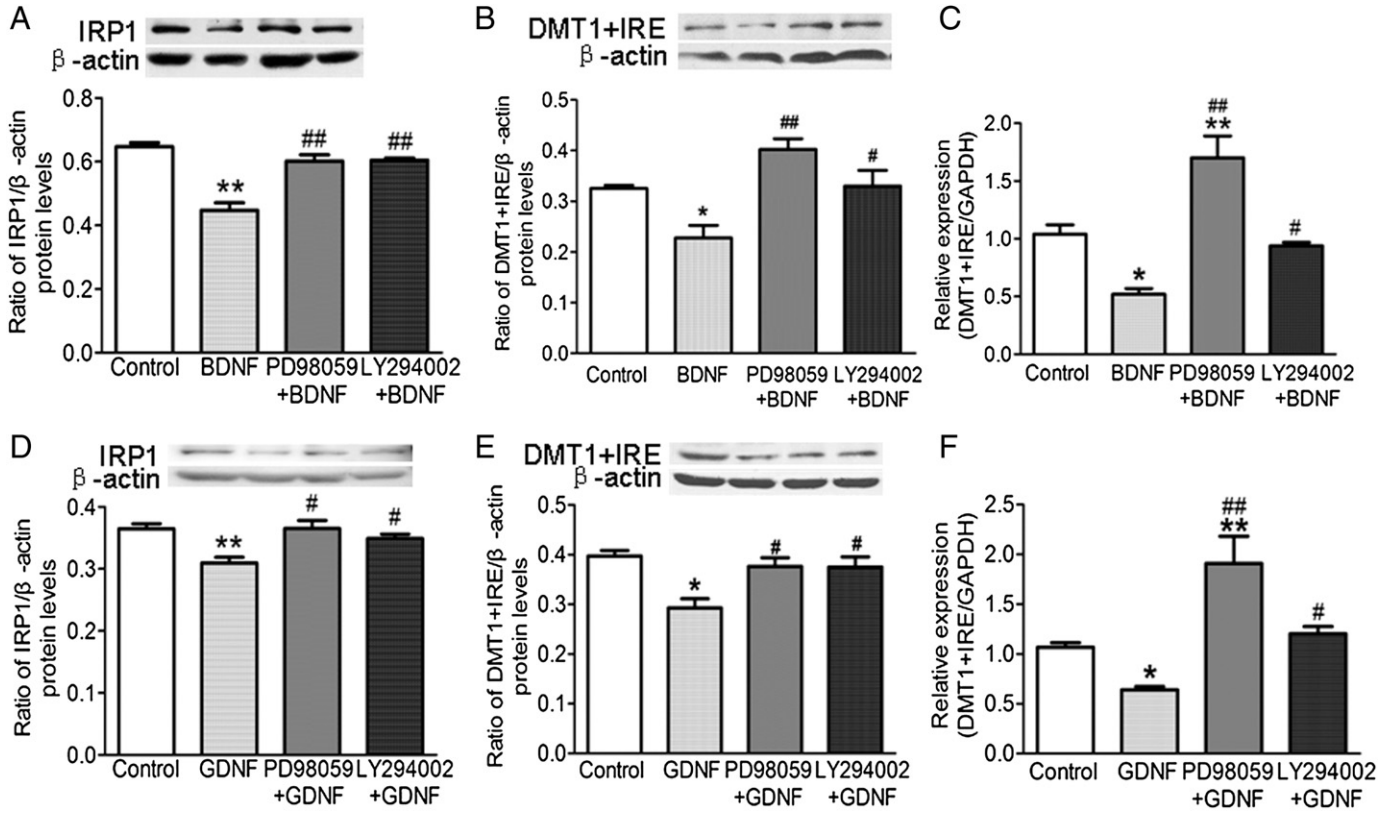

Fig. 6. MEK/ERK and PI3K/Akt signaling pathways mediated IRP1 and DMT1 + IRE regulation by BDNF and GDNF. Primary cultured VM neurons were treated for 30 min with either 5 μM PD98059 or 2.5 μM LY294002 before 10 ng/ml BDNF or GDNF incubation for another 24 h. Western blotting and real-time PCR were conducted to investigate the expression of IRP1 and DMT1 + IRE. A and D: Pharmacological inhibitors of the MEK/ERK or PI3K/Akt pathways fully blocked the BDNF- or GDNF-induced down-regulation of IRP1. B and E: Pharmacological inhibitors of the MEK/ERK or PI3K/Akt pathways abolished the BDNF- or GDNF-induced down-regulation of DMT1 + IRE. β-actin was used as a loading control. C and F: In the presence of PD98059 or LY294002, the BDNF- and GDNF-induced down-regulation of DMT1 + IRE mRNA was completely abrogated. The mRNA expression of DMT1 + IRE was even reversed in the groups pretreated with PD98059. Each bar represents the mean ± S.E.M. of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the BDNF or GDNF group.

up-regulation [52]. These results combined with the present study suggest a crucial role for DMT1 in the modulation of iron metabolism, which is regulated by BDNF and GDNF.

Most cellular iron transporters can be regulated at both the transcriptional and post-transcriptional levels. Iron-regulatory proteins (IRP1 and IRP2) can translationally regulate proteins involved in iron storage (ferritin), acquisition (TfR1, DMT1 + IRE) and export (ferroportin 1) by binding to iron-responsive elements (IREs) present in the untranslated regions (UTR) of their respective mRNAs [53,54]. IRPs bind to IREs located in the 3'-UTR of DMT1 + IRE mRNA, thereby stabilizing it against endonucleolytic cleavage and thus increasing the uptake of NTBI. In this report, we present data demonstrating that the down-regulation of DMT1 + IRE in VM neurons is consistent with decreased IRP expression after BDNF and GDNF incubation. Pretreatment with BDNF and GDNF could also abolish the 6-OHDA-induced up-regulation of IRP1, in accordance with the expression of DMT1 + IRE. In parallel with DMT1 + IRE, 6-OHDA-induced dysregulation of TfR1 was blocked by BDNF and GDNF treatments. These results suggest that both NTFs may participate in the regulation of iron transport in primary cultured VM neurons by directly influencing the expression of IRP1.

The intracellular mechanisms of BDNF and GDNF required for IRP1 down-regulation must be elucidated. Mature BDNF binds with picomolar affinity to its specific TrkB [55]. Upon ligand binding, Trk autophosphorylates tyrosine residues and stimulates intracellular signaling pathways by activating a variety of enzymes and effectors, including phospholipase C-γ (PLC-γ), PI3K, and MEK [56]. GDNF belongs to the transforming growth factor-β (TGF-β) superfamily. By binding to a multicomponent receptor complex termed GFRα, GDNF aggregates two rearranged membrane Ret protein molecules and triggers a number of intracellular signaling cascades, including the MAPK and PI3K pathways [57]. Both the MEK/ERK and PI3K/Akt pathways have multiple effects on neuronal survival [58]. Our western blot and double immunofluorescence labeling data demonstrated rapid and prolonged activation of ERK1/2 and Akt in VM neurons after BDNF and GDNF treatment. Thus, we speculate an obligate requirement for ERK1/2 and Akt activation in the negative regulation of IRP1. Consistent with our results, a recent study showed that lead (Pb)-induced activation of ERK1/2 in the vascular endothelium *in vitro* may participate in IRP1 protein down-regulation [59]. To confirm our hypothesis, we measured the effects of an MEK inhibitor (PD98059) and PI3K inhibitor (LY294002) on IRP1 and DMT1 + IRE levels. The inhibition of the MEK/ERK and PI3K/Akt pathways was effective in attenuating the down-regulation of IRP1 and sequent expression of DMT1 + IRE induced by BDNF and GDNF, suggesting that these pathways are involved in the regulation of iron homeostasis after NTF treatment. Remarkably, although IRP1 was expressed at basal levels in BDNF- and GDNF-treated cells by pretreatment with PD98059, DMT1 + IRE mRNA expression was up-regulated, indicating a direct modulation of the MAPK pathway in BDNF- and GDNF-regulated DMT1 mRNA expression. Further work is needed to elucidate this mechanism.

In conclusion, BDNF and GDNF protect VM neurons against 6-OHDA-induced iron accumulation through down-regulation of IRP1, which post-transcriptionally regulates DMT1 + IRE expression. Both NTFs activated the ERK and Akt signaling pathways, which may participate in these effects. These findings of the specific interaction between BDNF, GDNF and iron homeostasis provide novel insights into iron homeostasis in the SN and may have implications for therapeutic strategies for treating PD.

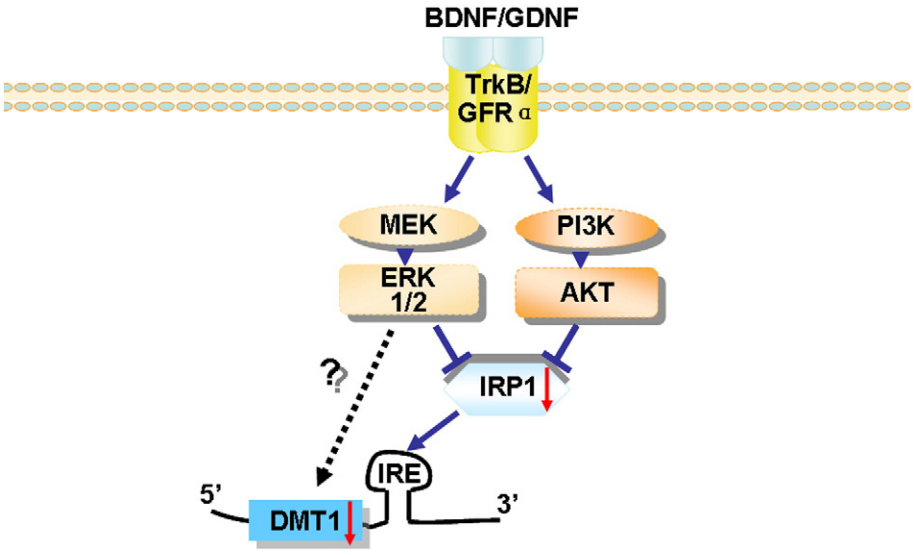

Fig. 7. Schematic diagram illustrating the signal transduction pathway involved in BDNF- and GNDF-mediated DMT1 + IRE expression. BDNF/GDNF-induced activation of TrkB or GFRα results in signal propagation through ERK1/2 and Akt, leading to the down-regulation of IRP1, thereby regulating the expression of DMT1 + IRE post-transcriptionally. In addition, other transcriptional regulatory mechanisms may be involved in the BDNF- and GDNF-related MEK/ERK pathway of direct regulation of DMT1 + IRE.

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