Brain-derived neurotrophic factor inhibits hyperglycemia-induced apoptosis and downregulation of synaptic plasticity-related proteins in hippocampal neurons via the PI3K/Akt pathway

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Received April 29, 2018; Accepted October 4, 2018

DOI: 10.3892/ijmm.2018.3933

Abstract. It is not known whether brain-derived neurotrophic factor (BDNF) protects hippocampal neurons from high glucose-induced apoptosis and/or synaptic plasticity dysfunction. The present study aimed to assess whether BDNF exerted a neuroprotective effect in rat hippocampal neurons exposed to high glucose and examine the underlying mechanisms. The apoptosis of primary hippocampal neurons was assessed by Annexin V-fluorescein isothiocyanate/propidium iodide staining. The mRNA and protein expression levels were measured by reverse transcription-quantitative polymerase chain reaction and western blot experiments, respectively. Synaptic plasticity was evaluated by the immunolocalization of synaptophysin (Syn). Exposure of the hippocampal neurons to high glucose (75 mM for 72 h) resulted in cell apoptosis, decreased mRNA and protein expression levels of three synaptic plasticity-related proteins (Syn, Arc and cyclic AMP response element-binding protein), and changes in the cellular distribution of Syn, indicating loss of synaptic density. These effects of high glucose were partially or completely reversed by prior administration of BDNF (50 ng/ml for 24 h). Pre-treatment with wortmannin, a phosphatidylinositol-3-kinase (PI3K) inhibitor, suppressed the ability of BDNF to inhibit the effects of high glucose. In addition, BDNF significantly upregulated the tropomyosin-related kinase B, its cognate receptor, Akt and phosphorylated Akt at the protein levels under high glucose conditions. In conclusion, high glucose induced apoptosis and downregulated synaptic plasticity-related proteins in hippocampal neurons. These effects were reversed by BDNF via the PI3K/Akt signaling pathway.

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Key words: brain-derived neurotrophic factor, rat hippocampal neurons, hyperglycemia, apoptosis, synaptic plasticity

Introduction

Diabetes mellitus is estimated to affect >366,000,000 individuals worldwide and is characterized by chronic hyperglycemia (1). Studies in humans and animal models have reported an association between diabetes and neurological conditions that affect learning and memory, including Alzheimer's disease (AD) (2-4). Diabetic encephalopathy is now recognized as a complication of diabetes (5). Hyperglycemia has been shown to significantly decrease cell viability and induce apoptosis and loss of hippocampal neurons. The effect of high glucose accumulation involves the intracellular accrual of reactive oxygen species (ROS) (6,7). Therefore, it is necessary to develop neuroprotective strategies to inhibit diabetic encephalopathy. One avenue of investigation has focused on neurotrophic factors, which are important for neuronal survival and regeneration and are considered potential therapeutics for AD and other neurodegenerative diseases (8).

Brain-derived neurotrophic factor (BDNF) is a specific neurotrophic factor that is expressed in neurons and is involved in the growth and differentiation of new neurons and synapse development. BDNF binds to two receptors, namely tropomyosin-related kinase B (TrkB) and low-affinity nerve growth factor receptor, and is involved in the process of long-term memory. BDNF provides trophic support to neurons and exerts a neuroprotective effect against brain injury. In addition to its well-established role in the survival, differentiation and plasticity of neurons (9), BDNF and its cognate receptor TrkB are implicated in the regulation of energy and glucose homeostasis through their effects on the central nervous system (10). Perturbed BDNF signaling in the brain triggers hyperphagia and obesity in mice, suggesting that BDNF acts as an anorexigenic signaling factor (10). Studies have suggested that BDNF regulates glucose metabolism by improving insulin sensitivity and increasing pancreatic insulin production (11,12). In addition, in rodents with impaired leptin signaling through diet-induced obesity and/or deficient leptin signaling (db/db mice), systemic or central BDNF administration has been shown to reduce food intake and body weight (11) in a dose-dependent manner (13).

Synaptic plasticity is defined as the ability of synapses to reinforce and/or weaken their connections over time, depending on their relative activity levels. Synaptic plasticity is considered as one of the most important neurochemical

processes involved in learning and memory (14). In animals, diabetes can cause changes in synaptic proteins, and hyperglycemia is one of the factors contributing to these alterations (15). Synaptophysin (Syn) is a key protein involved in the biogenesis and exo-endocytosis of synaptic vesicles. Syn is considered to be a specific marker of synaptic density and is closely associated with activity-dependent synapse formation and synaptic plasticity (16,17). Syn is mainly degraded through the ubiquitin-proteasome system (18), and evidence suggests that reduced expression levels of Syn may contribute to hyperglycemia-induced cognitive impairment in mice (19). Other factors have also been implicated in synaptic plasticity and memory. Cyclic AMP response element-binding protein (CREB) is a nuclear transcription factor that is essential for the formation and retention of memory. The activation of CREB occurs by phosphorylation at serine residue 133 and is required for neuronal growth and survival (20). CREB is necessary for the maintenance of normal synapses in hippocampal neurons (21), and reduced levels of phosphorylated CREB (p-CREB) have been observed in the postmortem brains of patients with AD and experimental models of AD (22,23). Arc protein, which is transcribed from the Arc/Arg3.1 gene, is another factor associated with the progression of AD (24-26). The dysregulation of Arc in cerebral neurons interferes with their normal activity and causes synaptic damage and neuron loss, leading to the degradation of specific neural circuit functions and a decrease in neuronal network activity that may be involved in AD (24-26).

Previous studies have investigated the interaction of the TrkB receptor with hyperglycemia and neuronal function (27,28). BDNF upregulates TrkB and increases the phosphorylation levels of TrkB and ERK in retinal neurons exposed to hyperglycemic conditions (29). A previous study demonstrated that the phosphorylation levels of the cell signaling molecule Akt (protein kinase B) and transcription factor CREB are reduced in diabetic rats compared with those in control animals (30), suggesting that these factors may be involved in diabetes-induced cognitive dysfunction. Chen *et al* (31) demonstrated that the neuroprotective effects of BDNF, acting via the TrkB receptor, were induced by activation of the phosphatidylinositol-3-kinase (PI3K)-Akt pathway and the increased expression of Arc.

However, whether BDNF protects hippocampal neurons from high glucose-induced apoptosis and/or synaptic plasticity dysfunction remains to be fully elucidated. Therefore, the aim of the present study was to evaluate whether long-term elevated glucose, which mimics prolonged hyperglycemia, causes significant changes in neuronal survival and synaptic plasticity, and whether exogenous BDNF exerts neuroprotective effects.

Materials and methods

Primary culture of rat hippocampal neurons. All animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Ethics Committee of Animal Experiments of the Shanghai Sixth People's Hospital affiliated to Shanghai Jiao Tong University [Shanghai, China; permit no. SYXK (Shanghai) 2011-0128].

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of 10 neonatal Sprague-Dawley rats within 24 h of birth (Shanghai Laboratory Animal Co., Ltd., Shanghai, China), weighing between 4.5-6.5 g, as described previously (32), with minor modifications. The hippocampi were dissected from the rat brain tissues and were placed on ice. Subsequently, the blood vessels and meninges were thoroughly removed, and the hippocampi were washed with phosphate-buffered saline (PBS). The tissues were then transferred into Eppendorf tubes containing 1 ml 0.123% trypsin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The hippocampi were cut into small pieces with sterile ophthalmic scissors (Kun Sheng Medical Instrument Co., Ltd., Shanghai, China). The hippocampal pieces were digested for 15 min at 37˚C with vortexing every 5 min. The digestion procedure was terminated by the addition of 5 ml Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 20% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The cell suspension was passed through a 200-mesh cell strainer and separated by centrifugation at 300 x g for 5 min at room temperature. The pellets were resuspended in 2 ml of DMEM containing 20% FBS, at ~70 cells per ml. The neurons were seeded on poly-D-lysine (0.1 mg/ml; Gibco; Thermo Fisher Scientific, Inc.)-coated glass coverslips (Corning Incorporated, Corning, NY, USA), 96-well plates and/or 6-well plates in 60-70 μl medium. Following 6-12 h of incubation, the cells were cultured in Neurobasal medium supplemented with B27 (1:50, Gibco; Thermo Fisher Scientific, Inc.). Half of the medium was replaced with fresh medium every 2 or 3 days.

Immunofluorescence staining. The primary hippocampal neurons were fixed with 4% paraformaldehyde (China National Medicines Corporation, Ltd., Beijing, China) for 1 h at room temperature and incubated in PBS containing 0.5% Triton for 20 min at room temperature. Non-specific antibody binding was blocked by incubation at room temperature for 30 min with normal goat serum (Gibco; Thermo Fisher Scientific, Inc.). Each coverslip was incubated with 20 μl rabbit anti-NeuN primary antibody (1:200; cat. no. ab177487, Abcam, Cambridge, MA, USA) or rabbit anti-synaptophysin primary antibody (1:200; cat. no. ab32127; Abcam) at 4˚C overnight. The coverslips were subsequently washed three times in PBS and incubated with donkey anti-rabbit secondary antibody (1:500; cat. no. A0453, Alex fluor 555; Beyotime Institute of Biotechnology, Shanghai, China) or goat anti-rabbit secondary antibody (1:500; cat. no. A0423; Alex fluor 488; Beyotime Institute of Biotechnology) for 30 min at room temperature. The coverslips were finally incubated with 20 μl of 4',6-diamidino-2-phenylindole (DAPI; Roche Diagnostics, Basel, Switzerland) for 5 min at room temperature in the dark. The cells were observed and images were captured using a Volocity Demo imaging system (PerkinElmer, Inc., Waltham, MA, USA).

High glucose exposure and experimental grouping. The hippocampal neurons in primary culture for 3 days were divided into four experimental groups, including the control group (CON), high glucose group (HG), high glucose + BDNF group (HG + BDNF) and high glucose + BDNF + wortmanin

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')	Product length (bp)	Temperature (˚C)
GAPDH	F: CAGGGCTGCCTTCTCTTGTG	111	60.70
	R: AACTTGCCGTGGGTAGAGTC		60.54
Arc	F: TATGTGGACGCTGAGGAGGA	77	60.77
	R: CGCAGAAAGCGCTTGAACTT		60.75
CREB	F: AGGGCCTGCAGACATTAACC	88	60.03
	R: TGTCCATCAGTGGTCTGTGC		60.04
Syn	F: TCGTGTTCAAGGAGACAGGC	78	60.80
	R: CAGGTGCTGGTTGCTTTTCC		60.82

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CREB, cyclic AMP response element-binding protein; Syn, synaptophysin; F, forward; R, reverse.

group (HG + BDNF + wort). The hippocampal neurons were seeded in 96-well plates at a density of 5,000-10,000 cells in each well and maintained at 37˚C in a humidified incubator supplemented with 5% CO_2. Each of the four groups was exposed to different intervention measures. The control group was exposed to normal medium containing 25 mM glucose. The primary hippocampal neurons were exposed to 75 mM glucose (China National Medicines Corporation, Ltd.) for 72 h (33), which has no effect on normal metabolism. To establish the HG + BDNF group, the primary hippocampal neurons were incubated for 24 h with 50 ng/ml BDNF (Sigma; Merck KGaA, Darmstadt, Germany) prior to stimulation with 75 mM glucose for 72 h. Primary hippocampal neurons in the HG + BDNF + wort group were pretreated with 0.5 μM of wortmannin (Selleck Chemicals, Houston, TX, USA) to suppress PI3K for 2 h, and further treatments were the same as those for the HG + BDNF group.

Assessment of apoptosis by flow cytometry. The apoptotic rate was measured using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Gibco; Thermo Fisher Scientific, Inc.). Flow cytometric data were acquired on FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed with the use of FlowJo v10 software (FlowJo, LLC, Ashland, OR, USA). Following 72 h of incubation, the primary hippocampal neurons were washed twice with ice-cold PBS and stained with 190 μl Annexin V-FITC (Gibco; Thermo Fisher Scientific, Inc.) and 10 μl PI (Roche Diagnostics) according to the manufacturer's protocol. Following 30 min of incubation at 37˚C, the stained neurons were analyzed by flow cytometry, and the rate of cellular apoptosis was determined. Annexin V was set as the horizontal axis and PI as the vertical axis. Apoptotic or necrotic cells were indicated in the upper right quadrant of the flow-cytogram, whereas early apoptotic cells were indicated in the lower right quadrant.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from the primary hippocampal neurons using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed at 37˚C for 15 min followed by 85˚C for 5 sec using the PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Specific mRNA quantification was performed by real-time PCR using SYBR® Premix Ex Taq™ II (Tli RNase H Plus; Takara Biotechnology Co., Ltd.) in a FTC3000HT real-time PCR system (Funglyn Biotech, Inc., Toronto, ON, Canada). The gene-specific primers used are presented in Table I. Each PCR mixture contained 1 μl cDNA, 0.8 μl each primer (10 μmol/l), 7 μl ddH_2O, 0.4 μl ROX and 10 μl SYBR Green Premix (Takara Bio, Inc., Shiga, Japan). All reactions involved initial denaturation at 95˚C for 30 sec followed by 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec. The $2^{-\Delta\Delta Cq}$ method (34) was used to determine the relative mRNA expression of the target genes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Western blot analysis. The cells were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) containing phenylmethylsulfonyl fluoride (final concentration 1 mM; Ameresco, Inc., Framingham, MA, USA) and centrifuged at 10,000 x g for 5 min at 4˚C. The protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Equal quantities of protein (60 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Burlington, MA, USA). The membranes were subsequently incubated in blocking buffer for 1 h, 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) in Tris-buffered saline with 0.1% Tween-20, followed by overnight incubation at 4˚C with rabbit anti-CREB (1:1,000; cat. no. ab32515; Abcam), rabbit anti-Arc/Arg3.1 (1:1,000; cat. no. ab183183; Abcam), rabbit anti-TrkB (1:1,000; cat. no. 4603; Cell Signaling Technology, Inc.), rabbit anti-pAkt (1:1,000; cat. no. cst-4060s; Cell Signaling Technology, Inc.), rabbit anti-Akt (1:1,000; cat. no. cst-4691s; Cell Signaling Technology, Inc.), rabbit anti-Syn (1:5,000; cat. no. ab32127; Abcam) or mouse anti-GAPDH (1:1,000; cat. no. sc-293335; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies. The membranes were subsequently incubated for 1 h at room temperature with goat anti-rabbit (1:5,000; cat. no. sc-2030)

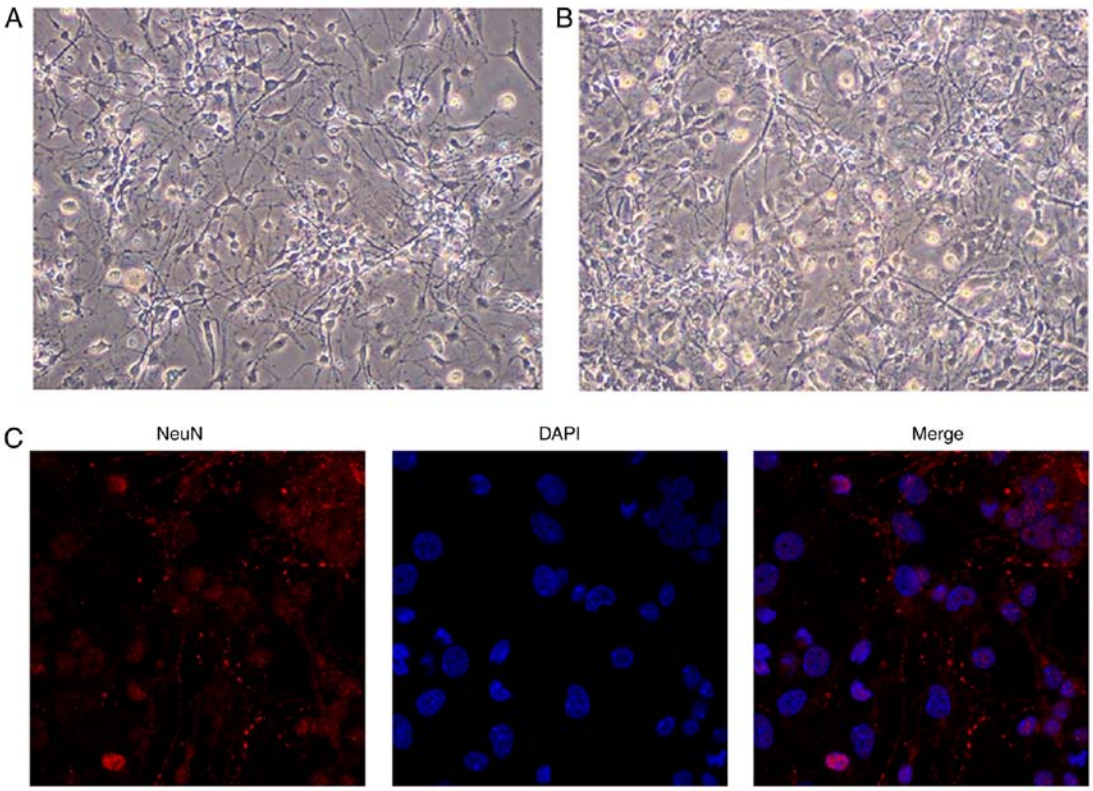

Figure 1. Identification of primary rat hippocampal neurons by immunofluorescence detection of NeuN protein. (A) Hippocampal neurons cultured for 3 days (magnification, x200). (B) Hippocampal neurons cultured for 5 days (magnification, x200). (C) Immunofluorescence detection of NeuN protein (red) and staining of the nuclei with DAPI (blue). The images were obtained by scanning laser confocal microscopy (magnification: x400). NeuN protein, neuron-specific nuclear protein; DAPI, 4',6-diamidino-2-phenylindole.

or anti-mouse (1:5,000; cat. no. sc-516180) IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc.). The detection of specific bands was achieved with enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.), and the immunoreactive bands were visualized on an ImageQuant LAS4000 mini apparatus (GE Healthcare, Chicago, IL, USA). Semi-quantification was performed using Image-Pro Plus v6.0 software (www.mediacy.com).

Statistical analysis. Statistical analyses were performed using Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard deviation of three or four independent experiments. Statistical significance was determined by two-way analysis of variance followed by the Newman-Keuls post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of hippocampal neurons. Previous studies have used hippocampal neurons from fetal tissues obtained from 18 days of pregnancy (35) or newborn rats (36,37). Chen *et al* (38) demonstrated no difference in the neuronal survival rates between hippocampal neurons from fetal rats and those from corresponding newborn rats. In the present study, hippocampal neurons from newborn rats were selected for culture *in vitro*. On days 3 and 5 of culture, the neurites were observed to interconnect with each other to form a loose network of cells (Fig. 1A and B), which is a typical function of cultured hippocampal neurons. Nuclear staining of the neurons was achieved using DAPI, and neurite growth was demonstrated by immunofluorescence staining of NeuN (red staining, Fig. 1C). The purity of the neurons, calculated as the ratio of the number of positive cells (identified by nuclear staining) to the total number of cells, was estimated to be ~95% (Fig. 1C).

BDNF inhibits the high glucose-induced apoptosis of hippocampal neurons, and wortmannin reverses this effect. The apoptotic rate was significantly higher in hippocampal neurons treated with high glucose than in neurons exposed to normal glucose (36.32±1.80, vs. 2.68±0.60%, $P<0.001$; Fig. 2A and B). BDNF suppressed the apoptotic rate of neurons exposed to high glucose (11.75±1.10, vs. 36.32±1.80%, $P<0.001$; Fig. 2A and B). However, this effect of BDNF was attenuated by wortmannin, an inhibitor of PI3K (24.72±1.06, vs. 11.75±1.10%, $P<0.01$; Fig. 2A and B). These data indicated that high glucose induced the apoptosis of hippocampal neurons cultured *in vitro*, which was suppressed by BDNF via PI3K signaling.

High glucose suppresses the expression levels of synaptic plasticity-related proteins, and BDNF reverses these effects. To examine the mechanism underlying the protective effect of BDNF on hippocampal neurons under hyperglycemic conditions, RT-qPCR and western blot experiments were performed to assess the expression levels of the synaptic plasticity-related proteins, CREB, Arc and Syn. The RT-qPCR experiments revealed that the mRNA expression levels of Syn, Arc and

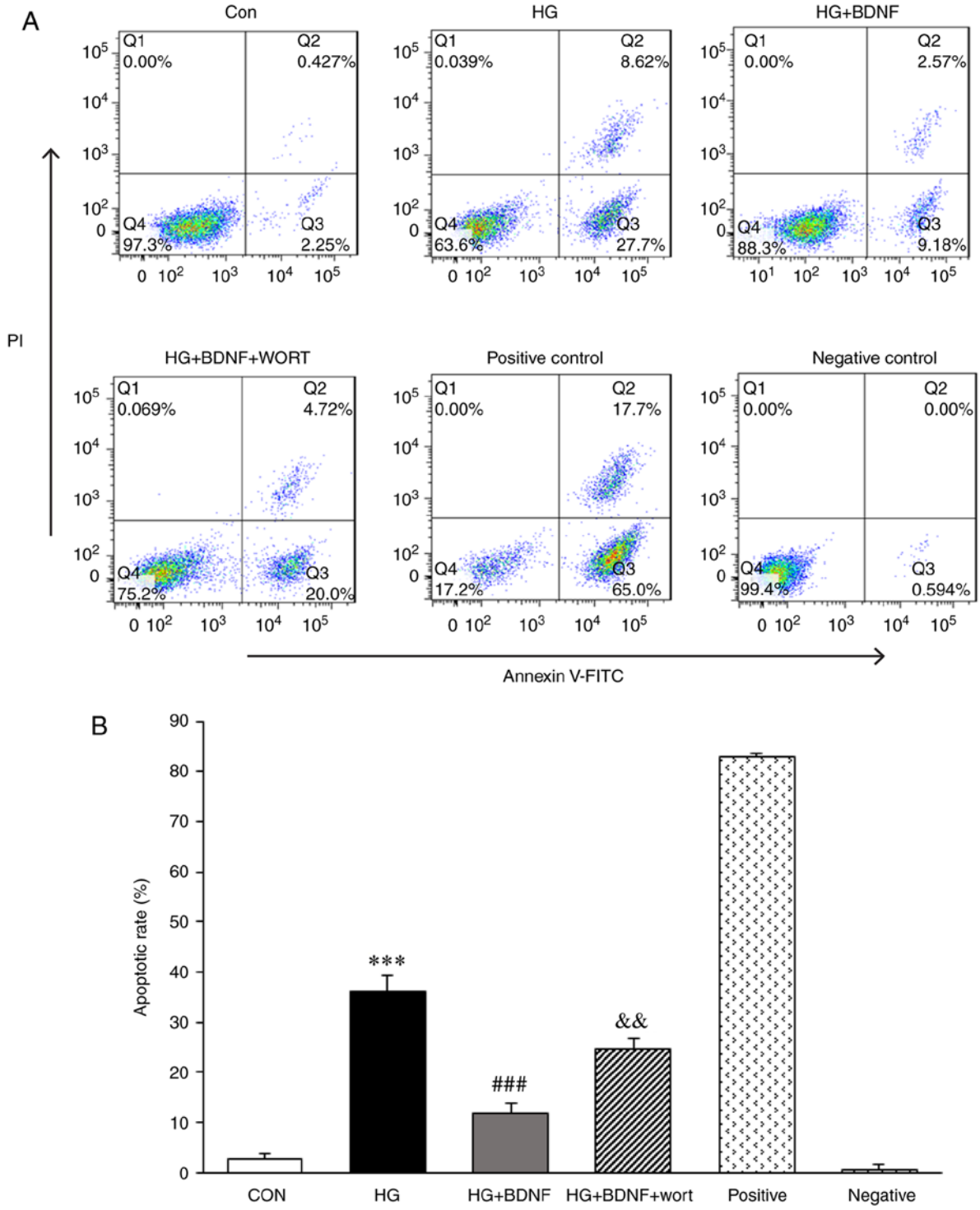

Figure 2. Effect of BDNF on HG-induced neuronal apoptosis. (A) Neuronal apoptosis was assayed by flow cytometry (Annexin V-FITC/PI staining). CON: 25 mM glucose; HG: 75 mM glucose for 72 h; HG + BDNF: 50 ng/ml BDNF for 24 h followed by 75 mM glucose for 72 h; HG + BDNF + wort: 0.5 μM wort pretreatment for 2 h to suppress PI3K, followed by ng/ml BDNF for 24 h and then 75 mM glucose for 72 h. (B) Data are presented as the mean ± standard deviation of three independent triplicate experiments. ***P<0.001, vs. CON group; $^{\#\#\#}$P<0.001, vs. HG group; $^{\&\&}$P<0.01, vs. HG + BDNF group. FITC, fluorescein isothiocyanate; PI, propidium iodide; CON, control; BDNF, brain-derived neurotrophic factor; HG, high glucose; wort, wortmannin.

CREB were significantly reduced on exposure to high glucose (all P<0.001; Fig. 3A-C). BDNF significantly inhibited the effects of high glucose on the mRNA expression levels of Syn, Arc and CREB (all P<0.01; Fig. 3A-C). In addition, prior administration of wortmannin significantly attenuated the ability of BDNF to reverse the effects of high glucose on the mRNA expression levels of Syn (P<0.001), Arc (P<0.05) and CREB (P<0.01; Fig. 3A-C). When the protein levels of Syn, Arc and CREB were assessed by western blotting (Fig. 4A-D), the results were consistent with those of the RT-qPCR experiments. Taken together, these data indicated that high glucose may lead to an imbalance in the synaptic plasticity of hippocampal neurons, and that this effect is suppressed by BDNF via PI3K signaling.

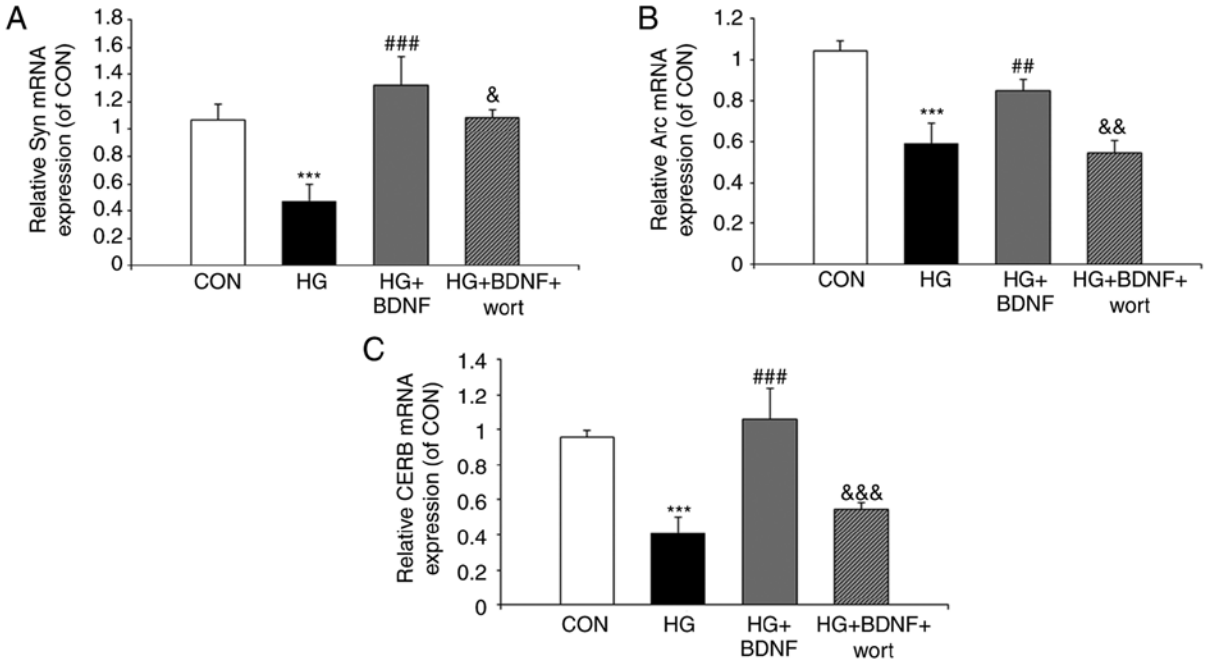

Figure 3. Effects of BDNF on the mRNA expression levels of synaptic plasticity-related proteins in primary hippocampal neurons under high glucose conditions. The mRNA expression of (A) Syn, (B) Arc and (C) CREB (C) in primary hippocampal neurons was monitored by reverse transcription-quantitative polymerase chain reaction analysis. GAPDH was used as an internal control. Data are presented as the mean ± standard deviation of three independent experiments. ***P<0.001, vs. CON group; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001, vs. HG group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001, vs. HG + BDNF group. Syn, synaptophysin; CREB, cyclic AMP response element-binding protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CON, control; BDNF, brain-derived neurotrophic factor; HG, high glucose; wort, wortmannin.

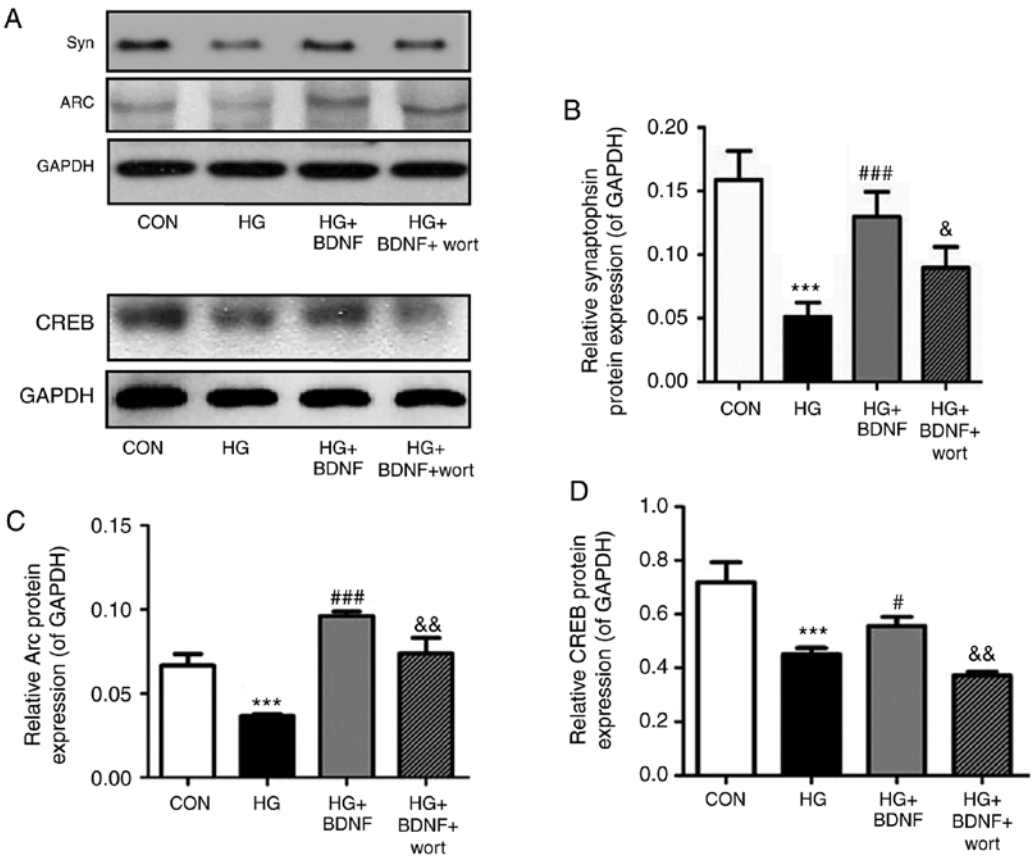

Figure 4. Effects of BDNF on the protein expression levels of synaptic plasticity-related proteins in primary hippocampal neurons under high glucose conditions. (A) Western blot analysis was used to measure the protein expression levels of (B) Syn, (C) Arc and (D) CREB in primary hippocampal neurons. GAPDH was used as an internal control. Data are presented as the mean ± standard deviation of three independent experiments. ***P<0.001, vs. CON group; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001, vs. HG group; $^{\&}$P<0.05 and $^{\&\&}$P<0.01, vs. HG + BDNF group. Syn, synaptophysin; CREB, cyclic AMP response element-binding protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CON, control; BDNF, brain-derived neurotrophic factor; HG, high glucose; wort, wortmannin.

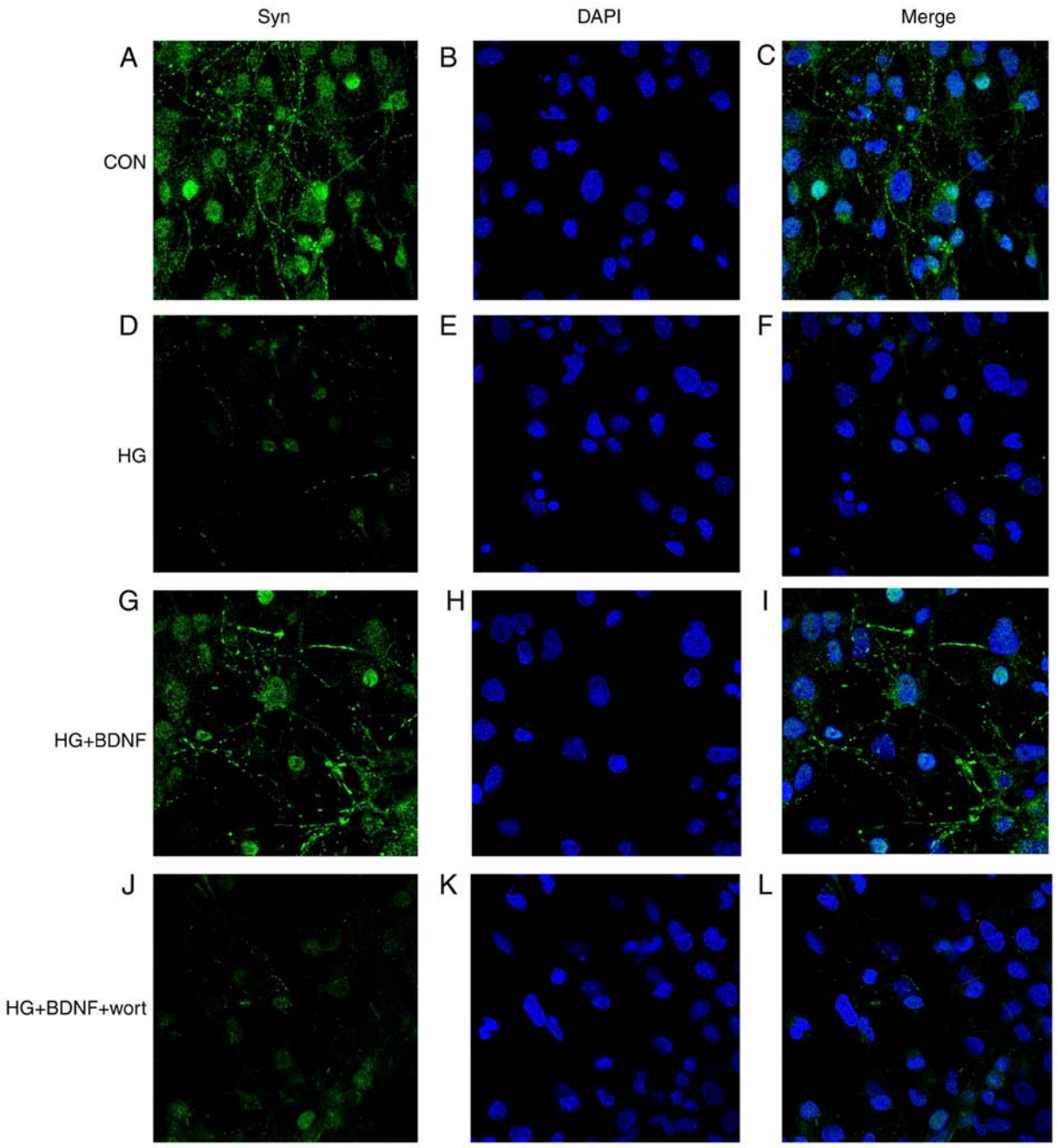

Figure 5. Effects of BDNF on the expression and distribution of Syn in primary hippocampal neurons under high glucose conditions. The expression and distribution of Syn were determined using immunofluorescence techniques. Syn is stained green, nuclei are stained blue with DAPI. (A) Syn, (B) DAPI and (C) Merge staining in the CON group; (D) Syn, (E) DAPI and (F) Merge staining in the HG group; (G) Syn, (H) DAPI and (I) Merge staining in the HG + BDNF group; (J) Syn, (K) DAPI and (L) Merge staining in the HG + BDNF + wort group. Magnification, x630. CON, control; BDNF, brain-derived neurotrophic factor; HG, high glucose; wort, wortmannin; DAPI, 4',6-diamidino-2-phenylindole.

BDNF regulates the level and distribution of Syn in primary hippocampal neurons under high glucose conditions. The level and distribution of Syn can be used as an indirect measure of synaptic density (19). Therefore, the distribution of Syn in primary hippocampal neurons was assessed using immunofluorescence techniques. In the CON group, Syn protein was expressed in the neurites of hippocampal cells (Fig. 5A-C). However, the expression level of Syn was markedly reduced in hippocampal neurons in the HG group, with loss of expression in the neurites (Fig. 5D-F). BDNF normalized the levels and distribution of Syn protein in the neurons, and this effect of BDNF was prevented by wortmannin (Fig. 5G-L). These findings indicated that high glucose reduced synaptic density in primary hippocampal neurons, and that BDNF was able to reverse this effect via PI3K signaling.

BDNF upregulates protein expression levels of TrkB, Akt and p-Akt in primary hippocampal neurons under high glucose conditions. The expression levels of the downstream signaling proteins of BDNF were assessed to further investigate the molecular mechanisms underlying the neuroprotective effect of BDNF on hippocampal neurons under high glucose conditions (Fig. 6A). The protein expression levels of TrkB in the HG group were similar to those in the CON group, whereas

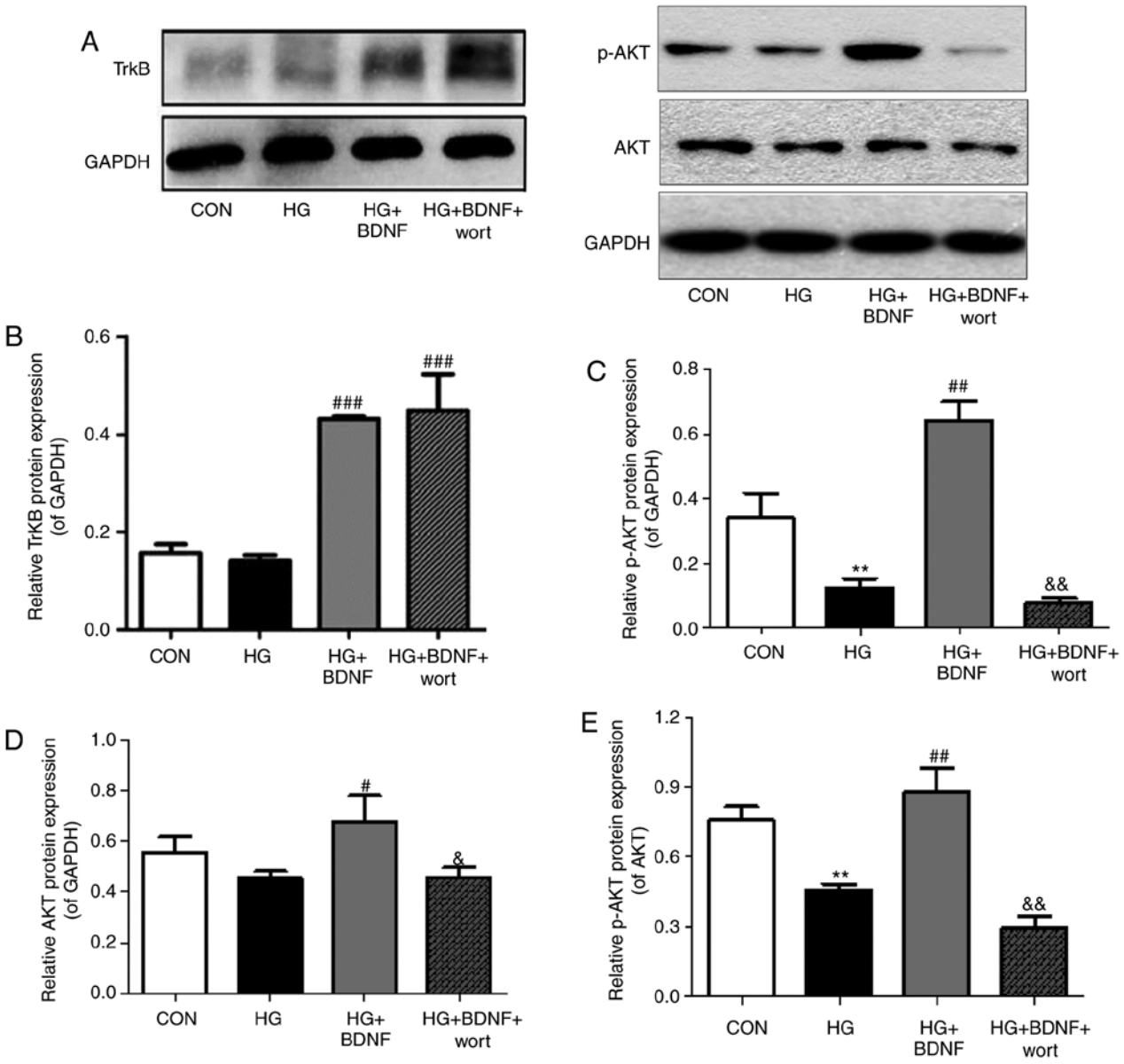

Figure 6. Effects of BDNF on protein expression levels of TrkB, Akt and p-Akt in primary hippocampal neurons under high glucose conditions. (A) Expression levels of TrkB, Akt and p-Akt were determined by western blot analysis, with data for (B) TrkB, (C) p-Akt, (D) Akt and (E) p-Akt/Akt presented as the mean ± standard deviation of three independent experiments. **P<0.01, vs. CON group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001, vs. HG group; $^{\&}$P<0.05, $^{\&\&}$P<0.01, vs. HG + BDNF group. CON, control; BDNF, brain-derived neurotrophic factor; HG, high glucose; wort, wortmannin; TrkB, tropomyosin-related kinase B; p-, phosphorylated; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

combined treatment of HG + BDNF resulted in increased protein expression levels of TrkB compared with those in the HG group (P<0.001; Fig. 6B). The expression levels of TrkB were significantly increased in the HG + BDNF + wort group compared with those in the HG group (P<0.001; Fig. 6B), but did not differ significantly compared with those in the HG + BDNF group. The expression levels of p-Akt, Akt, and p-Akt/Akt were significantly decreased in the HG + BDNF + wort group compared with those in the HG + BDNF group (P<0.01, P<0.05 and P<0.01, respectively; Fig. 6C-E), whereas the HG + BDNF group exhibited significantly higher protein expression levels of p-Akt, AKT and p-Akt/Akt compared with those in the HG group of cells (P<0.01, P<0.05 and P<0.01, respectively; Fig. 6C-E). The expression levels of p-Akt and p-Akt/Akt were significantly decreased in the HG group compared with those in the CON group (both P<0.01; Fig. 6C and E), Taken together, these data suggested that high glucose may lead to abnormal plasticity in hippocampal neuronal synapses, which is reversed by BDNF via its cognate receptor TrkB and the downstream signaling protein Akt.

Discussion

In the present study, an *in vitro* model of rat hippocampal neurons was established from newborn rats, and neurite growth was evaluated by immunostaining for NeuN (39). Furthermore, hyperglycemic conditions were established for neuronal growth and it was demonstrated that BDNF protected against neuronal cell apoptosis induced by high glucose. In addition, BDNF increased the expression levels of

synaptic plasticity-related proteins under high glucose conditions. Notably, the various effects of BDNF in rat hippocampal neurons treated with high glucose were dependent on PI3K-Akt signaling.

In the present study, BDNF protected neuronal cells from high glucose-induced apoptosis as demonstrated by flow cytometry. The neuronal apoptotic rate was decreased markedly (11.49, vs. 38.86%) when the neurons were exposed to 75 mM glucose for 72 h and administered with 50 ng/ml BDNF. A previous study demonstrated that the addition of BDNF prior to anoxia resulted in neuroprotective effects (40). The protective effect of BDNF was decreased following prolonged anoxia and irreversible neuronal injury. Therefore, in the present study, the hippocampal neurons were pretreated with BDNF for 24 h prior to their culture in the presence of high glucose, and a BDNF concentration of 50 ng/ml was selected based on the previous studies (41,42). Consistent with the findings in the present study, Bathina *et al* (43) reported that RIN5F cells exhibited reduced viability following treatment with streptozotocin, which was reverted by BDNF.

The present study also demonstrated that the mRNA and protein expression levels of Syn, Arc and CREB were lower in hippocampal neurons exposed to high glucose than in neurons of the control group. Notably, the immunofluorescence experiments revealed a decrease in the protein expression of Syn in the neurites following treatment with high glucose, and this abnormal distribution of Syn was consistent with a reduction in synaptic density. These findings suggested that high glucose may lead to abnormal plasticity in hippocampal neuronal synapses via alterations in the levels, and thus functions, of proteins that are closely associated with synaptic plasticity. Consistent with these observations, Zhao *et al* (18) demonstrated that Syn was downregulated in primary neuronal cultures subjected to high glucose and hypoxia. Furthermore, a previous study demonstrated that the combination of hyperglycemia and hypoxia in mice resulted in cognitive impairment and was associated with significantly reduced protein levels of Syn in the hippocampus (19). It was suggested that the effects of high glucose and hypoxia on the protein levels of Syn may result from the enhanced degradation of Syn involving the E3 ubiquitin ligase, siah family (19). Another report found abnormal levels of certain synaptic proteins (synaptosomal-associated protein-25, synaptotagmin-1 and vesicular glutamate transporter-1) following long-term exposure of hippocampal neurons to hyperglycemia, suggesting that the trafficking of proteins to the synapse may be impaired (15).

In the present study, BDNF caused an increase in the mRNA and protein expression levels of Syn, Arc and CREB in hippocampal neurons treated with high glucose. BDNF also normalized the distribution of Syn in these cells. These observations suggested that the protective effect of BDNF on hippocampal neurons was achieved, at least in part, through enhancement of synaptic plasticity. Leal *et al* (44) demonstrated that BDNF can regulate hippocampal synaptic plasticity. In addition, a previous study found that rats fed on a high-fat, high-glucose diet to induce experimental diabetes exhibited impaired spatial learning, decreased hippocampal dendritic spine density and reduced long-term potentiation, and these changes were associated with a reduction in hippocampal BDNF levels (45). Arc has been demonstrated to exert a neuroprotective effect via decreased AMPA receptor current and glutamate receptor 2 internalization (46), therefore, the upregulation of Arc levels by BDNF may contribute to the neuroprotective effects of BDNF.

BDNF binds to TrkB and recruits proteins that activate several signal transduction cascades, including the sequential activation of insulin receptor substrate-1, PI3K and Akt (47). The BDNF signaling pathways activate CREB and CREB-binding protein, regulating the genes involved in neural plasticity (47). The PI3K-Akt signaling pathway is involved in synaptic plasticity, memory consolidation and synaptic morphogenesis (48,49). In terms of the role of this pathway in diabetes, asiaticoside, a glycosylated triterpene from *Centella asiatica*, has been shown to attenuate diabetes-induced cognitive impairment and upregulate the expression of synaptic proteins via PI3K/Akt signaling (50). However, no previous reports have examined the role of the PI3K-Akt pathway in mediating the effects of BDNF in diabetic encephalopathy. In the present study, BDNF was demonstrated to activate PI3K-Akt signaling under high glucose conditions, as the levels of p-Akt and Akt were increased. In addition, BDNF enhanced the mRNA and protein expression levels of Arc, Syn and CREB, all of which can influence synaptic plasticity through the PI3K-Akt pathway as the effects of BDNF were inhibited by wortmannin. These findings indicated that BDNF-TrkB activates Akt under hyperglycemic conditions to reverse the abnormalities in synaptic plasticity and inhibit apoptosis. Taken together, these data indicated that BDNF protects hippocampal neurons partially through the upregulation of CREB and Arc, which is mediated through the PI3K-Akt signaling pathway.

In the present study, the expression of TrkB was increased following treatment with BDNF under high glucose conditions. Although it has been reported that the expression of TrkB is regulated by the cyclic AMP/CREB pathway in neurons (51), the administration of PI3K inhibitor did not decrease the expression level of TrkB, despite a reduction in CREB and p-Akt/Akt levels. This suggests that the regulation of the expression of TrkB by BDNF occurs upstream of Akt.

Of note, BDNF has also been demonstrated to protect retinal neurons from hyperglycemia via the TrkB/ERK/MAPK pathway and attenuate diabetic hyperglycemia via an insulin-independent mechanism in rats (13,29). The regulation of long-term synaptic plasticity and memory formation by Arc are dependent on its phosphorylation by ERK protein, suggesting that MAPK kinases are important in the memory process (52). The findings of the present study indicate the possibility of potential interplay between the ERK/MAPK and PI3K-Akt pathways in the regulation of neuronal plasticity by BDNF.

In conclusion, the present study demonstrated that BDNF can activate the PI3K-Akt signaling pathway and induce the expressions of synaptic plasticity-related proteins in hippocampal neurons cultured under high glucose conditions. This improves synaptic plasticity in the hippocampal neurons and protects them from high glucose-induced apoptosis. These findings provide a theoretical basis for subsequent investigations on the mechanism of BDNF-mediated hippocampal neuroprotection. In addition, the present study provides novel insights into therapeutically targeting BDNF and PI3K-Akt signaling for the prevention of diabetic encephalopathy.

Acknowledgements

Not applicable.

Funding

The present study was funded by the Shanghai Sixth People's Hospital Group Science Foundation, the Shanghai Science and Technology Commission Foundation Research Project (grant no. 13JC1401504) and the Chinese National Natural Science Foundation (grant no. 81300933).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YZ, YM and YTZ performed the experiments, were involved in data collection and drafted the manuscript. TH and QL performed the statistical analyses and were involved in study design. WL assisted in drafting the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Ethics Committee of Animal Experiments of The Shanghai Sixth People's Hospital affiliated to Shanghai Jiao Tong University [permit no. SYXK (Shanghai) 2011-0128].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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