Substantial Neuroprotection Against K⁺ Deprivation-Induced Apoptosis in Primary Cerebellar Granule Neurons by Novel Dimer Bis(propyl)-Cognitin Via the Activation of VEGFR-2 Signaling Pathway

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Keywords

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SUMMARY

Background: Neuronal loss via apoptosis in CNS is the fundamental mechanism underlying various neurodegenerative diseases. Compounds with antiapoptotic property might have therapeutic effects for these diseases. In this study, bis(propyl)-cognitin (B3C), a novel dimer that possesses anti-AChE and anti-N-methyl-D-aspartate receptor activities, was investigated for its neuroprotective effect on K⁺ deprivation-induced apoptosis in cerebellar granule neurons (CGNs). **Methods:** Cerebellar granule neurons were switched to K⁺ deprived medium with or without B3C. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, fluorescein diacetate (FDA)/propidium iodide (PI) staining, Hoechst staining, and DNA laddering assays were applied to detect cytotoxicity and apoptosis. Additionally, the expression of p-VEGFR-2, p-Akt, p-glycogen synthase kinase 3 β (GSK3 β), and p-extracellular signal-regulated kinase (ERK) was examined in CGNs. **Results:** Switching CGNs to K⁺ deprived medium resulted in remarkable apoptosis, which could be substantially blocked by B3C treatment (IC₅₀, 0.37 μ M). Moreover, a rapid decrease in p-Tyr1054-VEGFR-2 was observed after the switch. B3C significantly reversed the inhibition of p-Tyr1054-VEGFR-2 as well as Akt and ERK pathways. VEGFR-2 inhibitor PTK787/ZK222584, as well as PI3-K inhibitor LY294002 and MEK inhibitor PD98059, each abolished the neuroprotective effect of B3C. **Conclusions:** Our results demonstrate that B3C blocks K⁺ deprivation-induced apoptosis in CGNs through regulating VEGFR-2/Akt/GSK3 β and VEGFR-2/ERK signaling pathways, providing a molecular insight into the therapeutic potential of B3C for the treatment of neurodegenerative diseases.

Introduction

As the population ages, a growing number of people are suffering from neurodegenerative disorders such as Alzheimer's disease due to a combination of genetic and environmental factors. There is still no effective medication for preventing, halting, or curing these devastating disorders, and they can only be modified symptomatically. Regardless of the variety of pathogenesis, neurodegenerative disorders are characterized by a progressive neuronal loss in specific neuronal populations, which correlates with the neurological dysfunctions of the nervous system. Accumulating lines of evidence suggest that neuronal loss is caused, at least partially, by inappropriate activation of apoptotic mechanisms [1–3]. Because apoptotic neurons could still be rescued after a period of challenge time, neuroprotective agents with antiapoptotic proper-

ties might have potential therapeutic significance in neurodegenerative disorders [4].

In the developing nervous system, apoptosis is of vital importance and can be observed as early as during neural tube formation and persists throughout terminal differentiation of the neural network. Apoptosis is crucial for the establishment of appropriate neuronal circuitry in the development of the vertebrate nervous system, during which approximately 50% or more of the vertebrate neurons die of this programmed cell death process [5]. However, excessive apoptosis occurs in association with the neurological dysfunctions of the nervous system, as suggested for neurodegenerative diseases [1]. A young neuron-fate decision involves its interaction with the ever-changing microenvironment in the developing nervous system. Normally, the two most important factors that affect neuronal fate are the

presence and the amount of neurotrophic factors and appropriate neuronal activity. More specifically, either the lack of appropriate neurotrophic factors or the blockage of electrical activity of specific types of neurons can trigger excessive apoptosis before differentiation.

Primary cultured cerebellar granule neurons (CGNs) from post-natal rat represent a highly homogeneous neuron population that provides an excellent experimental model to study the molecular mechanisms underlying neuronal apoptosis. Normally, dissociated CGNs survive, differentiate, and mature *in vitro* in the presence of serum and/or depolarizing concentrations of K⁺ (25 mM). Removing serum or reducing K⁺ to 5 mM (K⁺ deprivation) would trigger apoptotic cell death [6]. Although the mechanisms underlying CGNs' apoptosis remain to be fully characterized, inhibition of PI3-K/Akt [7] and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase pathways [8,9] due to the lack of neurotrophic support, activation of caspases [7], and generation of reactive oxygen species [10,11] have all been implicated in the apoptotic process of CGNs.

Bis(propyl)-cognitin, also known as B3C, is a novel dimer derived from tacrine and has been reported by us as a promising agent against neurodegenerative disease on the basis of MEF2D activation [12], uncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonism [13,14], and acetylcholinesterase (AChE) inhibition [15]. Moreover, B3C was shown to offer neuroprotective effect with low toxicity in scopolamine [15] and middle cerebral artery occlusion-induced brain damage [13]. In this study, we further showed that B3C conferred neuroprotective effect against K⁺ deprivation-induced apoptosis in CGNs involving the pathway that sequentially requires activation of vascular endothelial growth factor receptor-2 (VEGFR-2)/Akt/glycogen synthase kinase 3 β (GSK3 β) and VEGFR-2/ERK signaling pathways.

Materials and Methods

Chemicals and Reagents

B3C was synthesized as we previously described [16]. Unless otherwise mentioned, all media and supplements used for cell culture were obtained from Gibco (Carlsbad, CA, USA). LY294002, PD98059, SB415286, and U0126 were purchased from Calbiochem (San Diego, CA, USA). PTK787/ZK222584 (PTK/ZK) was purchased from LC laboratories (Woburn, MA, USA). Fluorescein diacetate (FDA), propidium iodide (PI), and Hoechst 33342 were obtained from Sigma Chemicals (St Louis, MO, USA).

Primary Rat Cerebellar Granule Neurons Culture

Rat CGNs were prepared as we previously described [13]. Briefly, cell suspensions dissociated from 8-day-old Sprague–Dawley rats' cerebellum were seeded at a density of 2.0×10^6 cells/mL in basal modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 25 mM KCl, 100 units/mL penicillin/streptomycin, and 2 mM glutamine. Cytosine arabinoside (10 μ M) was added into the culture medium 16–24 h after seeding to limit the replication of non-neuronal cells. Cultures prepared by this procedure were enriched in CGNs with more than 95%, as evaluated by GAP-43 (a neuron marker) and

GFAP (an astrocyte marker) immunocytochemistry. CGNs were used at Day 8 *in vitro* (8 DIV) for all the experiments.

Treatment of CGNs

At 8 DIV, the culture medium in which CGNs were grown (10% FBS and 25 mM KCl) was replaced with a fresh serum-free medium containing 25 mM KCl (25K, control group), or 5 mM KCl (5K, K⁺ deprivation), or 5 mM KCl and the tested compounds. When pharmacological inhibitors were used for signaling assays, CGNs were pretreated with the inhibitors in the serum-free medium containing 25 mM KCl 0.5 h before the switch of CGNs to K⁺ deprived medium in the presence or absence of the tested compounds. B3C, tacrine, donepezil, memantine, and MK801 were dissolved in distilled water. For those dissolved in dimethyl sulfoxide (DMSO), the final concentration of DMSO added into the cell cultures was <0.1% and did not affect cell viability.

Measurement of Cell Viability

Cell viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay as previously described [17]. Briefly, CGNs (1.5×10^5 cells/well) were cultured in 96-well plates. After 24 h of treatment, 10 μ L of MTT solution (5 mg/mL in PBS) was added to each well, and then, the plates were incubated for 4 h at 37°C. All culture media were then discarded, and the formazan crystals were dissolved in 150 μ L DMSO. Cell viability was analyzed by testing colorimetric changes using a Bio-Rad Microplate Reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA) at a test wavelength of 570 nm with 655 nm as reference. Data were presented as a percentage of the control.

Image Assessment of FDA/PI Viability Staining

Simultaneous double staining with FDA and PI was applied to further differentiate between living and dead cells based on membrane integrity. FDA penetrates through living cell membranes and is hydrolyzed by intracellular esterase to produce fluorescein. Fluorescein accumulates inside the cell and emits green. However, PI passes through dead cell membranes and intercalates with DNA to form a bright red fluorescent complex. As a consequence, when a population of cells is stained with FDA/PI, living cells will be FDA positive and PI negative, dead cells will be PI positive but FDA negative. Briefly, after incubation with 10 μ g/mL FDA and 5 μ g/mL PI at room temperature for 15 min, the neurons were washed twice, incubated in PBS containing 5% glucose, and then photographed using fluorescence microscopy.

Morphological Evaluation of Nuclear Changes by Hoechst 33342 Staining

Chromatin condensation was analyzed by nucleus staining with Hoechst 33342 as described in our previous publication with some modifications [17]. After 24 h of treatment, CGNs (4.0×10^6 cells/well) plated in 6-well plates were washed twice with ice-cold PBS containing 5% (w/v) glucose and then stained with Hoechst 33342 (5 μ g/mL) for 5 min at 4°C. The nuclei were visualized using a fluorescence microscope at $\times 200$ magnification.

Analysis of DNA Fragmentation

DNA fragmentation was examined by agarose gel electrophoresis as previously reported [17] with slight modifications. After 24 h of treatment, cells were lysed in lysis buffer [10 mM Tris-HCl (pH 7.6), 20 mM EDTA, 0.5% v/v Triton X-100, 0.5 mg/mL proteinase K] for 30 min on ice. The lysate was centrifuged at $21,000 \times g$ for 30 min. The supernatant of lysate was extracted once with equal volume of phenol and once with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol at -20°C overnight. The extracted DNA (10 μg) was loaded onto 1.2% agarose gels and run at 80 V for 45 min in Tris-borate-EDTA buffer. The DNA bands were then visualized with ethidium bromide staining and photographed.

Western Blot

Western blot was performed as previously described [17]. Briefly, after treatment, cells were washed with PBS twice and then lysed on ice for 10 min in lysis buffer. Protein samples (20–40 μg) were separated by 12% SDS-PAGE and then transferred to polyvinylidene fluoride membranes for 2 h at 100 V. Subsequently, the membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h at room temperature. The blots were incubated with 1:1000 diluted anti-p-ERK, anti-ERK, anti-p-Ser-473Akt, anti-Akt, anti-p-Ser9-GSK3 β , anti-GSK3 β (rabbit monoclonal; Cell Signaling Technology Inc, MA, USA), 1:500 diluted anti-p-Tyr1054-VEGFR-2 (rabbit polyclonal; Abcam Inc., MA, USA), and 1:1000 diluted β -actin (goat monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Blots were developed using a Super Enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL, USA) for 5 min, and the protein bands were visualized after exposure of the blots to autoradiography X-ray film (Eastman Kodak, Rochester, NY, USA). The relative levels of each protein to housekeeping protein were determined by densitometry analysis using Gel-Pro analyzer 4.0 software (Gel media system, Beijing, China).

Statistical Analysis

Data, representative of at least three independent experiments carried out with different neuronal preparations, were presented as means $\pm$ SEM. Analysis of variance (ANOVA) followed by Bonferroni's post-test was used for statistical comparisons, and $P < 0.05$ or less was considered to be statistically significant.

Results

B3C Blocks Cell Death in CGNs Induced by K⁺ Deprivation

A dose–response curve was plotted after incubation with gradually increasing concentrations of B3C for 24 h (Figure 1A). It was found that B3C effectively blocked K⁺ deprivation-induced cell

death in CGNs in a concentration-dependent manner with an IC₅₀ of 0.37 μM (Figure 1A). With the increase in exposure time, the neuroprotective effect of B3C gradually decreased and disappeared completely 60 h after the K⁺ deprivation challenge (Figure 1B). Furthermore, B3C provided diminishing but significant protection when added 10 h after the switch to K⁺ deprived medium (Figure 1C).

B3C Inhibits Apoptosis in CGNs Induced by K⁺ Deprivation

To further investigate the neuroprotective effect of B3C, FDA/PI double staining, Hoechst staining, and DNA fragmentation assays were performed in succession. As observed from phase contrast microscopy (Figure 2A), B3C significantly blocked the loss of neurons and reversed the morphological changes caused by 24 h of K⁺ deprivation, including unhealthy bodies and broken extensive neuritic network. Similarly, results of FDA/PI double staining revealed that treatment with B3C restored the viable neurons as evidenced by the increase in the number of FDA-labeled neurons and the decrease in the number of PI-labeled neurons. To distinguish the apoptotic neurons from the necrotic ones, Hoechst staining and DNA fragmentation assay, two typical methods for detecting apoptosis were applied. The counts of apoptotic bodies stained by Hoechst 33342 indicated that B3C substantially reversed nuclear condensation induced by K⁺ deprivation (Figure 2B). Similarly, DNA fragmentation gel assays conducted on the above-mentioned CGNs suggested that B3C remarkably blocked DNA fragmentation (DNA “ladder”), another hallmark of apoptosis (Figure 2C).

The Neuroprotective Effect of B3C Against K⁺ Deprivation is Independent of AChE Inhibition and NMDA Receptor Antagonism

It was unclear whether the neuroprotective effect of B3C was produced by inhibiting AChE enzymatic activity. Therefore, B3C, together with the FDA-approved AChE inhibitors, tacrine and donepezil, was used to investigate whether they could block K⁺ deprivation-induced neuronal death. With MTT assay, it was observed that both tacrine and donepezil at 1–50 μM failed to attenuate neuronal death in CGNs induced by K⁺ deprivation in contrast to B3C (Figure 3A).

Next, both atropine (a muscarinic cholinergic receptor antagonist) and dihydro- β -erythroidine (a nicotinic cholinergic receptor antagonist) were applied to test whether the neuroprotective effect of B3C was produced through the activation of cholinergic receptors. By the same conditions as mentioned above, it was shown that atropine at 10 μM , dihydro- β -erythroidine at 50 μM , or their combination failed to block the neuroprotective effect of B3C against K⁺ deprivation-induced apoptosis in CGNs (Figure 3B).

Finally, two NMDA receptor antagonists, MK801 and memantine, were used in our *in vitro* model to investigate the blockade of NMDA receptor would contribute to the neuroprotective effect of B3C against K⁺ deprivation. With MTT assay, it was observed that neither MK801 at 5 μM nor memantine at 30 μM could attenuate neuronal death induced by K⁺ deprivation (Figure 3C).

Figure 1 B3C remarkably blocks neuronal death in cerebellar granule neurons (CGNs) induced by K^+ deprivation. **(A)** At Day 8 *in vitro* (8 DIV), CGNs were switched to K^+ deprived medium in the absence or presence of B3C (0–10 μM). At 24 h after K^+ deprivation, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. IC_{50} value was calculated using Origin Pro 8 statistical software from the sigmoidal inhibition curve. $^{##}P < 0.01$, compared with 5K group. **(B)** CGNs were switched to K^+ deprived medium in the absence or presence of 3 μM B3C for different times as indicated. Cell viability was measured at 24 h after treatment. $^{##}P < 0.01$, compared with 5K group. **(C)** 3 μM B3C was added at different times after K^+ deprivation, and cell viability was determined at 24 h after treatment. Cell viability was expressed as percentages of that of control (25K group). $^{##}P < 0.01$, compared with 5K group.

B3C Reverses Inhibition of the Activation of VEGFR-2 by K^+ Deprivation

To investigate whether the VEGF/VEGFR-2 system was involved in the neuroprotective effect of B3C in our system, PTK/ZK, a specific VEGFR-2 tyrosine kinases inhibitor, was applied in our experiments. With MTT assay, pretreatment with 10 μM PTK/ZK for 0.5 h abolished the neuroprotective effect of B3C against K^+ deprivation-induced cell death (Figure 4A). Furthermore, the activation of VEGFR-2 was examined in our system by determining the level of p-Tyr1054-VEGFR-2. It was found that K^+ deprivation caused a rapid decrease in p-Tyr1054-VEGFR-2, which peaked at 1 h (Figure 4B), and treatment with 3 μM B3C reversed the decrease in p-Tyr1054-VEGFR-2 induced by K^+ deprivation (Figure 4C).

B3C Reverses the Inhibition of Akt and ERK Pathways Caused by K^+ Deprivation

To determine whether PI3-K/Akt/GSK3 β pathway was involved in the K^+ deprivation in our CGNs, SB415286, a specific inhibitor of GSK3 β , was used to pretreat CGNs for 0.5 h before the K^+ deprivation challenge. It was shown that SB415286 at 3 and 10 μM blocked neuronal death caused by K^+ deprivation (Figure 5A). Thereafter, with the use of Western blot, we examined the time course of phosphorylated Akt and its downstream target phosphorylated GSK 3 β after K^+ deprivation. It was found that K^+ deprivation caused a significant decrease in p-Ser473-Akt and p-Ser9-GSK 3 β (Figure 5B,C), which peaked at 1 h. 3 μM B3C effectively reversed the decrease in p-Ser473-Akt and p-Ser9-GSK 3 β caused by K^+ deprivation at 1 h (Figure 5D). In addition, pharmacological inhibition of PI3-K by LY294002 (10 and 30 μM) as well as inhibition of VEGFR-2 by PTK/ZK (10 μM) abolished the neuroprotective effect (Figure 5A) and the reversal of decline in p-Ser473-Akt and p-Ser9-GSK 3 β caused by B3C (Figure 5D).

To examine whether activation of ERK pathway was involved in the neuroprotective effect of B3C in our model, PD98059, a specific inhibitor of MEK, was applied to pretreat CGNs 0.5 h before they were switched to K^+ deprived medium in the absence or presence of B3C. It was observed that PD98059 at the concentration of 30 and 50 μM abolished the neuroprotective effect of B3C, while PD98059 itself was unable to block apoptosis

Figure 2 B3C substantially attenuates apoptosis in cerebellar granule neurons (CGNs) induced by K^+ deprivation. **(A)** At Day 8 *in vitro* (8 DIV), CGNs were switched to K^+ deprived medium in the absence or presence of 3 μ M B3C. At 24 h after treatment, CGNs were assayed with a phase contrast microscope, fluorescein diacetate (FDA)/propidium iodide (PI) double staining, and Hoechst 33342 staining. Scale bar = 100 μ m. **(B)** The number of apoptotic nuclei with condensed chromatin was randomly and double blindly counted from **(A)** ($\times 200$). $^{**}P < 0.01$, compared with control (25K group), $^{##}P < 0.01$, compared with 5K group. **(C)** Under the same treatment conditions as **(B)**, DNA fragmentations were extracted from the CGNs, and then, agarose gel electrophoresis and ethidium bromide staining were used to visualize DNA extracted from the above samples.

(Figure 6A). With the use of Western blot, CGNs showed a significant decrease in ERK phosphorylation in the first 4 h after K^+ deprivation, an observation consistent with a previous study [18]. B3C was able to significantly increase the phosphorylation of ERK at 1 and 2 h after K^+ deprivation (Figure 6B). Pharmacological inhibition of MEK1/2 with PD98059 or inhibition of VEGFR-2 with PTK/ZK both returned p-ERK to 5K levels (Figure 6C).

Discussion

Cell death via apoptosis plays a positive role in mammalian neural development [19]. However, unregulated excessive apoptosis is

Figure 3 The neuroprotective effect of B3C is independent of acetylcholinesterase (AChE) inhibition and *N*-methyl-D-aspartate (NMDA) receptor antagonism. **(A)** At Day 8 *in vitro* (8 DIV), cerebellar granule neurons (CGNs) were switched to K^+ deprived medium containing 1, 10, or 50 μ M tacrine or donepezil. Cell viability was measured at 24 h after K^+ deprivation. $^{**}P < 0.01$, compared with 25K group. **(B)** At 8 DIV, CGNs were incubated with 10 μ M atropine (Atr) or/and 50 μ M dihydro- β -erythroidine (DHE) for 0.5 h in serum-free medium containing 25 mM KCl and then switched to K^+ deprived medium containing 3 μ M B3C. At 24 h after treatment, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. $^{**}P < 0.01$, compared with 25K group, $^{##}P < 0.01$, compared with 5K group. **(C)** At 8 DIV, CGNs were switched to K^+ deprived medium containing 1, 10, or 50 μ M memantine (MEM) or 0.1, 1, and 10 μ M MK801. Cell viability was measured at 24 h after K^+ deprivation. $^{**}P < 0.01$, compared with 25K group.

Figure 4 B3C reverses the activation of vascular endothelial growth factor receptor-2 (VEGFR-2) inhibited by K^+ deprivation. **(A)** VEGFR inhibitor abrogates the neuroprotective effect of B3C. Cerebellar granule neurons (CGNs) were incubated with 3 and 10 μ M PTK/ZK for 0.5 h in serum-free medium containing 25 mM KCl and then switched to K^+ deprived medium containing 3 μ M B3C. At 24 h after K^+ deprivation, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. ** P < 0.01, compared with control (25K group), ## P < 0.01, compared with 5K group; $\Delta\Delta P$ < 0.01, compared with 5K plus B3C group. **(B)** K^+ deprivation time dependently decreases the level of pTyr1054-VEGFR-2. CGNs were switched to K^+ deprived medium at the indicated time points, and the extracted proteins were analyzed using specific antibodies. ** P < 0.01, compared with control (25K group). **(C)** B3C reverses K^+ deprivation-induced decrease in p-Tyr1054-VEGFR-2. CGNs were switched to K^+ deprived medium in the absence or presence of 3 μ M B3C. At 1 h after treatment, cells were harvested and the extracted proteins were detected with the specific antibodies. ** P < 0.01, compared with control (25K group), ## P < 0.01, compared with 5K group.

probably the fundamental mechanism underlying various neurodegenerative disorders. Therefore, neuroprotective agents targeting apoptotic signaling pathways may have therapeutic use [4,20]. B3C, a novel dimer derived from tacrine, was investigated for its neuroprotective effect and signaling pathways in this study. Here, we mainly report two novel points: (1) B3C substantially blocks K^+ deprivation-induced apoptosis in rat CGNs in a concentration-dependent manner with an IC_{50} value of 0.37 μ M and (2) the neuroprotective effect of B3C is mediated by VEGFR-2/Akt/GSK3 β and VEGFR-2/ERK pathways.

Cerebellar granule neurons are widely used as a model system in neuroscience due to their homogeneity and suitability for the study of molecular mechanisms involved in apoptosis. K^+ deprivation induces matured CGNs to degenerate and die, prevalently via apoptosis [21]. In our system, typical hallmarks of apoptosis, such as condensation and aggregation of nuclear chromatin and internucleosomal DNA fragmentation, have been induced by 24 h of K^+ deprivation in CGNs (Figure 2), an observation in agreement with previous studies [18,22,23]. Moreover, the gradual recovery of phosphorylated proteins over time with the switch of CGNs to K^+ deprived medium indicates that our treatment with K^+ deprivation is not irreversible and neurons may recover quickly from this treatment (Figures 4–6). Taken together, these results suggest that this model is suitable for the study of the molecular mechanisms underlying neuronal apoptosis.

Using this *in vitro* model, we have analyzed the neuroprotective effect and the underlying mechanisms of B3C. It was found that B3C substantially attenuated K^+ deprivation-induced apoptosis in CGNs with an IC_{50} value of 0.37 μ M (Figure 1). Because B3C is derived from its monomer tacrine and has comparable AChE inhibition to tacrine [16], it is reasonable to investigate whether AChE inhibition contributes to its robust neuroprotective effect. However, other AChE inhibitors, including tacrine and donepezil, were unable to block K^+ deprivation-induced apoptosis (Figure 3A). On the other hand, the activation of cholinergic receptors has been demonstrated to be able to prevent apoptosis induced by several apoptotic inducers [24]. In our study, neither atropine nor dihydro- β -erythroidine could affect the neuroprotective effect of B3C (Figure 3B). Taken together, these results suggest that the inhibition of K^+ deprivation-induced apoptosis is independent of AChE inhibition and cholinergic transmission. In addition, two other NMDA receptor antagonists used in our study, MK801 and memantine, hardly showed any neuroprotective effect against neuronal apoptosis induced by K^+ deprivation (Figure 3C), an observation indicative of the independence of NMDA receptor antagonism in the neuroprotective effect of B3C.

K^+ deprived CGNs need neurotrophic support to survive. Initially identified as an angiogenic and vessel-permeability factor [25], VEGF has been recently demonstrated to be directly neurotrophic to CNS neurons in culture via activation of VEGFR-2 signaling pathway [26,27]. VEGFR-2 is the major VEGF receptors expressed on neuronal cells such as CGNs [28]. VEGF can be secreted by CGNs or the small quantity (5%) of contaminated astrocytes and oligodendrocytes in the culture [26]. Based on these, we hypothesize that B3C exerts its neuroprotective effect by modulating the activities of the VEGF/VEGFR-2 system either by enhancing the secretion of VEGF or by acting as a VEGFR-2 agonist. In our study, we have found for the first time that K^+ deprivation

Figure 5 B3C reverses the inhibition of the PI3-K/Akt/glycogen synthase kinase 3 β (GSK3 β) pathway caused by K⁺ deprivation. **(A)** PI3-K specific inhibitor LY294002 abolishes the neuroprotective effect of B3C. Cerebellar granule neurons (CGNs) were incubated with LY294002 for 0.5 h in serum-free medium containing 25 mM KCl and then switched to K⁺ deprived medium containing 3 μ M B3C. For the study of the involvement of GSK3 β pathway in our model, CGNs were switched to K⁺ deprived medium containing 3 or 10 μ M SB415286. At 24 h after K⁺ deprivation, cell viability was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. ***P* < 0.01, compared with control (25K group), ##*P* < 0.01, compared with 5K group, and $\Delta\Delta$ *P* < 0.01, compared with 5K plus B3C group. **(B)** K⁺ deprivation time dependently decreases the level of p-Ser473-Akt. CGNs were switched to K⁺ deprived medium at the indicated time points, and the extracted proteins were analyzed using specific antibodies. ***P* < 0.01, compared with control (25K group). **(C)** K⁺ deprivation time dependently decreases the level of p-Ser9-GSK3 β . CGNs were switched to K⁺ deprived medium at the indicated time points, and the extracted proteins were analyzed using the specific antibodies. ***P* < 0.01, compared with control (25K group). **(D)** B3C reverses K⁺ deprivation-induced decrease in p-Ser473-Akt and p-Ser9-GSK3 β . CGNs were switched to K⁺ deprived medium in the absence or presence of 3 μ M B3C. At 1 h after treatment, cells were harvested and the extracted proteins were detected with the specific antibodies. ***P* < 0.01, compared with control (25K group), ##*P* < 0.01, compared with 5K group, and $\Delta\Delta$ *P* < 0.01, compared with 5K plus B3C group.

vation induces a decrease in phosphorylated VEGFR-2 in CGNs (Figure 4B) and that B3C is able to reverse the activation of VEGFR-2 inhibited by K⁺ deprivation (Figure 4C). Moreover, MTT assay and Western blot analysis have shown that the neuroprotective effect of B3C is abolished by the VEGFR-2 inhibitor PTK787/ZK222584. These results indicate that the activation of VEGFR-2 contributes to the neuroprotective effect of B3C.

To further confirm the role of VEGFR-2 in the neuroprotective effect of B3C, we have analyzed Akt and ERK pathways, two critical pathways that are downstreams of VEGFR-2 involved in survival [26,28,29]. For CGNs in particular, the PI3-K/Akt mediated by growth factors appears to be the predominant survival pathway [30]. A rapid, reversible, and time-dependent decrease in phosphorylated Akt and its downstream target GSK3 β , but not protein expression, was induced by K⁺ deprivation (Figure 5B,C), an observation consistent with other previous

studies [8,9,18,23,31,32]. B3C significantly reversed these phosphorylated proteins inhibited by K⁺ deprivation. Furthermore, VEGFR inhibitor PTK/ZK, as well as PI3-K inhibitor LY294002, abolished the neuroprotective effect and regulation of VEGFR-2/Akt pathway induced by B3C (Figure 5A,D). These results taken together suggest that the neuroprotective effect of B3C is dependent on the VEGFR-2/Akt/GSK3 β pathway.

Several studies have suggested that an early increase in ERK activation is involved in the neuroprotective effect provided by extracellular factors to CGNs cultured in K⁺ deprived medium [33]. On the other hand, some reports have indicated that delayed activation of ERK is associated with CGNs' death in K⁺ deprived medium [34]. Thus, such neuroprotective effect against K⁺ deprivation would probably need an early positive and a delayed negative regulation of the ERK pathway. Accordingly, we have observed that treatment with B3C induced an early activation of

Figure 6 B3C reverses the inhibition of the extracellular signal-regulated kinase (ERK) pathway inhibited by K^+ deprivation. **(A)** Pharmacological inhibition of MEK with specific inhibitor PD98059 abolishes the neuroprotective effect of B3C. Cerebellar granule neurons (CGNs) were incubated with PD98059 for 0.5 h in serum-free medium containing 25 mM KCl and then switched to K^+ deprived medium containing 3 μ M B3C. At 24 h after K^+ deprivation, cell viability was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. $**P < 0.01$, compared with control (25K group); $##P < 0.01$, compared with 5K group; $\Delta P < 0.05$, compared with 5K plus B3C group. **(B)** B3C treatment induces ERK phosphorylation. CGNs were switched to K^+ deprived medium in the absence or presence of 3 μ M B3C. Cell lysates were obtained at the indicated time points after treatment and subjected to Western blot analysis with p-ERK and ERK. $*P < 0.05$, $##P < 0.01$, compared with 5K group. **(C)** B3C reverses K^+ deprivation-induced decrease in p-ERK. CGNs were switched to K^+ deprived medium in the absence or presence of 3 μ M B3C. At 1 h after treatment, cell lysates were obtained and subjected to Western blot analysis with phospho-ERK and ERK. $**P < 0.01$, compared with control (25K group); $##P < 0.01$, compared with 5K group; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, compared with 5K plus B3C group.

ERK as demonstrated by the increase in phosphorylated ERK. Although it remains to be studied whether B3C treatment would block the delayed K^+ deprivation-mediated increase in ERK phosphorylation, the fact that pharmacological inhibition of MEK was able to block the B3C-dependent increase in neuronal survival and phosphorylated ERK clearly indicates that B3C protects CGNs against K^+ deprivation-induced apoptosis by a MEK/ERK signaling-dependent mechanism. Moreover, B3C-mediated ERK pathway also depends on the activation of VEGFR-2 because PTK/ZK abolishes the increased phosphorylated ERK of B3C. Although B3C-mediated activation of PI3-K/Akt and ERK pathway was mainly dependent on the activation of VEGFR-2 in our study, we do not rule out the existence of other possible upstream targets on the cell surface, such as epidermal growth factor receptor-2 (EGFR-2) [35]. Experiments concerning these possible upstream targets will be carried out in our future studies.

Although the exact target still remains unclear, our study have demonstrated that B3C substantially blocks K^+ deprivation-induced apoptosis in CGNs via activation of VEGFR-2 and subsequent Akt and ERK pathways. The neuroprotective effect of B3C appears to be independent of AChE inhibition and NMDA receptor blockage. Because activation of VEGFR-2 is mainly regulated by receptor dimerization and autophosphorylation after specific ligand binding [36], we speculate that B3C might either directly interact with VEGFR-2 as a potential agonist or indirectly facilitate the activation of VEGFR-2 by stabilizing the dimerization or increasing the endogenous VEGF from elevating its translation, transcription, or post-transcription. To validate the hypothesis that B3C directly acts on VEGFR-2, further experiments such as whole-cell ligand dependent autophosphorylation assays are being undertaken in our laboratory. Moreover, we will not rule out the possibility that B3C itself enhances the endocrine secretion of VEGF. The precise mechanisms of the neuroprotective effect of B3C would be revealed by our further studies.

VEGF has been considered as a therapeutic agent against neurodegenerative disorders owing to its neuroprotective property [37]. However, side effects such as pro-inflammation, pro-angiogenesis,

and procarcinogenesis limit the clinical applicability of the drug [38]. Our findings that B3C exhibits strong neuroprotective effect via activating VEGFR-2 and subsequent signaling pathways provide a molecular insight into the therapeutic potential of B3C for the prevention and treatment of neurodegenerative disorders.

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Conflict of Interest

The authors declare no conflict of interest.

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