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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