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Cellular and Molecular Characterization of Multiple Neuroprotections by Bis(propyl)-cognitin, A Promising Anti-Alzheimer's Dimer

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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CERTIFICATE OF ORIGINALITY

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HU Shengquan

Abstract

Neurodegenerative diseases such as Alzheimer's have emerged as a global issue, resulting in cognitive and behavioral problems, disability and economic burdens. Though their exact pathology remains unknown, neurodegenerative diseases are caused by, at least, inappropriate apoptosis as well as impairments in neurogenesis and neuronal differentiation. Compounds with anti-apoptotic property and neuronal differentiation-promoting activities could potentially have therapeutic significance against these devastating disorders.

Bis(propyl)-cognitin (B3C), a novel dimer derived from tacrine, has been shown as a multifunctional agent effectively against the key processes in neurodegenerative diseases on the basis of AChE inhibition and uncompetitive NMDA receptor antagonism. Moreover, B3C has been demonstrated to exert neuroprotections in middle cerebral artery occlusion-induced brain damage *in vivo*. However, the detailed molecular mechanisms of neuroprotection remain to be further elucidated. In this research, the multiple neuroprotections and underlying mechanisms by which B3C protects against glutamate or potassium (K^+) deprivation-induced apoptosis in primary cultured cerebellar granule neurons (CGNs) were examined. In addition, the neuronal differentiation-promoting activity of B3C was also investigated using PC12 cells and primary cultured cortical neurons.

In primary cultures of CGN, B3C (IC_{50}, 0.45 μM) substantially prevents glutamate-induced apoptosis with a potency approximately 10 times stronger

than memantine (IC_{50}, 4.58 μM), an effective anti-Alzheimer's drug approved by the FDA on the basis of NMDA receptor blockade. The neuroprotection of B3C is found to be associated with the blockade of NMDA receptors and subsequent regulation of NO, ERK and PI3-K/Akt/GSK3β pathways. In addition, B3C remarkably protects against K^+ deprivation-induced apoptosis in CGNs independent of its AChE inhibition and NMDA receptor antagonism, but through reversing the inhibition of vascular endothelial growth factor receptor-2 (VEGFR-2) /Akt/ GSK3β and VEGFR-2/ERK signaling pathways. Furthermore, B3C effectively induced neurite outgrowth in PC12 cells and primary cultured cortical neurons in a concentration- and time-dependent manner, as evidenced by the up-regulation of two neuron-specific protein markers growth-associated protein-43 and βIII-tubulin, and the neurite outgrowth-promoting activity of B3C correlates with the activation of α7-type nicotinic acetylcholine receptor (α7-nAChR).

In conclusion, B3C provides powerful neuroprotections by blocking NMDA receptor or activating VEGFR-2, and subsequently regulating pro-survival downstream signaling pathways. Moreover, B3C promotes neuronal differentiation by activating α7-nAChR. All these results, taken together, offer not only novel molecular insights into the therapeutic potential of B3C, but also a new experimental approach for developing new agents to modify or slow down the disease progression of Alzheimer's and other related neurodegenerative disorders.

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Publications arising from the thesis

1. **Hu S.,** Cui W., Xu D., Mak S., Tang J., Choi C., Pang Y. and **Han Y.** (2013) 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. ***CNS Neurosci Ther 19,*** 764-72

2. **Hu S.,** Cui W., Mak S., Tang J., Choi C., Pang Y. and **Han Y.** (2013) Bis(propyl)-cognitin protects against glutamate-induced neuro-excitotoxicity via concurrent regulation of NO, MAPK/ERK and PI3-K/Akt/GSK3beta pathways. ***Neurochem Int*** **62,** 468-477.

3. **Hu S.,** Han R., Mak S. and **Han Y.** (2011) Protection against 1-methyl-4-phenylpyridinium ion (MPP^+)-induced apoptosis by water extract of ginseng (Panax ginseng C.A. Meyer) in SH-SY5Y cells. ***J Ethnopharmacol*** **135,** 34-42.

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5. Cui W., **Hu S.,** Chan H. H., Luo J., Li W., Mak S., Choi T. C., Rong J., Carlier P. R. and **Han Y.** (2012) Bis(12)-hupyridone, a novel acetylcholinesterase inhibitor, protects against glutamate-induced neuronal excitotoxicity via activating alpha7-nAChR/PI3-K/Akt cascade. ***Chem Biol Interact*** *203, 365-70*. **(Co-first author).**

6. Cui W., Zhang Z., Li W., **Hu S.,** Mak S., Zhang H., Han R., Yuan S., Li S., Sa F., Xu D., Lin Z., Zuo Z., Rong J., Ma E. D., Choi T. C., Lee S. M. and **Han Y.** (2012) The anti-cancer agent SU4312 unexpectedly protects against MPP($^{+}$) -induced neurotoxicity via selective and direct inhibition of neuronal NOS. ***Br J Pharmacol*** **168,** 1201-1214.

7. Cui W., Zhang Z., Li W., Mak S., **Hu S.**, Zhang H., Yuan S., Rong J., Choi T. C., Lee S. M. and **Han Y.** (2012) Unexpected neuronal protection of SU5416 against 1-Methyl-4-phenylpyridinium ion-induced toxicity via inhibiting neuronal nitric oxide synthase. ***PLoS One*** **7,** e46253.

8. Han R. W., Zhang R. S., Chang M., Peng Y. L., Wang P., **Hu S.,** Choi C. L., Yin M., Wang R. and **Han Y.** (2012) Reversal of scopolamine-induced spatial and recognition memory deficits in mice by novel multifunctional dimers bis-cognitins. ***Brain Res*** **1470,** 59-68.

9. Cui W., Cui G. Z., Li W., Zhang Z., **Hu S.,** Mak S., Zhang H., Carlier P. R., Choi C. L., Wong Y. T., Lee S. M. and **Han Y.** (2011) Bis(12)-hupyridone, a novel multifunctional dimer, promotes neuronal differentiation more potently than its monomeric natural analog huperzine A possibly through alpha7 nAChR. ***Brain Res*** **1401,** 10-17.

10. Zhang H., Mak S., Cui W., Li W., Han R., **Hu S.**, Ye M., Pi R. and **Han Y**. (2011) Tacrine(2)-ferulic acid, a novel multifunctional dimer, attenuates 6-hydroxydopamine-induced apoptosis in PC12 cells by activating Akt pathway. ***Neurochem Int*** **59,** 981-988.

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List of abbreviations

α7-nAChR	Alpha7-type nicotinic acetylcholine receptor
Aβ	Amyloid β peptide
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer’s disease
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Atr	Atropine
B3C	Bis(propyl)-cognitin
BSA	Bovine serum albumin
CGN	Cerebellar granule neuron
ChAT	Choline acetyltransferase
CNS	Central nervous system
dbcAMP	2'-O-dibutyryladenosine 3':5' cyclic monophosphate
DHE	Dihydro-β-erythroidine
DIV	Days *in vitro*
DMEM	Dulbecco’s Modified Eagle Medium
E2020	Donepezil
ERK	Extracellular signal-regulated kinase
FDA	Fluorescein diacetate

GABA	γ-aminobutyric acid
GAP-43	Growth associated protein 43
Glu	Glutamate
GSK3 β	Glycogen synthase kinase 3β
HD	Huntington's disease
JNK	c-Jun N-terminal kinase
L-NMMA	N^G-monomethyl-L-arginine
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MK801	5H-dibenzo[a, d]cyclohepten-5, 10-imine
MLA	Methyllycaconitine
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NGF	Nerve growth factor
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NSAIDS	Non-steroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PI3-K	Phosphatidylinositol 3-kinase

PS	Presenilin
PTK	PTK787/ZK222584
PVDF	Polyvinylidene fluoride
UFO	Uncompetitive NMDA receptor antagonist with Fast Off-rate
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
V	Voltage
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2

Chapter 1

Introduction

Overview

As the population ages, numerous people tend to get Alzheimer's disease (AD) and other related neurodegenerative diseases. There are still no effective treatments for preventing, slowing down or curing the devastating diseases, and they can only be symptomatically modified. Regardless of the variety of pathology, neurodegenerative disorders are characterized by a selective and progressive cell death in specific neuronal populations in the central nervous system (Chaudhuri and Paul 2006; Lin and Beal 2006). Increasing lines of evidence indicate that the neuronal death is caused, at least partially, by inappropriate initiation of apoptotic signaling pathways (Mattson 2000; Ekshyyan and Aw 2004). Neuroprotection is an approach or strategy adopted to prevent neurons against neurotoxin insults through either directly antagonizing neurotoxins or indirectly blocking the downstream signals of neurotoxins. Based on the fact that apoptotic neurons could be recovered even after the neurotoxins' insult, molecules that display anti-apoptotic activities could provide potential therapeutic use against these devastating diseases (Schober 2004; Jana and Paliwal 2007).

In addition to neuroprotection by the inhibition of apoptosis, neurogenesis is considered to be another potential therapeutic strategy for these diseases. Several endogenous neurotrophic factors, including nerve growth factor (NGF) (Pardridge 2002), brain derived neurotrophic factor (BDNF) (Mattson 2008) and vascular endothelial growth factor (VEGF) (Jin et al. 2002), have been identified

as a contributor to enhance neurogenesis or neuronal differentiation, and thus are likely to be promising agents for ameliorating neurodegeneration. However, the peptidyl properties of neurotrophic factors have brought about a few serious difficulties to be overcome, *e.g.*, poor penetration through the blood-brain barrier (Thoenen and Sendtner 2002), and therefore limit its further developments as anti-neurodegenerative drugs. Accordingly, compounds that could enhance neurogenesis or neuronal differentiation may have potential therapeutic usage for these neurodegenerative diseases.

Bis(propyl)-cognitin (B3C), a multifunctional dimer derived from tacrine (Carlier et al. 1999) is designed and synthesized in our team. Recently, it has been demonstrated to be a multifunctional agent for the treatment of AD and other neurodegenerative diseases on the basis of MEF2D activation (Yao et al. 2012), uncompetitive NMDA receptor antagonism (Luo et al. 2010; Hu et al. 2013) and AChE inhibition (Carlier et al. 1999). In addition, B3C has been shown to exert neuroprotections in scopolamine (Han et al. 2012) and middle cerebral artery occlusion-induced brain damages (Luo et al. 2010) in rodents.

In the following study of literature review, AD, a representative of neurodegenerative diseases, is first presented on its pathology and current available pharmacological treatments. Subsequently, the relationship between AD and glutamate-mediated NMDA receptor over-activation, neuronal apoptosis and neuronal differentiation, in particular, are selected as research background in this study in detail. Second, the pathology of another two neurodegenerative

diseases (PD and HD) are briefly introduced. Most importantly, the research results on B3C will be analyzed and discussed in detail.

1.1 Alzheimer's disease

AD, first described by the German psychiatrist and neuropathologist Alois Alzheimer in 1906, is a progressive and degenerative brain disorder and represents the most common form of dementia among old adults (Kingwell 2013). It is estimated that approximately 35 million people get AD worldwide today, with 4.6 million new sufferers per year (Ferri et al. 2005; Selkoe 2012). China faces increasing numbers of AD and the number of AD patients is predicted to reach 26.1 million in 2040 (Ferri et al. 2005). The prevalence of AD rapidly increases with age, affecting approximately 9 % of people over age 65, and over 40 % of people aged 80 (Ferri et al. 2005). In addition, AD places a heavy burden on family and society both emotionally and financially. Costs for formal and informal care are estimated to reach to $ 62,189 per patient (Rice et al. 2001).

AD could be classified into two forms: familial and sporadic forms (Piaceri et al. 2013). Generally, AD is considered to be familial when one or more family members suffer, while sporadic refers to those when no other cases have been identified in extended family members. No more than 10 % of AD is familiar with the rest being sporadic. AD could be also divided into early- and late-onset forms (Joshi et al. 2012; Pievani et al. 2013). Early-onset AD denotes onset of

the disease when individuals develop AD before the age of 65, while late-onset AD means onset after 65. It is estimated that 90 % of familial AD belongs to late-onset forms, while almost all cases of sporadic AD are considered to be late-onset.

AD is clinically characterized by a gradual decline in memory and cognitive function, especially damaged memory, problems with language and communication, and finally decreased ability in performing activities of daily living (Pereira et al. 2005). The neuropathological hallmarks of AD include senile plaques (SP, β–amyloid fibrils deposits), neurofibrillary tangles (NFT, self-assembled hyperphosphorylated tau proteins bundles) and progressive neuronal loss in hippocampus (Selkoe 2001). Despite the rapid and enormous progress made in AD research over the past few decades, the detailed mechanisms underlying the pathology of AD still remain to be further elucidated, and no effective drug therapy is yet available. Up to now, three acetylcholinesterase (AChE) inhibitors and one NMDA receptor antagonist have been approved by U.S. Food and Drug Administration (FDA) for the treatment of AD (Doraiswamy and Xiong 2006).

1.1.1 Risk factors

1.1.1.1 Age

Age is generally considered as one of the strongest risk factors responsible for the occurrence of AD. In sporadic AD, risk increases with age after 65. For

those over 80, the number dramatically increases to 40 %.

1.1.1.2 Genetics

The well-characterized link between AD and genetics is observed in familial early-onset AD (Herz 2007). There are totally three genes involved. The gene of β-amyloid precursor protein (APP) located on chromosome 21 is normally cleaved to form small amounts of β-amyloid (Aβ). While, mutations in APP cause incorrect cleavage of this gene, resulting in the generation of Aβ species that is more inclined to aggregate into amyloid plaques (Goedert and Spillantini 2006). APP mutation constitutes 10-15 % familial early-onset AD cases. Presenilin (PS) 1 and PS2 located on chromosome 14 and 1 translate PS1 and PS2 proteins to form γ–secretases complex for the cleavage of APP. Mutations in both PS1 and PS2 also result in incorrect cleavage of APP, in association with the occurence of familial early-onset cases, with the proportion of 30-70 % and 5 %, respectively. Familial early-onset AD is an autosomally inherited condition, suggesting that inheritance of any one mutant allele of APP, PS1 and PS2 will increase the chance of getting AD. Thus, children with an affected parent may get a 50 % chance of inheriting these mutations and developing this disease (Goedert and Spillantini 2006).

The ε4 allele of the apolipoprotein E (ApoE) gene, located on chromosome 19, has been identified as a biggest genetic risk factor for the occurrence of sporadic late-onset AD cases (Yamagata et al. 2001; Lambert et al. 2002).

Individuals carrying two ApoE ε4 alleles are considered to have a 30 % chance of developing sporadic late-onset AD (Herz 2007).

1.1.1.3 Education

A recent study from Aging Research Center of Sweden has demonstrated that poorly educated people appear to be at a high risk of developing AD (Ngandu et al. 2007). Regardless of the precise mechanism, it is theorized that a higher education facilitates the formation or strengthening of synaptic connections among neurons in the brain. The process creates a “synaptic reserve” in the brain, enabling individuals to compensate for the loss of neurons and thus decrease the likelihood of the occurrence of AD.

1.1.1.4 Coexisting health problems

Epidemiological findings have indicated that high blood pressure, high cholesterol level and/or heart disease are associated with the high risk of developing AD. This can be explained by the damage to many blood vessels in the brain and subsequent occurrence of brain tissue death. In addition, inefficiency or resistance of insulin may, to some extent, increase the risk of developing AD (Pereira et al. 2005).

1.1.1.5 Gender

Women are naturally more inclined to get AD than men due to estrogen

deficiency or other yet unknown reasons (Alberca et al. 2002).

1.1.2 Hypothesis for the pathogenesis of AD

1.1.2.1 Cholinergic dysfunction and AChE toxicity

The cholinergic hypothesis, on which most existing anti-AD drugs are based, indicates that AD is caused by the reduction in the synthesis or production of the neurotransmitter acetylcholine (ACh). In addition to the dramatic loss of cholinergic neurons, a significant decline in choline acetyltransferase (CHAT) and an remarkable increase in AChE are found in AD patients brains (Auld et al. 2002).

AChE is usually regarded as an enzyme that plays a vital role in hydrolyzing ACh in the synaptic cleft and terminating the action of ACh. However, recent studies show that the AChE is a multifunctional protein in additional to its hydrolyzation capability (Inestrosa et al. 2005). This involves that the intracellular AChE participates in neuronal apoptosis induced by neurotoxins insults (Zhang et al. 2002). In the brain of AD patients, the expression of AChE protein is decreased, while AChE activity, to a large extent, experiences a significant enhancement around the Aβ plaques, which may be explained by the interaction of AChE and Aβ (Talesa 2001). Furthermore, with the specific motif in the peripheral site, AChE is able to promote Aβ aggregation (Talesa 2001; Inestrosa et al. 2005). As such, AChE inhibitors could potentially slowdown the progress of AD through simultaneously restoring cholinergic

system and inhibiting Aβ aggregation.

1.1.2.2 Aβ cascade hypothesis

Aβ, an about 4 KD protein, is generated from APP through “amyloidogenic pathway” in which APP is sequentially cleaved by β- and γ-secretases rather than α-secretase (Pereira et al. 2005). The Aβ cascade hypothesis has maintained widespread support. It proposes that AD is fundamentally caused by Aβ deposits. The demonstration that the mutations in related genes enable the over-generation of Aβ, together with the *in vitro* and *in vivo* studies that Aβ aggregates, oligomers in particular, is very neurotoxic to neurons (Pereira et al. 2004; Feng et al. 2009), comprise the convincing and compelling evidence for supporting the role of Aβ hypothesis in AD.

Increasing lines of evidence demonstrate that soluble Aβ oligomers, rather than insoluble Aβ plaques, represent the primary pathogenic form of Aβ. Oligomeric Aβ assemblies (Aβ-derived diffusible ligands, ADDL), are blamed for the interruption of synaptic plasticity, the basis for cognitive and memory formation. Soluble Aβ oligomers are highly expressed in AD brains (Gong et al. 2003) within synaptic compartments (Takahashi et al. 2004) in the absence of insoluble Aβ plaques and neurofibrillary tangles, indicating that oligomeric Aβ accumulates at the beginning or very early stage of AD.

1.1.2.3 Tau phosphorylation

Tau protein is a microtubule-associated protein abundant in the central nervous system and maintains neuronal morphology. However, recent studies have demonstrated that abnormal tau proteins, which are hyperphosphorylated either by GSK3β or cyclin dependent-kinase 5 (Cdk5) (Pereira et al. 2005), lead to the formation of neurofibrillary tangles and the degradation of the cytoskeleton and finally neurodegeneration.

1.1.2.4 Glutamate excitotoxicity

Glutamate, a major excitatory neurotransmitter, is responsible for the formation of memory. In the physiological process, glutamate binds to its specific receptors, either ionotropic (NMDA, AMPA and KA receptors) or metabotropic receptors, to fulfill numerous physiological functions in learning and memory. However, over-activation of NMDA receptors by excessive glutamate results in the continuous influx Ca^{2+}, and subsequently leads to oxidative stress or mitochondria dysfunction and ultimately neuronal death (Fig. 1.1) (Forder and Tymianski 2009). In addition, since glutamatergic neurons are also affected by other neurotransmitters, e.g. serotonin, γ-aminobutyric acid (GABA) or ACh, which are also abnormal in AD process, different synaptically intercalated neurotransmitter systems might produce a synergistic effect against cognitive deficits or memory loss.

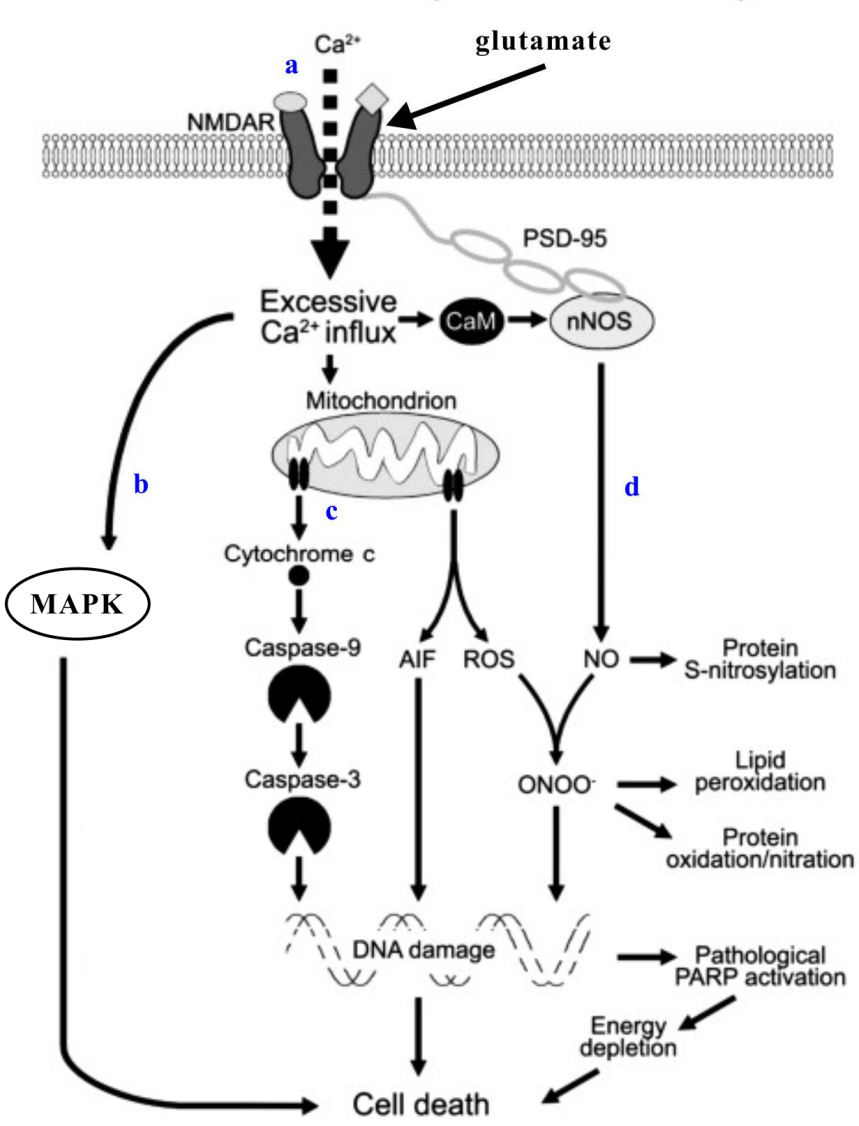


Fig. 1.1 Schematic diagram for the pro-apoptotic signaling pathways induced by the over-activation of NMDA receptor.

The glutamate excitotoxicity cascade includes (a) over-activation of NMDA receptor by excessive glutamate; (b) activation of MAPK pathway; (c) initiation of apoptosis-inducing enzymes, including caspases and apoptosis inducing factors (AIF); (d) activation of harmful free radicals including NO and ROS (Seki and Lipton 2008).

1.1.2.5 Oxidative stress and metal ion homeostasis

A mass of oxidative stress markers, accompanying SP and NFT, are found in AD patients brains (Reddy 2009), strongly suggesting the involvement of oxidative stress in the pathogenesis of AD. Increasing lines of evidence have demonstrated that Aβ not only acts as a source, but also as a consequence of oxidative stress in the pathogenesis of AD. Aβ toxicity correlates well with the increase in intracellular reactive oxygen species (ROS) (Varadarajan et al. 2000), oxidative stress also enables APP processing in an amyloidogenic way, leading to the over-production of Aβ species (Misonou et al. 2000). This suggests that the process can enter a potential vicious cycle whereby oxidative stress promotes the toxicity of Aβ, and Aβ facilitates the generation of more oxidative stress, neuronal homeostasis failure, and ultimately neuronal death.

Metal ion homeostasis is seriously dysregulated in AD brains and has been proved to be closely linked to oxidative stress. Zinc, iron and copper are highly increased within senile plaques in AD brains (Pereira et al. 2005). These metals bind to Aβ, promote Aβ aggregation, increase Aβ toxicity and enhance metal-catalysed oxidative stress.

1.1.2.6 Neurotrophic factor deficiency

Neurotrophic factors are small, versatile proteins that control neuronal survival, differentiation and synaptic plasticity, and play a vital role in cognition and memory formation (Schindowski et al. 2008). Neurotrophins are initially

synthesized as pro-neurotrophins, while they appears to function contradictorily: neurotrophins maintain survival and function to specific neuronal populations through binding to TrkA, TrkB, TrkC, specific tyrosine kinases receptors, while pro-neurotrophins trigger neuronal death by binding of $p75^{NTR}$ (Friedman 2000). For instance, pro-nerve growth factor (NGF) is increased in the frontal cortex and in hippocampus in the brain of AD patients, while NGF is decreased in the terminals of affected neurons in AD brains. In the absence of NGF, cholinergic neurons shrink and display down-regulation of neurotransmitter-associated enzymes such as ChAT, resulting in a dysfunction of cholinergic neurons (Svendsen et al. 1991). In parallel, AD rats showing a serious decline in ChAT and TrkA mRNA can be restored following NGF treatment (Venero et al. 1994).

1.1.3 Therapeutical strategies for AD

Though the precise mechanisms of AD still remain unclear, drug companies and institutions have been actively pursuing novel anti-AD drugs (Fig.1.2).

1.1.3.1 AChE inhibitors

By enhancing the concentration of neurotransmitter ACh in synaptic clefts, AChE inhibitors, which represent the main branch of anti-AD drugs, have been widely used for the treatment of AD. Totally, there are three currently available AChE inhibitors approved by FDA as anti-AD drugs, including Aricept® (donepezil, 1996), Exelon® (rivastigmine, 2000) and Razadyne® (galantamine,

2001) (Michaelis 2003). Cognex® (tacrine, 1993) is now rarely used due to its liver toxicity. More encouragingly, huperzine A, an alkaloid compound originally extracted from a Chinese medicinal herb *Huperzia serrata* (Qian ceng ta), has been approved to treat AD in China due to its strong AChE inhibition. However, huperzine A has not been widely used because of a lack of intellectual property protection, a shortage of natural supply and a difficulty in chemical synthesis (Wang et al. 2009).

1.1.3.2 Therapies targeting Aβ and tau

Since the rise of Aβ hypothesis in 1990, the use of β-secretase (BACE-1) and γ-secretase inhibitors have been actively pursued by the research institutions. However, the demonstration that β- and γ-secretases catalyze reactions involving multiple substrates (Notch receptor, ErbB4, p75NTR receptor) limits their further development as anti-AD drugs (Citron 2004). Due to these difficulties, there are no FDA approved drugs that target both enzymes and the discounted rate in clinical trial is high. Even so, a few β- and γ-secretase inhibitors, such as LY-2434074 (Eli Lilly) and avagacestat (BMS) are being actively developed and tested in clinical trials (Mangialasche et al. 2010).

In addition, both lithium and valproate, potent GSK3β inhibitors involved in the phosphorylation of tau protein, have been demonstrated to be effective in an AD animal model (Perez et al. 2003), and has been moved to clinical trial stage (Hampel et al. 2009). Other agents which could inhibit the aggregation of tau

protein or promote the disassembly of tau aggregates are also being actively pursued (Mangialasche et al. 2010).

1.1.3.3 NMDA receptor antagonist

In light of the glutamatergic system dysfunction hypothesis in the pathogenesis of AD, NMDA receptor antagonists are also designed and developed. Memantine (Namenda), approved by FDA in 2003, is an uncompetitive moderate affinity NMDA receptor antagonist with UFO property. Memantine preferentially blocks extrasynaptic over synaptic NMDA receptors in hippocampus (Xia et al. 2010), suggesting that it might prevent pathological excitotoxicity without affecting the physiological processes. Compared to AChE inhibitors, memantine shows a modest benefit against moderate-to-severe AD by slowing down the deterioration of cognitive functions (Rogawski and Wenk 2003; Hellweg et al. 2012).

1.1.3.4 Antioxidant

Oxidative stress markers is found within AD patients brains, treatment with antioxidants is therefore believed to be a promising approach in AD therapy (Gilgun-Sherki et al. 2003; Aliev et al. 2008; Mecocci and Polidori 2012). Recent findings indicate a tight link between antioxidant supplements and reduced occurrence of AD. A few antioxidants, including resveratrol, curcumin, green tea, vitamin C, E and ginkgo biloba extract, have been widely used in dementia

therapy (Frank and Gupta 2005). Ginkgo biloba has been demonstrated to be slightly, but significantly effective in comparison to placebo groups with AD (Oken et al. 1998; Carlson et al. 2007). Its standardized extract EGb761 has been widely used in Europe to improve memory and cognitive problems. In addition, vitamin E supplements have been added to the standard treatment regimen of AD by more and more practitioners (Kontush and Schekatolina 2004; Pham and Plakogiannis 2005; Isaac et al. 2008; Kontush and Schekatolina 2008).

1.1.3.5 Neurotrophic factors

Neurogenesis occurs in the adult brain in the hippocampus, the subgranular zone (SGZ) and the subventricular zone (SVZ), as a response to injury. Cholinergic neurons in basal forebrain need neurotrophic factors such as NGF for survival, neurite outgrowth, and modulation of transmitter-specific enzymes. Recent research have indicated a causal link between NGF imbalance and neurodegeneration in AD (Cattaneo et al. 2008), thus the boosting of NGF and other neurotrophins to combat against AD represents a new direction in the therapy of AD. NGF delivery was tested and an overall lower rate of cognitive decline was accordingly observed (Tuszynski et al. 2005).

1.1.3.6 Other potential treatments

Estrogen deficiency may contribute to the occurrence of AD in postmenopausal women (Levine and Battista 2004; Mulnard et al. 2004).

Estrogen supplement therapy has thus shown some benefits in women with familial AD through modulating ApoE gene, increasing the metabolism of APP or the formation of synapses, and inhibiting oxidative stress (Brinton et al. 2000; Miller et al. 2001).

Several lines of evidence indicate the involvement of the inflammation in the form of cytokines and microglial activation in AD pathogenesis (Ferencik et al. 2001), which is responded by a series of clinical trials with non-steroidal anti-inflammatory drugs (NSAIDs) (Gasparini et al. 2004; Imbimbo 2009).

Besides, there is evidence in support of several non pharmacological strategies, such as exercise, music and behavior therapy, which reverse, to some extent, the functional and behavioral deterioration (Nithianantharajah and Hannan 2009).

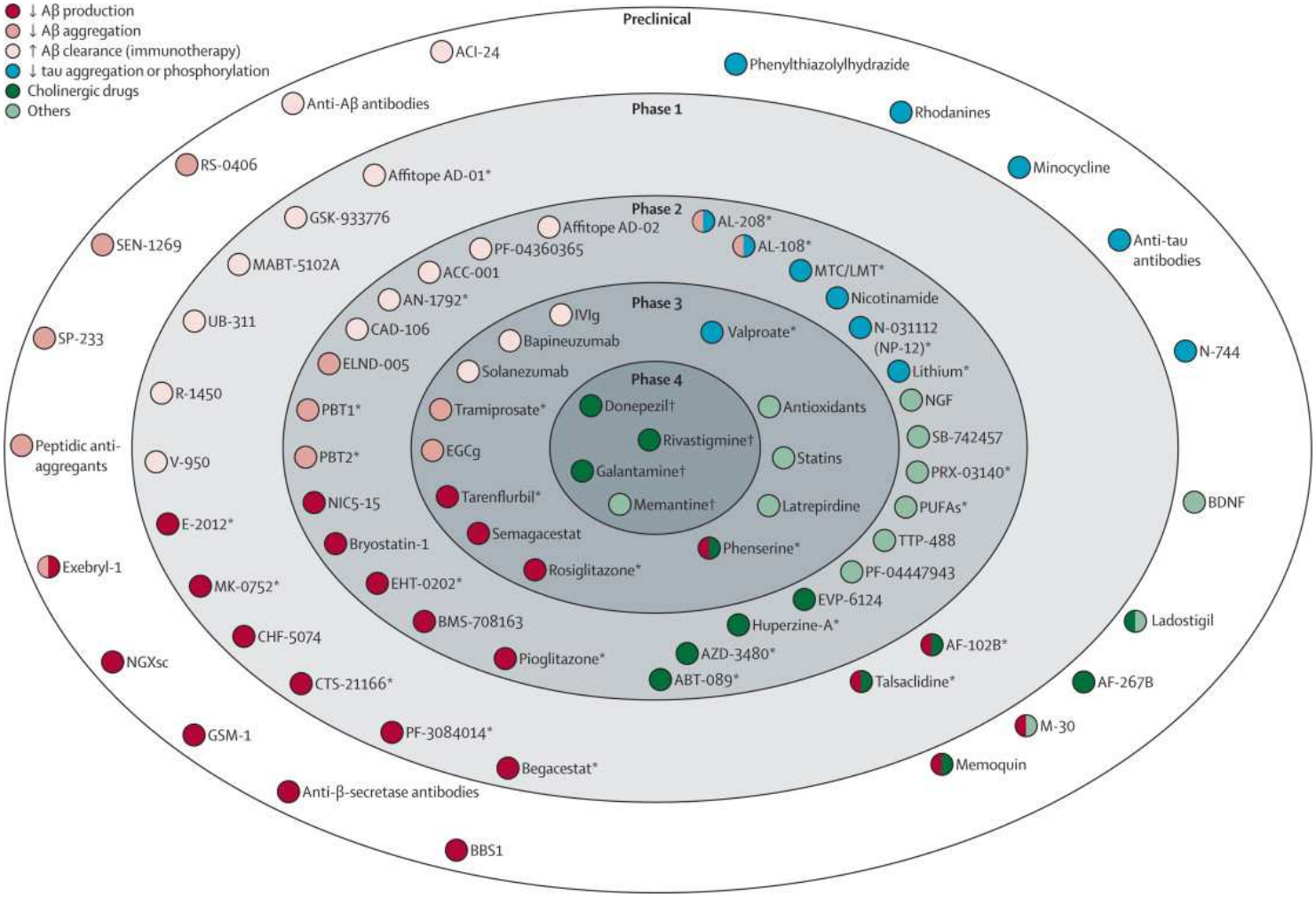


Fig. 1.2 Current drug development in AD (Mangialasche et al. 2010).

1.1.4 Apoptosis

1.1.4.1 Apoptosis in AD

In the developing nervous system, apoptosis maintains cell homoeostasis and plays a vital role in various biological processes, including neural tube formation and the establishment of appropriate neuronal circuitry (Raff et al. 1993). The other side of the coin, however, is that excessive apoptosis of neurons in adult brains results in the neurological dysfunctions of the nervous system, as suggested for AD and other neurodegenerative diseases (Mattson 2000). A growing body of evidence indicates apoptosis represents the final common pathway for neuronal degeneration (Raina et al. 2003). There are various pro-apoptotic neurotoxins and/or stimuli present in the pathogenesis of AD and other neurodegenerative diseases, which may either act by themselves or synergistically to result in an activation of the apoptotic death pathway, such as glutamate, Aβ, oxidative stress (Fang et al. 2005; Onyango et al. 2005). *In vivo* studies have shown that neurons, in hippocampus or cortex in particular, may die of apoptosis in AD (Shimohama 2000). Based on the fact that apoptotic neurons can still be recovered even after the neurotoxins insult, molecules displaying anti-apoptotic activities may constitute a promising approach in the therapy of AD (Bachis et al. 2001; Li et al. 2007).

1.1.4.2 Signaling pathways involved in glutamate-induced neuronal apoptosis

Glutamate excitotoxicity represents a vital contributing factor that underlies the

pathogenesis of AD (Butterfield and Pocernich 2003; Francis 2003). Pathologically, abnormal release of glutamate over-activates NMDA receptors on the postsynaptic neurons (Li and Tsien 2009), causes abnormal influx of Ca^{2+} and subsequent neuronal deaths. Several signaling pathways, including nitric oxide (NO) pathway (Li et al. 2007), extracellular signal-regulated kinase (ERK) pathway (Li et al. 2005) and phosphatidylinositol 3-kinase (PI3-K)/Akt/GSK3β pathway (Pi et al. 2004), are believed to play key role in glutamate-induced excitotoxicity and act as the downstream pathways of intracellular accumulation of Ca^{2+}.

The over-stimulation of NMDA receptor/NO pathway has been well documented in AD pathology (Fig. 1.1) (Li et al. 2007). Over-production of NO, generated by neuronal NO synthase (nNOS) anchored to NMDA receptor through postsynaptic density protein-95-NMDA receptor coupling protein, mediates the downstream pathways of NMDA receptor and leads to neuronal death (Boje 2004; Lipton 2004). Thus, nNOS inhibitors and NO scavengers have been demonstrated to be effective against glutamate insult and considered as potent anti-Alzheimer's candidates (Lipton 2004; Willmot et al. 2005).

ERK pathway involves a series of proteins within the neuron, which transfers an extracellular signal from the receptors on the membrane to the DNA in the nucleus. In AD condition (Fig. 1.3), the over-activation of NMDA receptor subsequently activates Ras via Ca^{2+}/calmodulin-dependent activation of Ras-GRF, and other core units, such as Raf (mitogen-activated protein kinase kinase kinase, MAPKKK), MEK1/2 (mitogen-activated protein kinase kinase, MAPKK) and ERK1/2

(mitogen-activated protein kinase, MAPK), are in turn activated. The activated ERK molecule then transfers the received signals into the nucleus, induces gene change and eventually leads to neuronal death (Kolch 2000).

On the contrary, PI3-K/Akt cascade is a crucial pro-survival pathway which is inhibited in glutamate-induced excitotoxicity (Fig. 1.3). Akt (Protein kinase B, PKB), acts as a downstream target of PI3-K and are responsible for multiple biological processes. Generally, activated Akt protein phosphorylates substrates in the cytoplasm, and then increases cell survival by inhibiting apoptotic processes. For instance, Akt is able to inhibit GSK3 activity through the phosphorylation of GSK-3 at Ser21 in GSK-3α or Ser9 in GSK-3β. Recently, much effort is actively directed towards the inhibition of GSK3 activity to protect neurons against neurotoxin insults. However, the relationship among NO, ERK and PI3-K/Akt/GSK3β pathways in glutamate-induced neurotoxicity has not yet been explored.

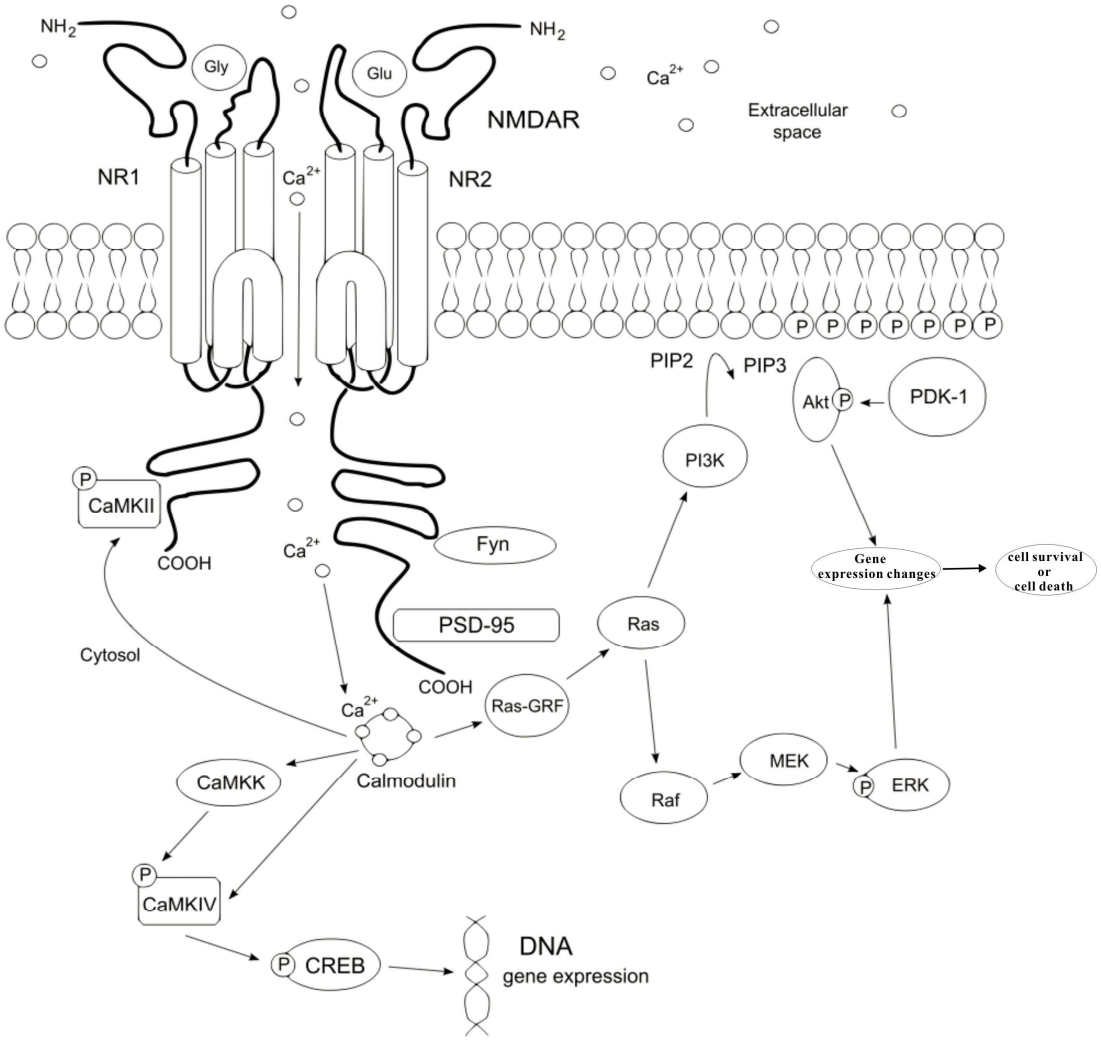


Fig. 1.3 Signaling pathways involved in glutamate excitotoxicity.

Some intracellular signaling pathways, namely Ca^{2+}/CaMK and ERK, are proposed to trigger neuronal apoptosis after glutamate insult. Other cascades, such as PI3-K/Akt pathway, are believed to protect against glutamate challenge (Chatterton et al. 2002).

1.1.4.3 Signaling pathways involved in potassium (K^+) deprivation-induced neuronal apoptosis

During early neural development, the neuron-fate decision involves its interaction with the changing microenvironment in CNS. Normally, the two most important factors affecting neuronal fate are the presence and the amount of neurotrophic factors as well as appropriate neuronal activity. More specifically, either the deficiency of neurotrophic factors or the blockage of electrical activity of specific types of neurons could induce excessive apoptosis.

Primary cultured rat cerebellar granule neurons (CGNs) represent a highly homogeneous neuronal population, which has been employed as a widely used *in vitro* model to study the molecular mechanisms underlying neuronal apoptosis. Normally, freshly dissociated CGNs survive, differentiate and mature *in vitro* when the neurons are subjected to medium containing serum and/or K^+ (depolarizing concentrations, 25 mM, 25K). On the contrary, withdrawing serum or reducing K^+ to 5 mM (K^+ deprivation, 5K) would induce apoptosis in CGNs (Ramiro-Cortes and Moran 2009). Though the precise mechanisms that underlie the apoptotic process of CGNs remain to be further explained, inhibition of PI3-K/Akt (Mora et al. 2001) and ERK pathways (Zhong et al. 2004; Severini et al. 2008), activation of caspases cascades (Mora et al. 2001) and abnormal generation of reactive oxygen species (ROS) (Atlante et al. 2010) are believed to be implicated in the neuronal apoptosis.

Though the process of K^+ deprivation-triggered neuronal death is not directly involved in the pathogenesis of AD, the molecular mechanisms underlying neuronal

apoptosis could be, to some extent, shared between the apoptotic processes induced by K^+ deprivation and that by neurotoxins associated with AD. Neuroprotective compounds that can protect against K^+ deprivation-induced apoptosis in CGNs are considered to be attractive candidates for AD and related neurodegenerative disorders (Uryu et al. 2002; Zhong et al. 2007).

1.1.5 Neuronal differentiation

Because of the tight link between neuronal loss and the pathogenesis of neurodegenerative disease, in addition to agents with anti-apoptotic property, novel compounds that can promote neural differentiation might have therapeutic significance against AD and other neurodegenerative diseases. During neural development and regeneration in response to these kind of compounds, the process of neurite outgrowth from the cell body is of particular importance for regeneration of the nervous system (Sarina *et al.*).

PC12 cells act as an excellent experimental *in vitro* model for investigating neuroscience, *e.g.* neuronal differentiation and the underlying molecular mechanisms (Sarina et al.; Das et al. 2004; Cui et al. 2012). In response to NGF, PC12 cells are differentiated into neuronal phenotype similar to those found in sympathetic neurons (Greene and Tischler 1976), characterized by condensed cell body and extended neurite outgrowth. These physiological changes involve various signaling transduction pathways. NGF induces a rapid increase in TrkA phosphorylation and subsequent stimulation of MEK/ERK pathway (Vaudry et al. 2002). In addition, an

intracellular elevated level of cyclic AMP (cAMP) usually employs cAMP-PKA-CREB pathway to induce differentiation in PC12 cells (Maruoka et al. 2010). Recent findings have demonstrated that nicotinic acetylcholine receptors (nAChR), α7-nAChR in particular, play a pivotal role in synaptic transmission and differentiation (Nery et al. 2010). Concordantly, with the activation of α7-nAChR, PC12 cells display condensed cell bodies and extended outgrowth of neurites (Utsugisawa et al. 2002), while depletion of α7-nAChR destroys hippocampal dendritic neurons maturation (Maggini et al. 2006; Campbell et al. 2010), indicative of the involvement of α7-nAChR for neuronal differentiation.

1.2 Other neurodegenerative disorders

Neurodegenerative disorders are caused by complicated and yet unknown reasons, however, their common typical histological feature is the neuronal loss in specific regions, such as the loss of cholinergic neurons in hippocampus and cortex in AD, the loss of dopaminergic (DA) neurons in the substantia nigra in Parkinson's disease (PD) and the loss of medium spiny neurons in the striatum in Huntington's disease (HD) (Srivastava et al. 2008). All of these neurodegenerative diseases, though with different clinical symptoms, share some common pathological processes. PD and HD are selected for brief introduction.

1.2.1 Parkinson's disease

PD, also known as idiopathic parkinsonism or paralysis agitans, is characterized

by rigidity, shaking, slowness of movement and difficulty with walking and gait. Cognitive and behavioral problems are also observed in the advanced stages of PD. Lewy body, an inclusion body found in the cytoplasm of neurons (Taylor et al. 2002), is the primary pathological hallmark of PD. Though the exact cause remains still unclear, it is generally accepted that PD is caused by the loss and degeneration of dopaminergic neurons in the substantia nigra of the midbrain and other monoaminergic neurons in the brain stem (Tatton et al. 2003; Witt and Flower 2006). A growing body of evidence indicates the involvement of glutamate-induced excitotoxicity in the pathology of PD (Armentero et al. 2006; Johnson et al. 2009; Meredith et al. 2009). In addition, a sharp decline in neurogenesis and neuronal differentiation in SGZ and SVZ, as well as a reduced number of new neurons in the olfactory bulb have been observed in the early stage of PD (Simuni and Sethi 2008), suggesting that compounds with neuroprotection and neuronal differentiation-promoting activities may have therapeutic significance against PD. Recent findings have indicated the involvement of genetic factors in the etiology of PD. Two mutations (A53T and A30P) of the α-synuclein gene cause autosomal dominante PD via a gain-of-function mechanism (Shimura et al. 2001; Outeiro et al. 2007). Recessive early-onset PD can be caused by mutations in the genes encoding parkin, DJ-1 or PINK1, presumably via a loss-of-function mechanism (Taylor et al. 2002).

1.2.2 Huntington's disease

Huntington's disease, clinically characterized by a distinctive phenotype, such as dystonia, cognitive dysfunction and behavioral problems, represents an inherited neurodegenerative disease. The characteristic symptoms result from the selective loss or degeneration of medium spiny GABAergic neurons in the striatum, the subcortical brain structure that controls body movements (Hickey and Chesselet 2003; Almeida et al. 2008). The gene responsible for the occurrence of HD is hungtinton, which contains a polymorphic stretch of repeated CAG trinucleotides that encodes a polyglutamine tract within hungtinton. There is evidence for the disturbance of NMDA receptor transmission in the early pathogenesis of HD (Milnerwood et al. 2012). Recent studies have demonstrated that the reduced ability of neural stem cells to differentiate into neurons is also observed in HD brains, indicating the tight link between neuronal differentiation and HD (Nakaguchi et al. 2011).

Though diverse neurodegenerative diseases display distinct phenotypes, they may share some common pathways, such as glutamate-induced excitotoxicity, leading to their pathology. Impairment of neurogenesis and neuronal differentiation are also associated with these devastating disorders. Compounds with anti-apoptotic property or neuronal differentiation-promoting activities may have therapeutic significance against these neurodegenerative diseases.

1.3 Bis(propyl)-cognitin--novel dimer derived from tacrine

Bis(propyl)-cognitin (1, 7-N-propylene-bis-9, 9'-amino-1, 2, 3, 4-tetrahydroacridine) (Fig. 1.4), also known as B3C, together with other

bis(n)-cognitins which are derived from tacrine linked with different lengths of methylene (-CH_2-) groups in our lab (Carlier et al. 1999), is designed and synthesized as a novel dimeric AChE inhibitor with dual binding sites of AChE. It is a yellowish-white powder which is slightly soluble in water and dissolves easily in organic solvent. B3C exhibits comparable potency (254 nM *versus* 223 nM) and selectivity (0.6 *versus* 0.4) for AChE in comparison to its monomer tacrine (Carlier et al. 1999), however, it possesses plenty of advantages distinctive from tacrine and other existing AChE inhibitors approved by FDA.

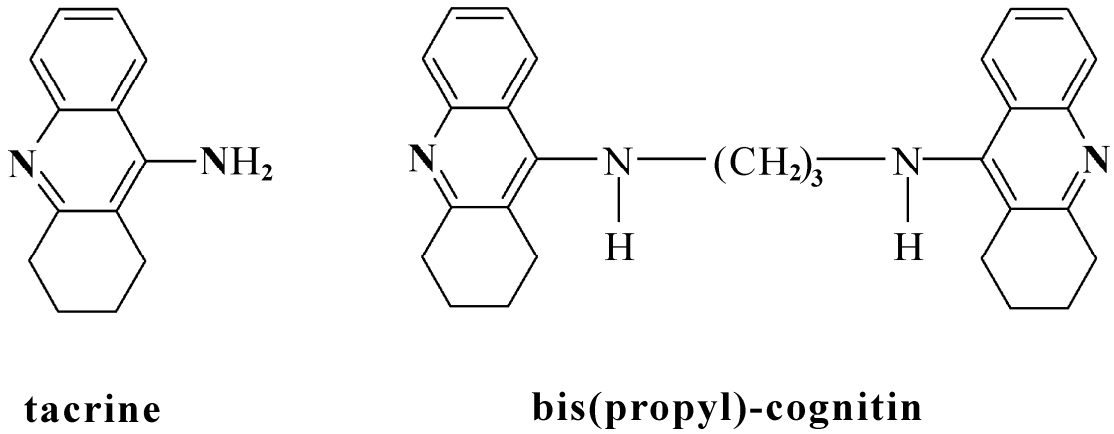


Fig.1.4 Structures of tacrine and bis(propyl)-cognitin (Hu et al. 2013).

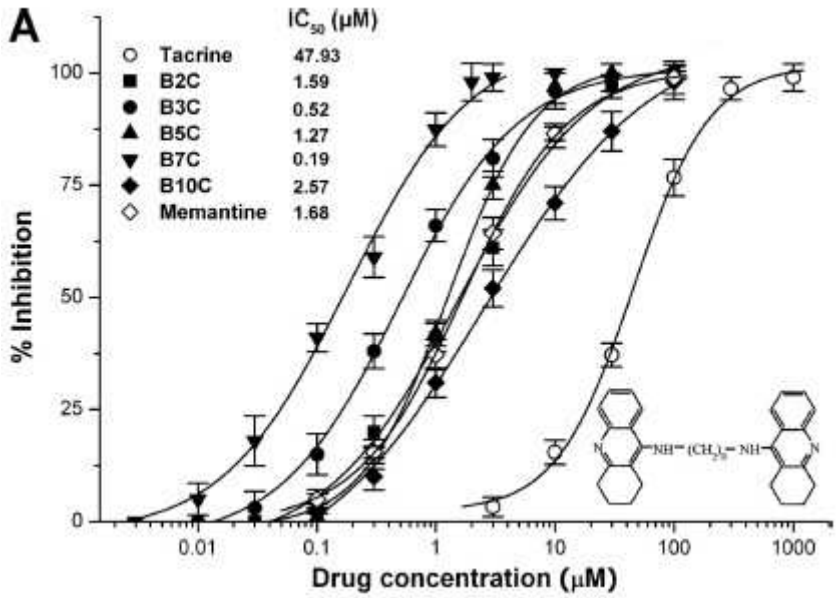


Fig.1.5 Tether length differential potency of bis(n)-cognitins (Luo et al. 2010).

1.3.1 B3C acts as an Uncompetitive NMDA receptor antagonist with Fact Off-rate (UFO)

It is well characterized by us that B3C moderately, voltage-dependently and selectively inhibited NMDA-activated currents in hippocampus using patch clamp (Fig. 1.5) (Luo et al. 2010). The inhibitory effects of B3C increased with the increase in the concentrations of glutamate. With the use of kinetics analysis, the B3C's inhibition is of fast onset and offset with an off-rate time constant of 1.9 s. Studies from molecular docking have further indicated B3C moderately blocks NMDA receptor at the MK-801 binding site. B3C has been further found to compete with [^{3}H]MK801 with a K_i value of 0.27 μM.

1.3.2 B3C provides neuroprotections in scopolamine and middle cerebral artery occlusion-induced brain damage

As assayed by Morris water maze test and novel object recognition tasks, treatment with B3C (1.5-2.5 μmol/kg) in mice dose-dependently mitigated the neurological deficits and memory impairments induced by scopolamine, with a potency that was 5 to 20 folds over that of tacrine (Han et al. 2012). In addition, B3C (0.65 μmol/kg) shows more pronounced improvement in neurological scores and reduction in infarct volume in rats subjected to MCAO than memantine does (Luo et al. 2010). These taken together suggest that B3C has high the blood-brain barrier penetrate when administrated.

1.4 The aim of the study

Neurodegenerative diseases are caused by, at least, inappropriate apoptosis induced by glutamate or other neurotoxic stimuli as well as impairments in neurogenesis and neuronal differentiation. Compounds with anti-property and neuronal differentiation-promoting activities could have therapeutic significance against these devastating diseases. B3C has been demonstrated as a promising agent against neurodegeneration on the basis of AChE inhibition, NMDA receptor antagonism and some neuroprotections in animal studies. However, more studies need to be carried out to confirm the therapeutic advantages, as well as to explore the mechanisms that underlie the potential therapeutics of B3C. In my research, we would like to achieve the following aims:

1> To investigate the molecular mechanisms of neuroprotection by which B3C protects against glutamate-induced excitotoxicity.

2> To elucidate the molecular mechanisms of neuroprotection by which B3C protects against K^+ deprivation-induced apoptosis

3> To explore the molecular mechanisms by which B3C promotes neuronal differentiation in PC12 cells and primary cortical neurons.

Chapter 2

Materials and Methods

2.1 Chemicals and reagents

B3C was designed as previously described (Carlier et al. 1999). Unless otherwise mentioned, all culture media and supplements used for cell cultures were purchased from Gibco (Carlsbad, CA, USA). LY294002, PD98059, U0126, SB415286, LiCl, SB203580, SP600125 and N^G-monomethyl-L-arginine (L-NMMA) were obtained from Calbiochem (San Diego, CA, USA). PTK787/ZK222584 (PTK/ZK) was from LC laboratories (Woburn, MA, USA). Glutamate, fluorescein diacetate (FDA), propidium iodide (PI), Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAF-FM), tacrine, donepezil, memantine, atropine, dibutyryl cAMP (dbcAMP), mecamylamine, MLA, K252a and tubocurarine were obtained from Sigma Chemicals (St Louis, MO, USA). 4-amino-5-methylamino-2', 7'-difluorescein diacetate (DAF-FM diacetate) were purchased from Invitrogen (Eugene, OR, USA). NGF was from R&D Systems (Minneapolis, MN, USA).

2.2 Primary culture of cerebellar granule neurons (CGNs)

Rat CGNs were prepared from 8-day-old Sprague-Dawley rat pups (15 to 19 g) as described previously (Hu et al. 2013). Briefly, fresh cerebella were removed and rinsed into ice-cold Kreb's buffer, then finely chopped in hood and digested with 0.25% trypsin at 37 ^{o}C for 15 min. Trypsin inhibitor and DNase A solution were added to terminate trypsin activity. After centrifugation, a single cell suspension was

obtained through resuspending the sedimented tissue. Following cell count using trypan blue exclusion test, neurons at a density of 2.0×10^6 cells / ml were seeded in basal modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS), 25 mM KCl, 2 mM glutamine and 100 units/ml penicillin/streptomycin. All Petri dishes and plates were coated with poly-D-lysine prior to seeding to enhance adhesion. Cytosine arabinonucleoside (10 μM) was added into the culture media 24 h after seeding to inhibit the growth of non-neuronal cells. With the use of this protocol, the CGN cultures consisted of > 95 % neurons (stained with anti-GAP-43 antibody), < 1 % astrocytes (stained with anti-GFAP antibody). All the experiments were carried out after 8 days in culture.

2.3 Primary culture of cortical neurons

Primary cortical neurons were obtained from 18-day-old Sprague-Dawley rat embryos as previously described by us with modification (Fu et al. 2006). Briefly, the cortex was mechanically fragmented, transferred to 0.25 % trypsin, and incubated in a water bath for 15 min at 37 ^{o}C. Following trypsinization, cells were washed twice with neurobasal medium and re-suspended in neurobasal medium containing 10 % fetal bovine serum, 0.5 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. The cells were then plated onto 35-mm cell culture dishes (150 cells/mm^2) pre-coated with poly-L-lysine and kept at 37 ^{o}C under an atmosphere of 95 % air and 5 % CO_2. 24 h after plating, half-medium was removed and replaced with neurobasal medium containing 1 % B-27, 0.5 mM glutamine, 100 U/ml penicillin,

100 U/ml streptomycin and 1 μM cytosine arabinonucleoside. After 2 days in culture, cortical neurons were incubated with increasing concentrations of B3C for 2 days.

2.4 Drug treatment of CGNs

For the study of neuroprotection against glutamate-induced excitotoxicity by B3C:

After 8 days *in vitro* (DIV), CGNs were pre-treated with B3C, tacrine, donepezil, memantine and L-NMMA at the desired concentrations for 2 h, and then incubated with glutamate for different lengths of time. When pharmacological inhibitors were used for signaling assays, CGNs were also pre-treated with these inhibitors 2 h before the addition of the tested compounds and/or the stimuli. Cells that did not receive drugs received a control vehicle (dimethyl sulfoxide for SB415286, U0126 and LY294002, and distilled water for other inhibitors), and the final concentration of DMSO was no more than 0.1 % .

For the study of neuroprotection against K^+ deprivation-induced excitotoxicity by B3C:

At 8 DIV, CGNs were switched from the culture medium (10 % FBS and 25 mM KCl) to 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 (5K + tested compounds),. When pharmacological inhibitors were used, CGNs were pre-treated with the selected inhibitors in the serum-free medium containing 25 mM KCl 0.5 h before the switch of CGNs to K^+ deprived medium with or without the tested

compounds. B3C, tacrine, donepezil, memantine and MK801 were dissolved in distilled water.

2.5 PC12 Cell Culture

PC12 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in high glucose DMEM medium supplemented with 6 % FBS, 6 % horse serum, 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin at 37 ^{o}C under an atmosphere of 95 % air and 5 % CO_2.

2.6 α7-nAChR gene knockdown

A pGPU6-GFP-neo shRNA expression vector containing DNA oligonucleotides (21 bp) specially targeting sequences (5'-GCAGTGCAAACTGAAGTTTGG-3') of α7-nAChR or the vector with non-sense oliglnucleotides as a negative control (GenePharma, Shanghai, China) was transfected into PC12 cells. Briefly, 24 h after seeding, PC12 cells were rinsed with PBS and transfected with indicated plasmids in serum-free DMEM for 6 h by using Lipofectamine 2000 at the ratio of 1:2.5 (plasmids/liposome). The culture media were subsequently substituted by DMEM containing 10 % FBS for an additional 48 h culture. GFP expression was monitored to evaluate transfection efficiency.

2.7 Quantitative Assessment of Neurite Outgrowth in PC12 cells

Morphological analysis and quantification of cells with neurites were performed

as previously described (Shi et al. 2006). Briefly, PC12 cells were seeded in growth medium at 1×10^4 cells/well in poly-L-lysine–coated 6-well plates and incubated overnight. The next day, cells were treated with various concentrations of chemicals, with fresh low serum medium (0.5 % FBS and 0.5 % horse serum) and chemicals renewed every other day. A light microscope equipped with a phase-contrast condenser, 10 × objective lens and a digital camera (Diagnostic Instruments) was used to capture images with the manual setting. For analyzing the number of neurite bearing cells, approximately 300 cells were counted from at least 5 randomly chosen visual fields for each culture. With the use of Image J 1.42 software, the cells were identified as differentiated if one or more neurites were longer than the diameter of cell body.

2.8 Determination of Cell Viability

Cell viability was measured using the classical MTT assay as described previously (Hu et al. 2011; Hu et al. 2013). Briefly, neurons were seeded and cultured in 96-well plates. After drug treatment, 10 µL of 5mg/mL MTT solution (dissolved in PBS) was added to each well, and the plates were then incubated at 37 ^{o}C for 4 h. The purple MTT formazan crystals were dissolved in 150 µL DMSO. Cell viability was measured using a BIO-RAD Microplate Reader (Model 680, BIO-RAD Laboratories, Hercules, CA, USA) with 570 nm as a test wavelength and 655 nm as a reference wavelength.

2.9 Assay of Lactate Dehydrogenase (LDH) release

Cytotoxicity in different drug treatment groups was quantified by observing the extracellular LDH in the culture media. Prior to assay, CGNs were incubated with 1 % (V/V) Triton X-100 in culture medium for 1 h at 37 ^{o}C to obtain a representative maximal LDH release, which serves as the positive control with 100 % toxicity. The released LDH was examined with the commercial kit (Roche Diagnostics, Indianapolis, IN). Briefly, after gentle centrifugation, culture media in 96-well plates were transferred to another new plates, and then the same volume of mixed reaction buffer supplied in the kit were added into these plates. 30 min after mixing at room temperature, the released LDH was determined using a BIO-RAD Microplate Reader (Model 680, BIO-RAD Laboratories, Hercules, CA, USA) with 490 nm as a wavelength of.

2.10 Image Analysis of FDA/PI staining

To acquire further information whether the treated cells are viable, a double staining procedure with FDA and PI, two specific fluorophores, was employed. FDA can easily penetrate through the membranes of living cells, and be hydrolyzed by intracellular esterase to generate fluorescein. The resulted fluorescein remains within the cells and emits green color. On the contrary, PI easily passes through the membranes of dead cells and then intercalates with DNA to form a bright red fluorescent complex. Briefly, CGNs were incubated with 10 μg/ml FDA and 5 μg/ml PI (diluted in PBS containing 5% glucose) for 15 min, then washed twice and

photographed using a fluorescence microscope. The captured pictures were then compared with pictures taken under phase contrast microscopy.

2.11 Morphological Observation of Nuclear Change by Hoechst 33342 staining

Chromatin condensation stained by Hoechst 33342 was analyzed as described previously (Hu et al. 2013). After drug treatment, CGNs (4×10^6 cells) grown in a 6-well plate were washed with ice-cold PBS containing 5 % glucose, and then stained with Hoechst 33342 (5 μg/ml) for 5 min at 4 ^{o}C. After the two washes with PBS, the stained nucleus were immediately photographed using a fluorescence microscope. Cells with bright blue condensed and fragmented nucleus (chromatin condensation and fragment) were taken as apoptotic cells. Condensed nucleus were scored by calculating more than 500 cells of four randomly chosen microscopic fields for each sample.

2.12 Analysis of DNA Fragmentation

Apoptotic nucleosomal DNA ladder was detected by agarose gel electrophoresis as previously described (Hu et al. 2011). After drug treatment, CGNs were collected and lysed in lysis buffer [10 mM Tris-HCI (pH 7.6), 0.5 % v/v Triton X-100, 20 mM EDTA, 0.5 mg/ml proteinase K]. Following the centrifugation at 12, 000 rpm for 10 min, the supernatant of lysate was extracted once with equal volume of phenol, and once with phenol: chloroform: isoamyl alcohol (v/v/v 25: 24: 1). With the incubation of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol at -20 °C overnight,

DNA was precipitated. The precipitate was then rinsed with 70 % ice-cold ethanol, re-suspend in Tris borate-EDTA buffer, and then incubated with RNase A (0.1 mg/ml) at 37 °C for 1 h. 8 μg DNA was loaded and electrophoresed in 1.2 % agarose gels for 1 h at 70 V and visualized with ethidium bromide staining.

2.13 Measurement of Intracellular NO

Intracellular NO was detected with DAF-FM, a fluorescent probe which emits increased fluorescence through the reaction with an active intermediate of NO formed during the spontaneous oxidation of NO to NO_2 (Sheng and Kim 2002). Briefly, after treatment, CGNs were incubated with 5 μM DAF-FM at 37 °C for 30 min, then washed twice with PBS, and finally the DAF-FM fluorescence in CGNs was captured using a Galaxy Microplate Reader (POLARstar Galaxy, BMG Labtech GmbH, Offenburg, Germany) with excitation and emission wavelengths of 495 nm and 515 nm, respectively.

2.14 Immunocytochemical staining

After removing the culture medium, primary cortical neurons were incubated with fixing buffer (10 % sucrose, 4 % paraformaldehyde and 15 μg/ml Hoechst-33342 in PBS) for 20 min. Thereafter, cells were blocked for 1 h in 5 % goat serum, 0.5 % BSA and 0.1 % Triton X-100 in PBS, and then incubated with mouse anti-βIII tubulin antibody (Cell Signaling Technology Inc, MA, USA) at 4 °C overnight. After two washes with PBS, Alexafluor-488 anti-mouse secondary antibody were added for 1

hour at room temperature.

2.15 Image acquisition and analysis

The pictures were acquired by the Nikon ECLIPSE Ti-U microscope (Nikon Instruments Inc). Image analysis was conducted with ImageJ Software according to the previous publications with slight modifications (Hirata et al. 2005; Evans et al. 2008). Briefly, images of nuclei stained with Hoechst-33342, which allowed identification of the correct focal plane for further image acquisition, were identified by ultraviolet excitation and emission wavelengths. Second excitation wavelengths were used to illuminate neurites with Alexa-488 secondary antibody. Neuronal cell bodies were labeled as Hoechst-33342-positive objects. Neurites were labeled as βIII tubulin-positive structures, analyzed by a NeuriteTracer program (Pool et al. 2008).

2.16 Western Blot

Western blot analysis was conducted as previously described (Hu et al. 2013). CGNs were lysed in 1× cell lysis buffer (Cell Signaling Technology Inc, MA, USA). Following the homogenization and centrifugation, the supernatant was collected, and the total protein content was determined using a Bradford assay (Bio-Rad, Hercules, CA, USA).

Protein samples were mixed with sample loading buffer at the ratio of 1:4 (Cell Signaling Technology Inc, MA, USA) and boiled for 10 min. 25-50 g of total proteins were loaded on 12 % SDS-PAGE, electrophoresed and subsequently transferred to

polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Thereafter, the membranes were blocked in 5 % nonfat milk solution, incubated with 1:1 000 diluted anti-MEK, anti-p-ERK, anti-ERK, anti-p-Ser-473Akt, anti-Akt, anti-p-Ser9-GSK3β, anti-GSK3β (rabbit monoclonal, Cell Signaling Technology Inc, MA, USA), 1:500 diluted anti-p-Tyr1054-VEGFR-2 (rabbit polyclonal, Abcam Inc., MA, USA), 1:1000 diluted anti-GAP43, anti-β-actin (goat monoclonal, Santa Cruz Biotechnology, Santa Cruz, USA), respectively. Following three washes with TBST solution (20 mM Tris, pH 7.6, 0.1 % Tween 20), the membranes were immersed in corresponding secondary antibodies conjugated to peroxidase (1:2000) for 45 min, and blots on the membranes were developed by using an Enhanced Chemiluminescence Detection kit (Thermo Scientific, Rockford, IL, USA). The relative levels of each protein to house-keeping protein were determined by densitometry analysis using Gel-Pro analyzer 4.0 software (Gel media system, China).

2.17 Data Analysis and Statistics

Data, presented as means ± S.E.M, are representative of three independent experiments. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test, and $p<0.05$ or less was taken as statistically significant.

Chapter 3

Results

3.1

B3C protects against glutamate-induced excitotoxicity in CGNs via concurrent regulation of NO, ERK and PI3-K/Akt/GSK3β pathways, downstream of NMDA receptors

3.1.1 B3C protects against glutamate-induced excitotoxicity more potently than memantine

Prior to the examination of neuroprotection of tested compounds, we established a cell model in which excitotoxicity was induced by glutamate in CGNs. At DIV 8, CGNs were incubated with various concentrations of glutamate for 24 h, followed by MTT assay (for measurement of cell viability) and LDH assay (for evaluation of cell membrane damage), respectively. As demonstrated in Fig. 3.1.1A, glutamate (25 μM to 500 μM) induced a significant neuronal death in CGNs in a dose-dependent manner. Since glutamate, at low concentrations, induces typical apoptosis rather than necrosis in primary CGNs (Du et al., 1997), 100 μM glutamate with approximately 50 % cell death was used for all the subsequent experiments.

Further, to compare the neuroprotective effects of B3C and memantine, two uncompetitive NMDA receptor antagonists, CGNs were pre-treated with B3C (0.01-20 μM) or memantine (0.01-100 μM) for 2 h, and then subjected to 100 μM glutamate in the presence of tested compounds for 24 h. As shown in Figs.3.1.1B and 3.1.1C, B3C prevents glutamate-induced excitotoxicity 10 times more potently than memantine, with the IC_{50} values of 0.45 μM, 4.58 μM for increasing cell survival and 0.46 μM, 4.63 μM for maintaining membrane integrity, respectively. Meanwhile, even when the concentrations was up to 100 μM, tacrine and donepezil (two commonly used AChE inhibitors) were unable to attenuate cell death induced by glutamate, indicating that the neuroprotective effects of B3C are independently associated with its AChE inhibition capacity.

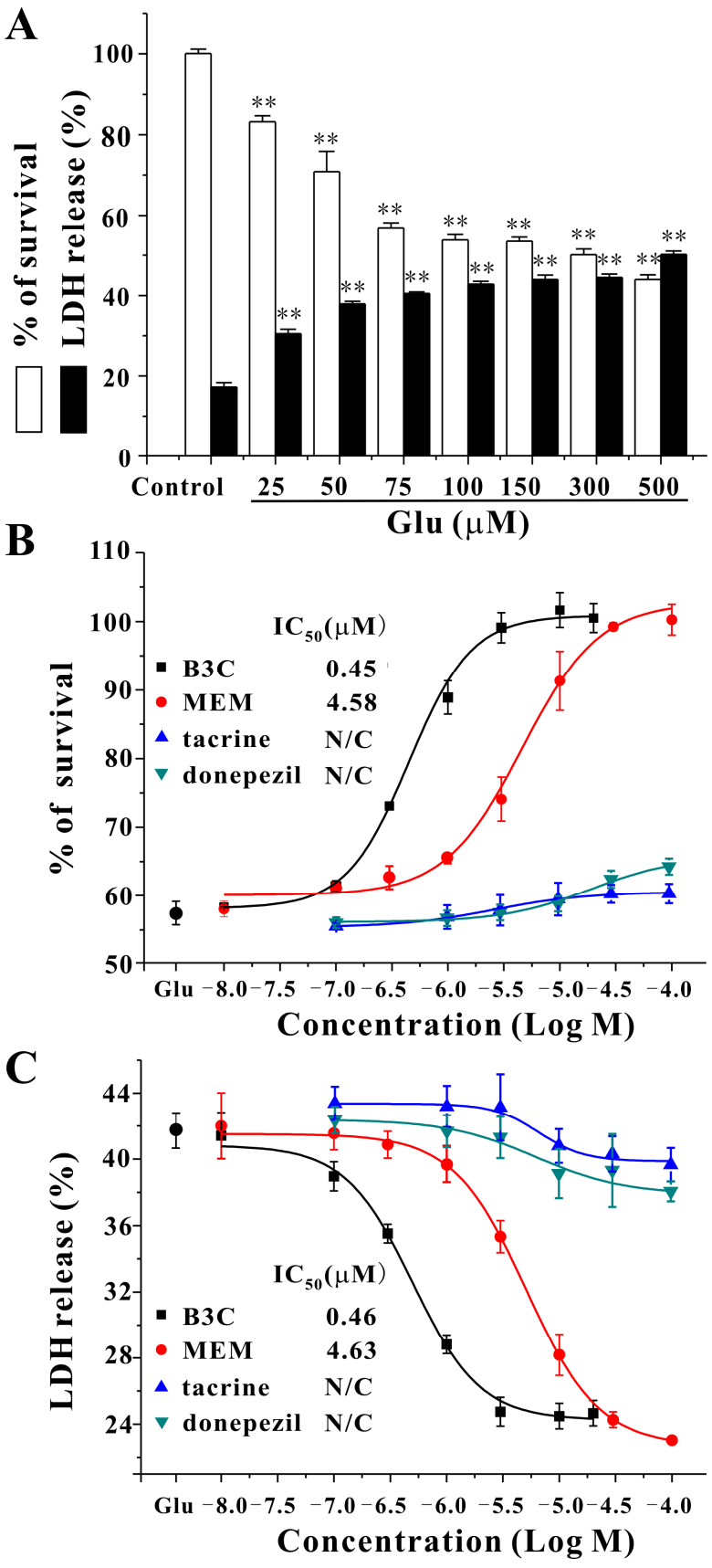

A
% of survival
LDH release (%)
100
80
60
40
20
0
**
Control
25
50
75
100
150
300
500
Glu (μM)
B
% of survival
110
100
90
80
70
60
50
IC50(μM)
B3C 0.45
MEM 4.58
tacrine N/C
donepezil N/C
Glu −8.0 −7.5 −7.0 −6.5 −6.0 −5.5 −5.0 −4.5 −4.0
Concentration (Log M)
C
LDH release (%)
44
40
36
32
28
24
IC50(μM)
B3C 0.46
MEM 4.63
tacrine N/C
donepezil N/C
Glu −8.0 −7.5 −7.0 −6.5 −6.0 −5.5 −5.0 −4.5 −4.0
Concentration (Log M)

Fig.3.1.1 B3C protects against glutamate-induced excitotoxicity 10 times more potently than memantine.

(A) Correlation between neuronal death and membrane damage induced by glutamate. At 8 DIV, primary CGNs were incubated with glutamate ranging from 25 μM to 500 μM for 24 h. MTT reduction assay and LDH release assay were then performed. **, $p<0.01$, compared to control group. (B, C) B3C protects against neuronal death induced by glutamate 10 times more potently than memantine (MEM). At 8 DIV, CGNs were pre-treated with B3C (0.01 μM to 20 μM), MEM (0.01 μM to 100 μM), tacrine (0.1 μM to 100 μM) or donepezil (0.1 μM to 100 μM) for 2 h, and then subjected to glutamate at 100 μM for 24 h in the presence of tested compounds. MTT reduction assay and LDH release assay were employed to measure the cell viability (B) and membrane integrity (C), respectively. IC_{50} values were calculated using Origin Pro 8 statistical software from the sigmoidal inhibition curve. N/C, not calculable.

3.1.2 B3C attenuates the typical apoptosis induced by glutamate more substantially than memantine

To further confirm the neuroprotective effects of B3C and memantine, FDA/PI double staining, Hoechst staining and DNA fragmentation assays were conducted in primary CGNs in succession. As can be seen from the phase contrast microscopy and FDA/PI double staining (Fig. 3.1.2A), cells displayed unhealthy bodies and broken extensive neuritic network in response to 24 h of glutamate challenge. However, B3C and memantine effectively inhibited the neuronal death and the morphological changes induced by glutamate. In addition, B3C and memantine remarkably blocked glutamate-induced nuclear condensation, as evidenced by the decrease in apoptotic bodies stained by Hoechst 33342 (Fig. 3.1.2B). In parallel, B3C and memantine effectively suppressed DNA fragmentation (DNA “ladder”), the hall marker of apoptosis (Fig. 3.1.2C), as assayed by DNA fragmentation gel assays. In all these cases, the neuroprotective effects offered by B3C at 3 μM was equivalent to that

offered by memantine at 30 µM.

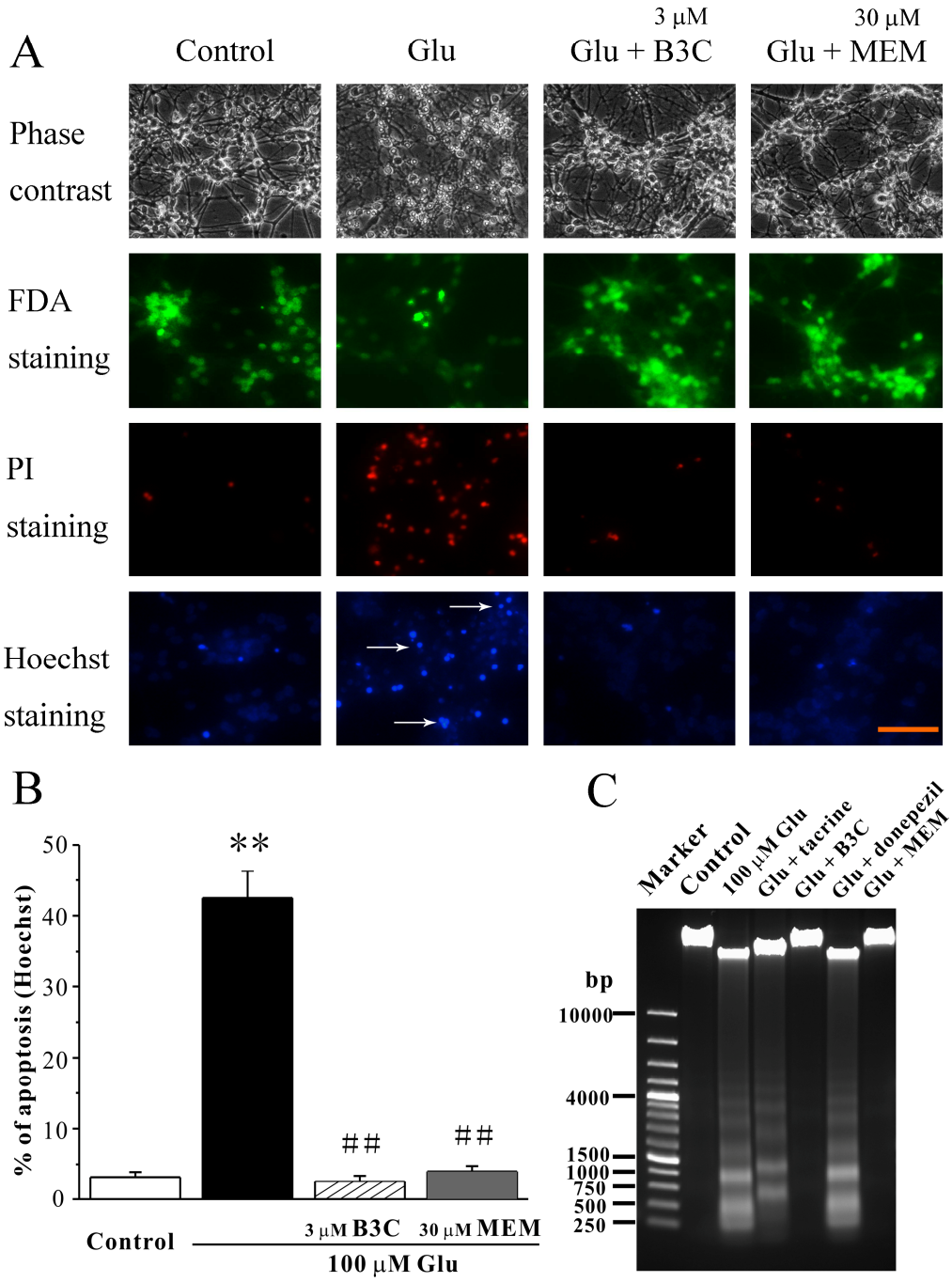


Fig.3.1.2 B3C attenuates glutamate-induced apoptosis more substantially than memantine in CGNs.

(A) At 8 DIV, CGNs were pre-treated with 3 μM B3C or 30 μM MEM for 2 h, and then incubated with 100 μM glutamate (Glu) in the presence of the tested compounds for 24 h, and finally assayed with a phase contrast microscope, FDA/PI staining and Hoechst 33342 staining. Scale bar = 100 μm. (B) The counts of apoptotic bodies from Hoechst staining as observed in (A). **, $p<0.01$, compared to control group; $^{\#\#}$,

p<0.01, compared to glutamate group. (C) The fragmented DNA in CGNs was extracted, and then agarose gel electrophoresis was used for visualization of DNA bands.

3.1.3 B3C potently suppresses the increased intracellular NO induced by glutamate

To analyze the inhibitory effects of B3C and memantine on glutamate-induced over-production of intracellular NO in our cell models, L-NMMA (a nitric oxide synthase inhibitor, 1-1000 μM), B3C (0.01-20 μM) or memantine (0.01-100 μM) were selected to pre-treat CGNs for 2 h before glutamate insult. As shown in Fig. 3.1.3, B3C (IC_{50}, 0.61 μM) was more potent in inhibiting the abnormal generation of NO caused by glutamate, as compared to L-NMMA (IC_{50}, 44.7 μM) and memantine (IC_{50}, 6.44 μM).

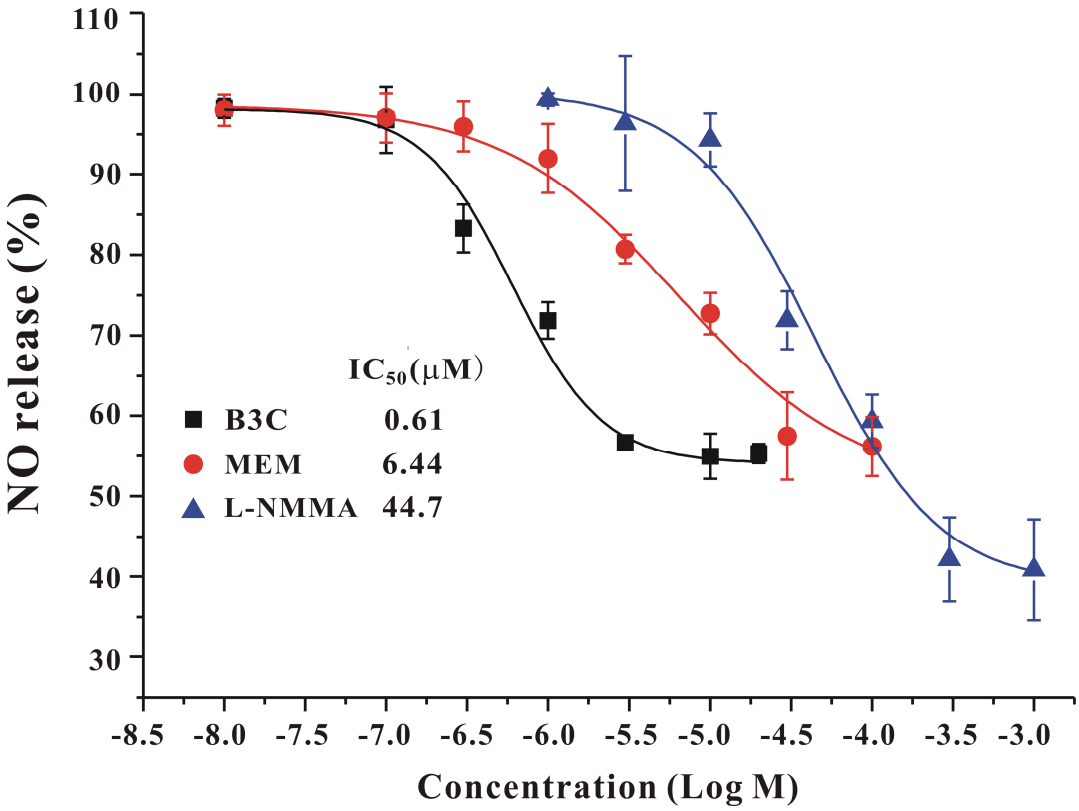


Fig.3.1.3 B3C potently suppresses the increase in the level of intracellular NO caused by glutamate.

At 8 DIV, CGNs were pre-treated with L-NMMA (1-1000 μM), B3C (0.01-20 μM) or memantine (0.01-100 μM) 2 h prior to 100 μM glutamate insult. Intracellular NO level was examined using DAF-FM diacetate (a fluorescence probe) 0.5 h after glutamate challenge. IC_{50} values were calculated from fits of the data by Origin Pro 8 software.

3.1.4 B3C potently inhibits the activation of ERK pathway caused by glutamate

To prove whether ERK pathway was involved in our cell models and to examine the inhibitory effects of B3C and memantine on the ERK pathway, the protein expression of p-MEK1/2 and p-ERK1/2 were analyzed in succession by western blot. As demonstrated in Fig. 3.1.4, as a response to 100 μM glutamate, the levels of both phosphorylation of kinases gradually increased, with a peak at 2 h. B3C at 3 μM, equivalent to memantine at 30 μM, substantially reversed the increased p-MEK1/2 and p-ERK1/2 caused by glutamate, whereas U0126 (MEK inhibitor) only inhibited the rise in p-ERK1/2 without affecting p-MEK1/2.

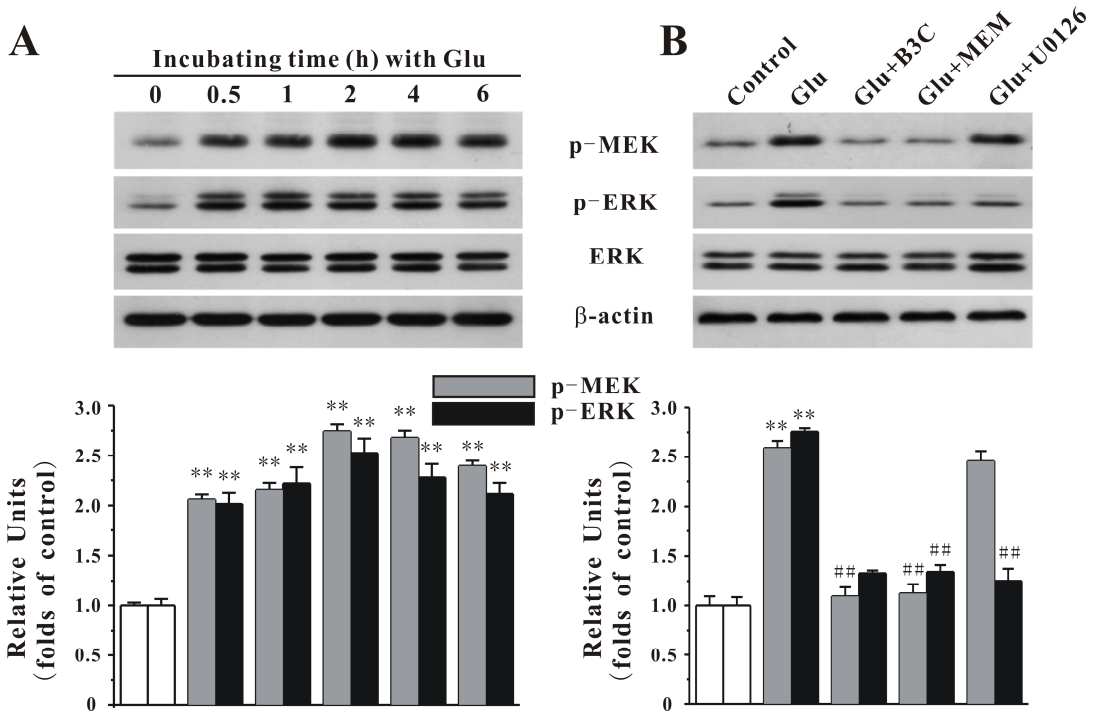


Fig.3.1.4 B3C potently inhibits the activation of ERK pathway caused by glutamate.

(A) CGNs were incubated with 100 μM glutamate (Glu) for 0.5, 1, 2, 4 and 6 h, respectively. The protein expression of p-MEK, p-ERK, total ERK and β-actin were examined using Western blot. **, $p<0.01$, compared to control group; ##, $p<0.01$, compared to glutamate group. (B) CGNs were pre-treated with 3 μM B3C or 30 μM memantine (MEM) for 2 h, and then incubated with 100 μM glutamate in the presence of the tested compounds for 2 h. The protein expression of p-MEK, p-ERK, total ERK and β-actin were examined using Western blot. **, $p<0.01$, compared to control group; ##, $p<0.01$, compared to glutamate group.

3.1.5 B3C potently reverses the inhibition of PI3-K/Akt/GSK3β pathway caused by glutamate

To prove whether GSK3β pathway was involved in our cell models, LiCl, a GSK3β inhibitor was used to pre-treated CGNs prior to the glutamate insult. It was found that LiCl well protected neurons against glutamate-induced neuronal death, indicative of the involvement of GSK3β pathway. Next, to evaluate the effects of B3C and memantine on the PI3-K/Akt/GSK3β pathway, the protein expression of p-Ser473-Akt and p-Ser9-GSK3β were also analyzed by Western blot. As observed in Figs. 3.1.5A and 3.1.5B, 100 μM glutamate time-dependently decreased the levels of p-Ser473-Akt and p-Ser9-GSK3β. As a response to 6 h of glutamate challenge, the level of p-Ser473-Akt and p-Ser9-GSK3β reached 60 ± 8 % and 21 ± 13 % of that of control group respectively, while pre-treatment with B3C dose-dependently reversed both suppressed kinases (Fig.3.1.5C). To further investigate whether the PI3-K/Akt/GSK3β pathway was associated with the neuroprotective effects of B3C, LY294002, a specific PI3-K inhibitor was used. It was shown that 50 μM LY294002 significantly abolished the neuroprotective effects of B3C (Fig.3.1.5D). In addition, LY294002 reversed the suppression of down-regulation of p-Ser473-Akt and p-Ser9-GSK3β levels caused by glutamate, as assayed by Western blot (Fig.3.1.5E). Similarly, B3C was 10 times more potent than memantine to reverse the suppression of p-Ser473-Akt and p-Ser9-GSK3β expression caused by glutamate in primary rat CGNs (Fig.3.1.5F).

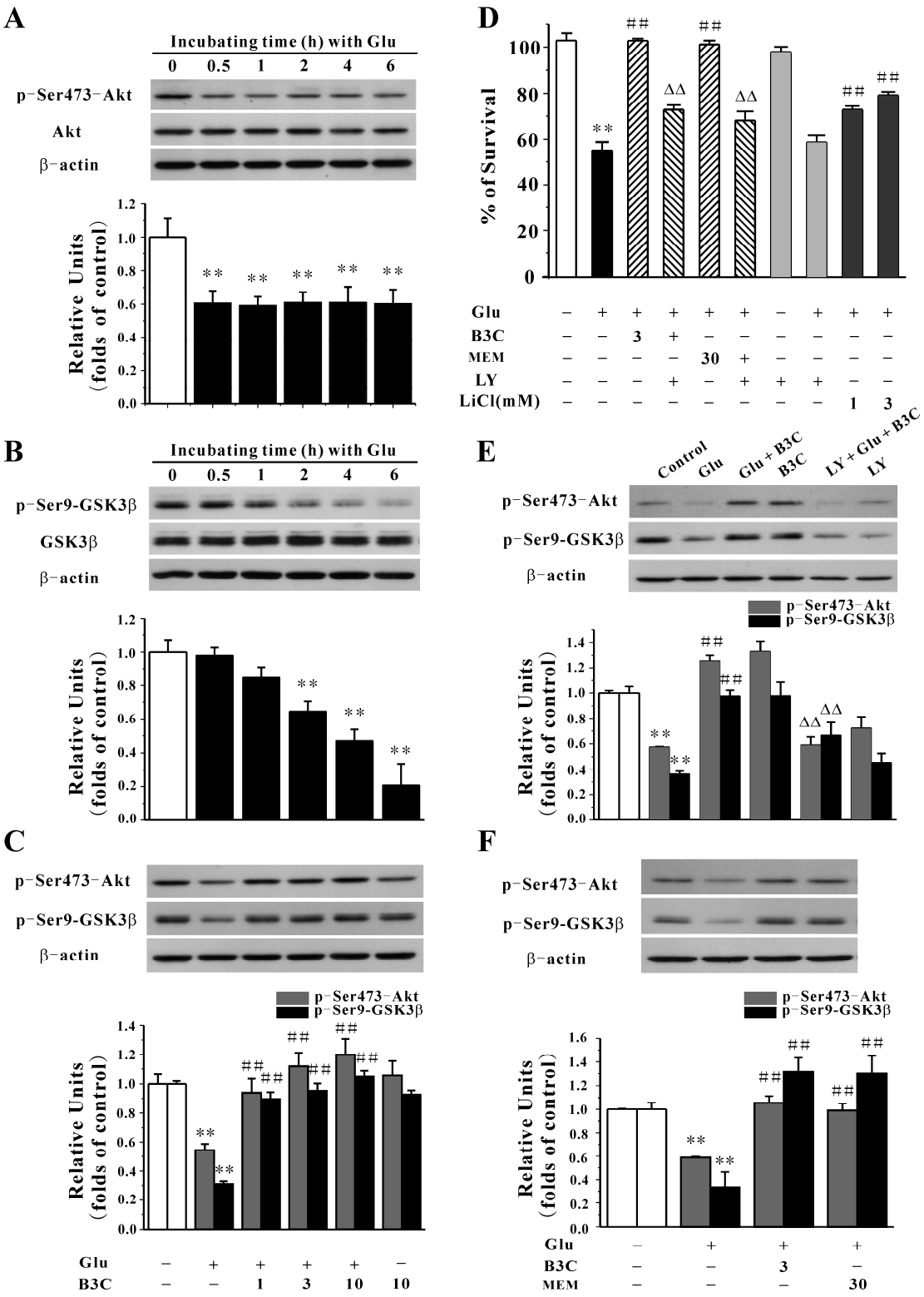


Fig.3.1.5 B3C potently reverses the attenuation of PI3-K/Akt/GSK3β pathway caused by glutamate.

(A) 100 μM glutamate significantly down-regulated the protein expression of p-Ser473-Akt in CGNs. **, $p<0.01$, compared to control group. (B) 100 μM glutamate time dependently suppressed the protein expression p-Ser9-GSK3β in CGNs **, $p<0.01$, compared to control group. (C) B3C dose-dependently reversed the inhibition of p-Ser473-Akt and p-Ser9-GSK3β caused by glutamate. **, $p<0.01$, compared to control group; $^{\#\#}$, $p<0.01$, compared to glutamate group. (D) PI3-K specific inhibitors partially abolished the neuroprotections of B3C and memantine against glutamate excitotoxicity. **, $p<0.01$, compared to control group; $^{\#\#}$, $p<0.01$, compared to glutamate group; $^{\triangle\triangle}$, $p<0.01$, compared to glutamate plus B3C group. (E) PI3-K specific inhibitors abolished the neuroprotection of B3C via PI3-K/Akt/GSK3β pathway. **, $p<0.01$, compared to control group; $^{\#\#}$, $p<0.01$, compared to glutamate group; $^{\triangle\triangle}$, $p<0.01$, compared to glutamate plus B3C group. (F) B3C at 3 μM, equal to memantine at 30 μM, almost completely reversed the inhibition of p-Ser473-Akt and p-Ser9-GSK3β caused by glutamate. **, $p<0.01$, compared to control group; $^{\#\#}$, $p<0.01$, compared to glutamate group.

3.1.6 Pharmacological inhibition of NOS, ERK and GSK 3 β provides a synergistic neuroprotective effect against glutamate-induced excitotoxicity

To investigate the relationship among NO, ERK and PI3-K/Akt/GSK3β pathways which are involved in glutamate-induced excitotoxicity in CGNs, the specific inhibitor of NOS (L-NMMA), that of MEK1/2 (U0126), and that of GSK 3β (SB415286) were selected, alone or in combination, to treat CGNs for 2 h before the glutamate insult. It was found that both single inhibitor and combinations of any two inhibitors partially blocked glutamate-induced apoptosis (Fig. 3.1.6A). However, the co-administration of these three inhibitors produced a synergistic and almost 100 % neuroprotection against glutamate insult. The full neuroprotective effect of these inhibitors is equivalent to that offered by B3C at 3 μM or memantine at 30 μM (Fig. 3.1.6B).

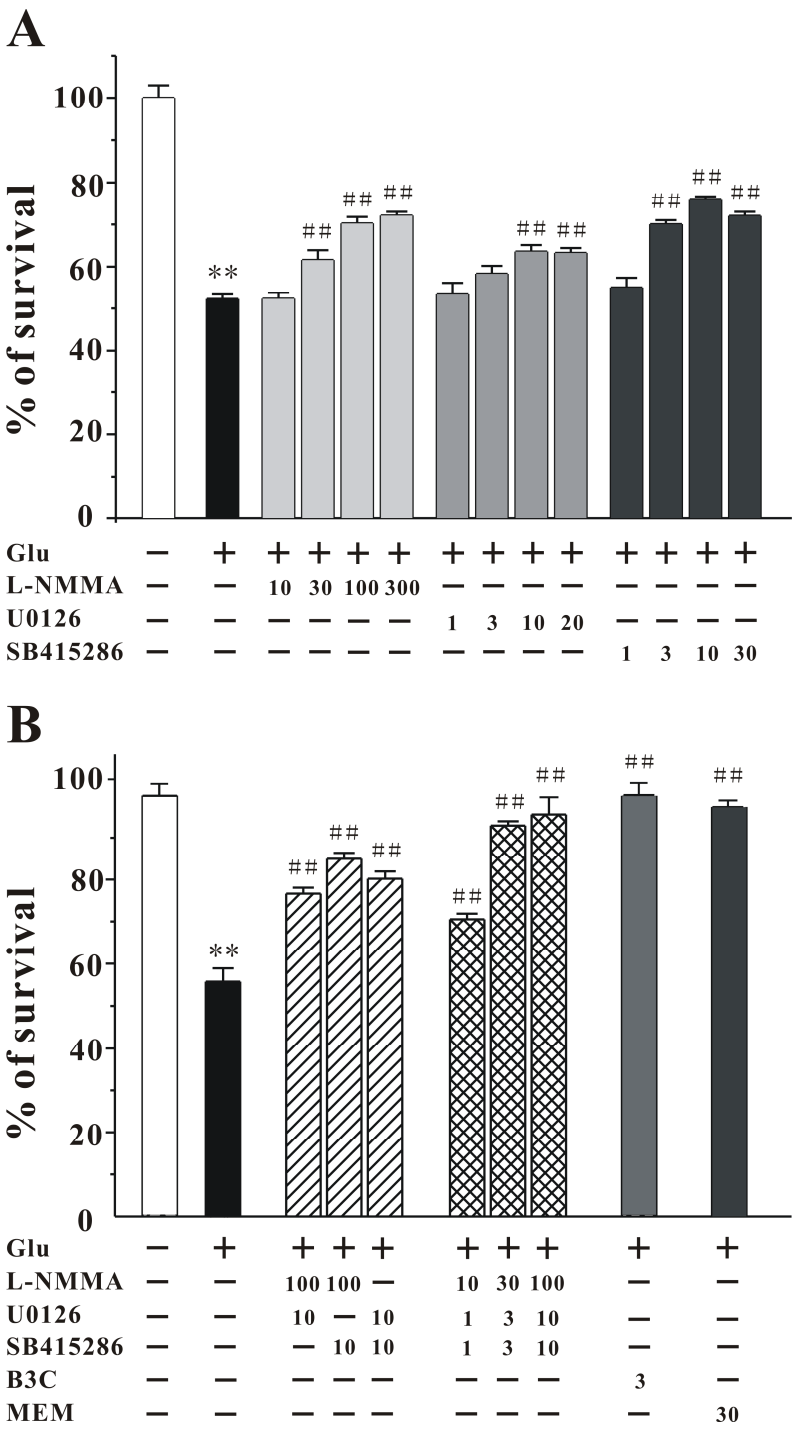


Fig.3.1.6 Pharmacological inhibition of NOS, ERK pathways and GSK3β produces a synergistic neuroprotective effect against glutamate-induced excitotoxicity in CGNs.

At 8 DIV, CGNs were pre-treated with various concentrations of L-NMMA (NOS inhibitor, μM), U0126 (MEK inhibitor, μM) or SB415286 (GSK3β inhibitor, μM),

alone (A) or in combination (B) for 2 h prior to 24 h of glutamate insult. MTT reduction assay was performed to measure the cell viability **, $p<0.01$, compared to control group; $^{\#\#}$, $p<0.01$, compared to glutamate group.

____(Hu S, *et al.* 2013, *Neurochem Int* 62, 468-477)

3.2

B3C protects against K^+ deprivation-induced apoptosis in CGNs via the activation of VEGFR-2 signaling pathways

3.2.1 B3C blocks neuronal death in CGNs induced by K^+ deprivation

As reported previously (Subramaniam et al. 2003), when switched from a serum-free medium containing a high depolarizing K^+ concentration (25K) to a serum-free medium containing a low K^+ concentration (5K), CGNs underwent apoptosis. As shown in Fig.3.2.1A, 24 h of K^+ deprivation killed 50 % cell death in CGNs, while B3C (IC_{50}, 0.37 μM) effectively blocked the cell death induced by K^+ deprivation (Fig.3.2.1A). In addition, with the increase of time exposure, the neuroprotective effect of B3C gradually declined and completely disappeared 60 h after K^+ deprivation insult (Fig.3.2.1B). More importantly, B3C offered diminishing, but significant neuroprotective effect when added 10 h after switching CGNs to K^+ deprived medium (Fig.3.2.1C).

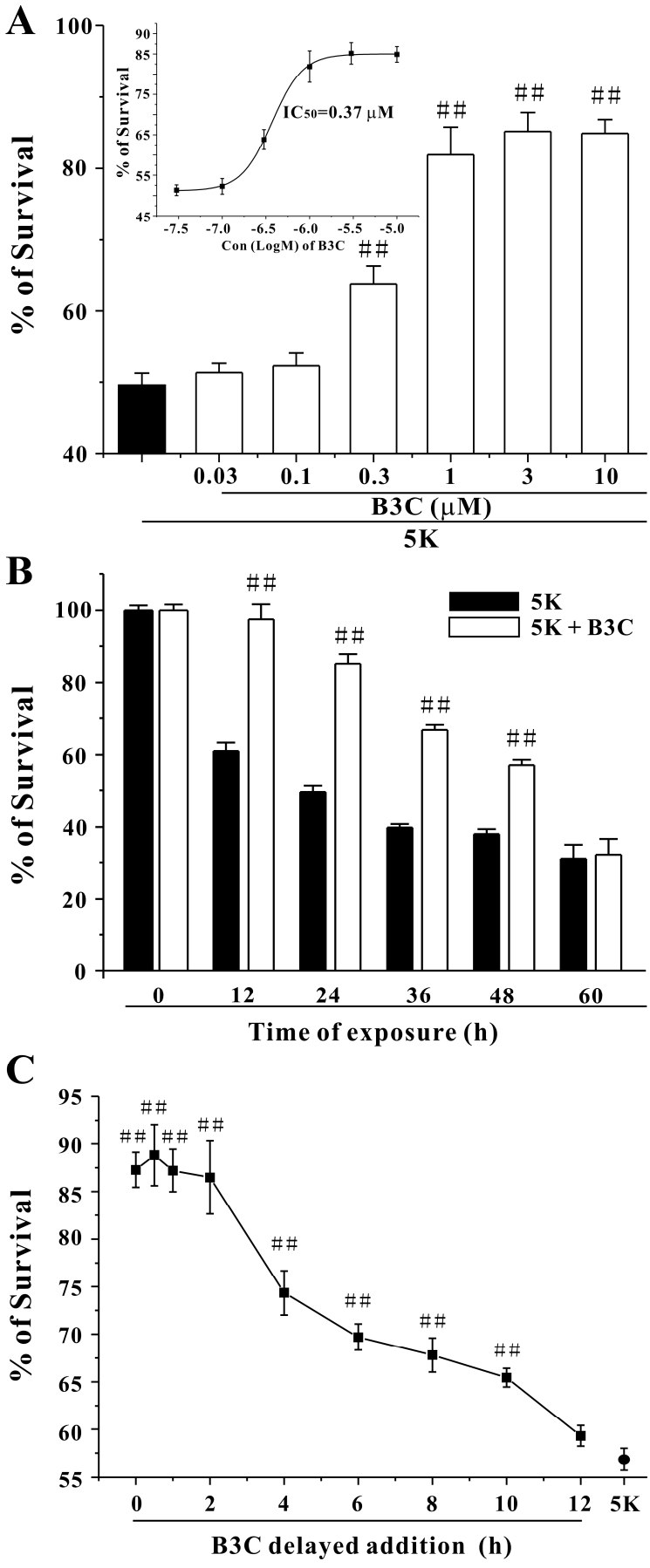

A
% of Survival
IC50=0.37 μM
Con (LogM) of B3C
B3C (μM)
5K
B
5K
5K + B3C
% of Survival
Time of exposure (h)
C
% of Survival
B3C delayed addition (h)
5K

Fig. 3.2.1 B3C effectively blocks neuronal death in CGNs induced by K^+ deprivation.

(A) At 8 DIV, CGNs were switched to K^+ deprived medium with or without B3C (0.03-10 μM). Cell viability was measured by MTT reduction assay 24 h after K^+ deprivation in primary CGNs. IC_{50} value was calculated using Origin Pro 8 statistical software. $^{\#\#}$, $p<0.01$, compared to 5K group. (B) CGNs were switched to K^+ deprived medium with or without 3 μM B3C for different lengths of time, and then subjected to MTT reduction assay. $^{\#\#}$, $p<0.01$, compared to 5K group. (C) B3C was added at different times after the switch of CGNs to K^+ deprived medium. MTT reduction assay was performed to measure the cell viability$^{\#\#}$, $p<0.01$, compared to 5K group.

3.2.2 B3C blocks the hallmark of apoptosis in CGNs induced by K^+ deprivation

Further, FDA/PI double staining, Hoechst staining and DNA fragmentation assays were conducted in succession to assess the neuroprotective effects of B3C. As shown by phase contrast microscopy (Fig. 3.2.2A), CGNs displayed a unhealthy bodies and broken neuritic network after 24 h of K^+ deprivation, while B3C significantly blocked the loss of neurons and reversed the morphological changes caused by K^+ deprivation insult. In addition, B3C prevented existing neurons from dying, as evidenced by the increase in the number of FDA-labeled neurons and the decrease in the number of PI-labeled neurons in FDA/PI double staining assays. Next, Hoechst staining and DNA fragmentation assay, two typical methods for detecting apoptosis, were applied to differentiate the apoptotic neurons from the necrotic ones. Results from Hoechst 33342 staining indicated that B3C substantially reversed K^+ deprivation-induced nuclear condensation, as evidenced by the decrease in the apoptotic bodies stained by Hoechst (Fig.3.2.2B). In parallel, results from DNA fragmentation gel assays suggested that B3C remarkably blocked DNA fragmentation (DNA “ladder”), another hallmark of apoptosis (Fig. 3.2.2C).

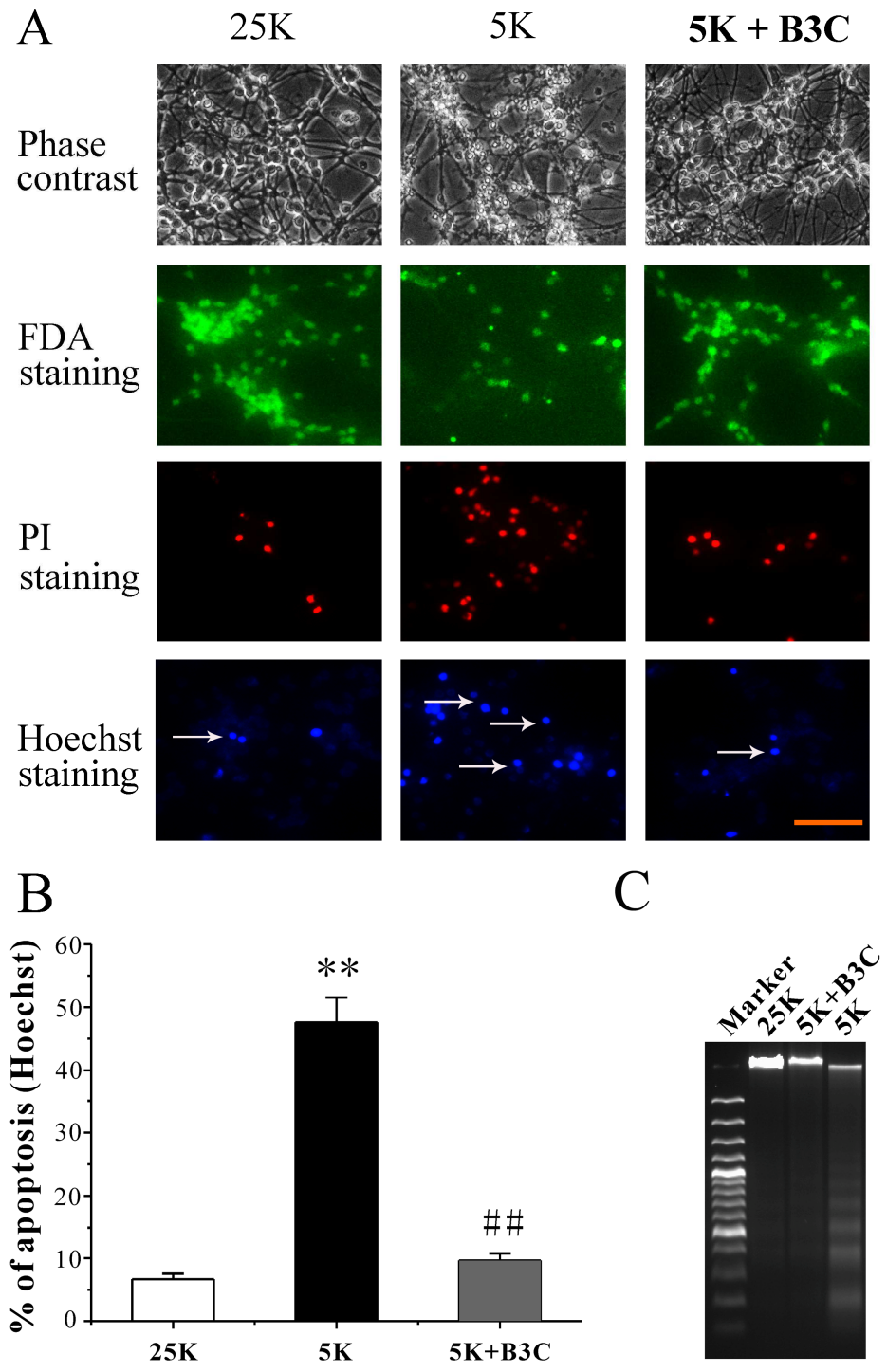


Fig. 3.2.2 B3C substantially attenuates the hallmark of apoptosis in CGNs induced by K^+ deprivation.

(A) At 8 DIV, CGNs were switched to K^+ deprived medium with or without 3 μM B3C. CGNs were assayed with a phase contrast microscope, FDA/PI double staining and Hoechst 33342 staining 24 h after K^+ deprivation,. Scale bar = 100 μm. (B) The number of apoptotic nuclei stained by Hoechst was randomly and double-blindly

calculated from (A). **, $p< 0.01$, compared to control (25K group), $^{\#\#}$, $p< 0.01$, compared to 5K group. (C) The fragmented DNA in CGNs was extracted, and then agarose gel electrophoresis was used for visualization of DNA bands.

3.2.3 The neuroprotection of B3C against K^+ deprivation is independent of AChE inhibition and NMDA receptor antagonism

Since B3C is derived from tacrine and has comparable AChE inhibition to tacrine (Carlier et al. 1999), it is reasonably asked whether the neuroprotective effect of B3C was associated with its inhibition on AChE. In this study, B3C, together with tacrine (the monomer of B3C) and donepezil (the commonly used AChE inhibitor), were selected to test their effects against K^+ deprivation-induced neuronal death in CGNs. By using MTT assay, it was found that both tacrine and E2020, even when their concentrations exceeded 50 μM, failed to block neuronal death in CGNs induced by K^+ deprivation (Fig.3.2.3A).

It has been reported that activation of cholinergic receptors could block neuronal death (Meyer et al. 2002). B3C inhibits the activity of AChE and indirectly improves the concentration of the neurotransmitter acetylcholine in the synaptic cleft, the latter of which may contribute to the therapeutic effects against neurodegenerative diseases in which cholinergic dysfunction is involved. As such, to test whether the neuroprotective effect of B3C was associated with its activation of cholinergic receptors, both atropine (a muscarinic cholinergic receptor antagonist) and dihydro-β-erythroidine (a nicotinic cholinergic receptor antagonist, DHE) were applied. With MTT assay, it was shown that 10 μM atropine, 50 μM DHE, or their combination, were unable to prevent K^+ deprivation-induced apoptosis in CGNs

(Fig.3.2.3B).

Further, since B3C has been proven to be an NMDA receptor antagonist (Luo et al. 2010), it is reasonably asked whether the neuroprotective effect of B3C against K^+ deprivation was associated with its blockage of NMDA receptor. Therefore, MK801 and memantine, two widely used NMDA receptor antagonists, were used in our model. With MTT assay, it was found that neither MK801 at 5 μM nor memantine at 30 μM could attenuate neuronal death induced by K^+ deprivation (Fig.3.2.3C).

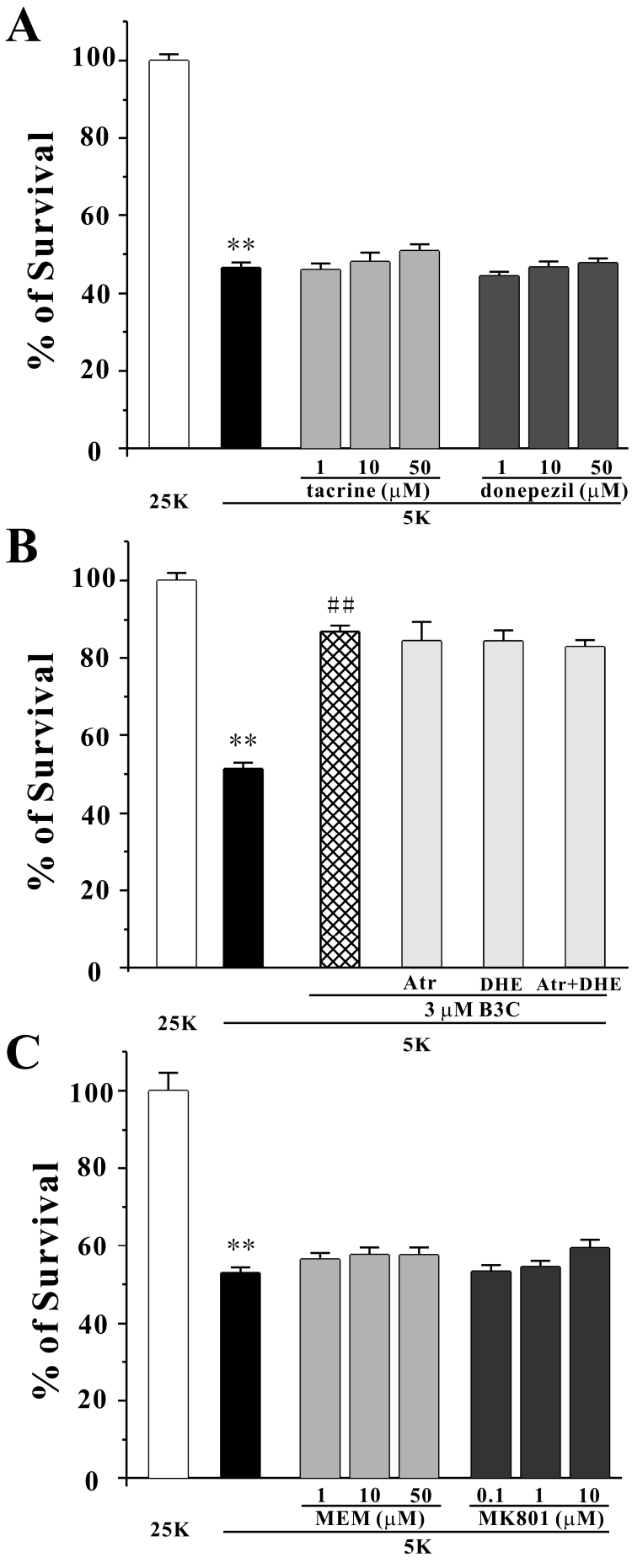

A
100
80
60
40
20
0
% of Survival
**
1 10 50
tacrine (μM)
1 10 50
donepezil (μM)
25K
5K
B
100
80
60
40
20
0
% of Survival
**
##
Atr
DHE
Atr+DHE
3 μM B3C
25K
5K
C
100
80
60
40
20
0
% of Survival
**
1 10 50
MEM (μM)
0.1 1 10
MK801 (μM)
25K
5K

Fig.3.2.3 The neuroprotection of B3C is independently associated with its AChE inhibition and NMDA receptor antagonism.

(A) At 8 DIV, CGNs were switched to K^+ deprived medium containing tacrine or E2020 (1, 10 or 50 μM). At 24 h after K^+ deprivation, MTT reduction assay was performed to measure cell viability. **, $p< 0.01$, compared to 25K group. (B) At 8 DIV, CGNs were pre-treated with 10 μM atropine (Atr) or/and 50 μM dihydro-β-erythroidine (DHE) for 0.5 h in a serum-free medium containing 25 mM KCl, then switched to K^+ deprived medium containing 3 μM B3C, and finally subjected to MTT reduction assay 24 h after treatment. **, $p< 0.01$, compared to 25K group, $^{\#\#}$, $p< 0.01$, compared to 5K group. (C) At 8 DIV, CGNs were switched to K^+ deprived medium containing memantine (MEM, 1, 10 or 50 μM) or MK801 (0.1, 1, 10 μM). and then subjected to MTT reduction assay at 24 h after K^+ deprivation. **, $p< 0.01$, compared to 25K group.

3.2.4 B3C reverses the inhibition of VEGFR-2 caused by K^+ deprivation

Recent studies have indicated that neurotrophic factors such as VEGF could protect against K^+ deprivation through the activation of receptor tyrosine kinases (Yu and Chuang 1997; Wick et al. 2002). To investigate whether the neuroprotective effect of B3C against K^+ deprivation was associated with VEGF/VEGFR-2 system, PTK/ZK, a specific VEGFR-2 tyrosine kinases inhibitor, was first used in our cell model. It was found that PTK/ZK at 10 μM for 0.5 h abolished the neuroprotective effects of B3C against K^+ deprivation-induced neuronal death in CGNs (Fig.3.2.4A). Moreover, the activation of VEGFR-2 produced by B3C was examined in our system by determining the protein expression of p-Tyr1054-VEGFR-2. Results from Western blot showed that K^+ deprivation caused a rapid decrease in p-Tyr1054-VEGFR-2, which reached a peak at 1 h (Fig. 3.2.4B), while treatment with 3 μM B3C reversed the decrease of p-Tyr1054-VEGFR-2 induced by K^+ deprivation (Fig. 3.2.4C).

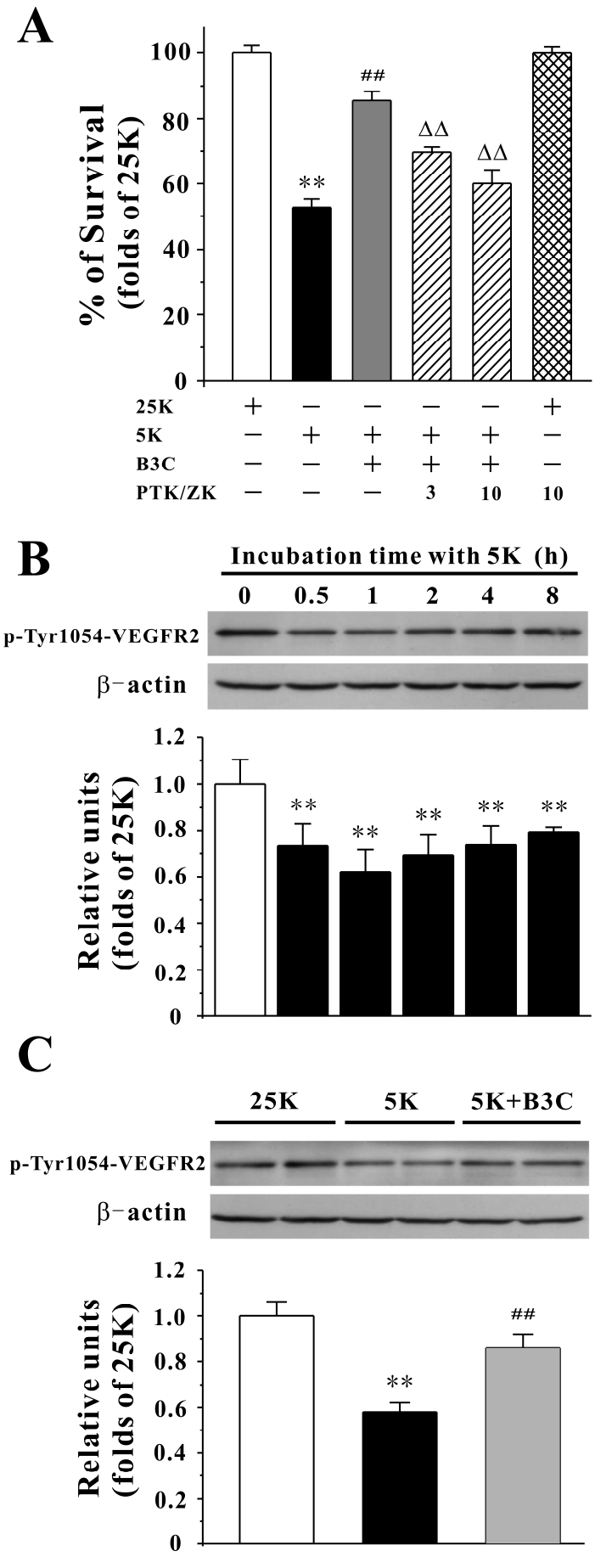

A
% of Survival
(folds of 25K)
100
80
60
40
20
0
**
##
ΔΔ
ΔΔ
25K + − − − − +
5K − + + + + −
B3C − − + + + −
PTK/ZK − − − 3 10 10
B
Incubation time with 5K (h)
0 0.5 1 2 4 8
p-Tyr1054-VEGFR2
β−actin
Relative units
(folds of 25K)
1.2
1.0
0.8
0.6
0.4
0.2
0
**
C
25K 5K 5K+B3C
p-Tyr1054-VEGFR2
β−actin
Relative units
(folds of 25K)
##

Fig.3.2.4 B3C reverses the inhibition of VEGFR-2 caused by K^+ deprivation.

(A) VEGFR inhibitor (PTK/ZK), partially abolished the neuroprotective effects of B3C against K^+ deprivation. CGNs were pre-treated with PTK/ZK (3 or 10 μM) for 0.5 h in serum-free medium containing 25 mM K^+, then switched to K^+ deprived medium containing 3 μM B3C, and finally subjected to MTT reduction assay 24 h after K^+ deprivation. **, $p< 0.01$, compared to control (25K group), $^{\#\#}$, $p< 0.01$, compared to 5K group; $^{\Delta\Delta}$, $p< 0.01$, compared to 5K plus B3C group. (B) K^+ deprivation decreased the protein expression of p-Tyr1054-VEGFR-2 in a time-dependent manner. CGNs were switched to K^+ deprived medium for 0.5, 1, 2, 4 and 8 h, and then subjected to Western blot for determining the protein expression of p-Tyr1054-VEGFR-2. **, $p< 0.01$, compared to control (25K group). (C) B3C reversed the decrease in p-Tyr1054-VEGFR-2 caused by K^+ deprivation. CGNs were switched to K^+ deprived medium with or without 3 μM B3C, and then subjected to Western blot at 1 h after treatment. **, $p< 0.01$, compared to control (25K group), $^{\#\#}$, $p< 0.01$, compared to 5K group.

3.2.5 B3C reverses the inhibition of Akt and ERK pathways caused by K^+ deprivation

Since the Akt and ERK signaling pathways, two well-studied survival-promoting signaling mechanisms in CGNs, have been demonstrated to be downstreams of VEGFR-2 (Kilic et al. 2006; Shen et al. 2012), it is reasonably asked whether these pathways were associated with the paradigm of B3C.

First, to determine whether GSK3β pathway was involved in K^+ deprivation-induced neuronal death in our CGNs, SB415286, a specific inhibitor of GSK3β, was selected to pre-treat the CGNs for 0.5 h prior to 24 h of K^+ deprivation insult. With MTT assay, it was found that 3 and 10 μM SB415286 blocked K^+ deprivation-induced neuronal death (Fig. 3.2.5A). Thereafter, we tested the time course of p-Ser473-Akt and its downstream target p-Ser9-GSK 3β with Western blot after K^+ deprivation. As can be seen from Figs. 3.2.5B and 3.2.5C, it was very

obvious that K^+ deprivation caused a significant decrease of p-Ser473-Akt and p-Ser9-GSK 3β, with a peak at 1 h. While, B3C at 3 μM effectively reversed the decrease of p-Ser473-Akt and p-Ser9-GSK 3β caused by 1 h of K^+ deprivation in CGNs (Fig. 3.2.5D). Furthermore, both pharmacological inhibition of PI3-K by LY294002 (10 and 30 μM) and inhibition of VEGFR-2 by PTK/ZK (10 μM) abolished the neuroprotective effects (Fig. 3.2.5A) and the reversal of decrease in p-Ser473-Akt and p-Ser9-GSK 3β mediated by B3C (Fig. 3.2.5D).

Second, to examine whether activation of ERK pathway was associated with the neuroprotective effects of B3C in our CGNs. PD98059, a specific inhibitor of MEK, was employed to pre-treat the CGNs 0.5 h before the switch of CGNs into K^+ deprived medium in the absence or presence of B3C. As expected, treatment with PD98059 (30 and 50 μM) abolished the neuroprotection of B3C, while PD98059 itself was unable to block apoptosis (Fig. 3.2.6A). With the use of western blot, CGNs showed a significant decrease in p-ERK in the first 4 h after K^+ deprivation, which is consistent with the previous study (Barneda-Zahonero et al. 2009). Meanwhile, B3C significantly increased the p-ERK at 1 h and 2 h after K^+ deprivation (Fig. 3.2.6B). Both pharmacological inhibition of MEK1/2 with PD98059 and inhibition of VEGFR-2 with PTK/ZK returned p-ERK to 5K levels (Fig. 3.2.6C).

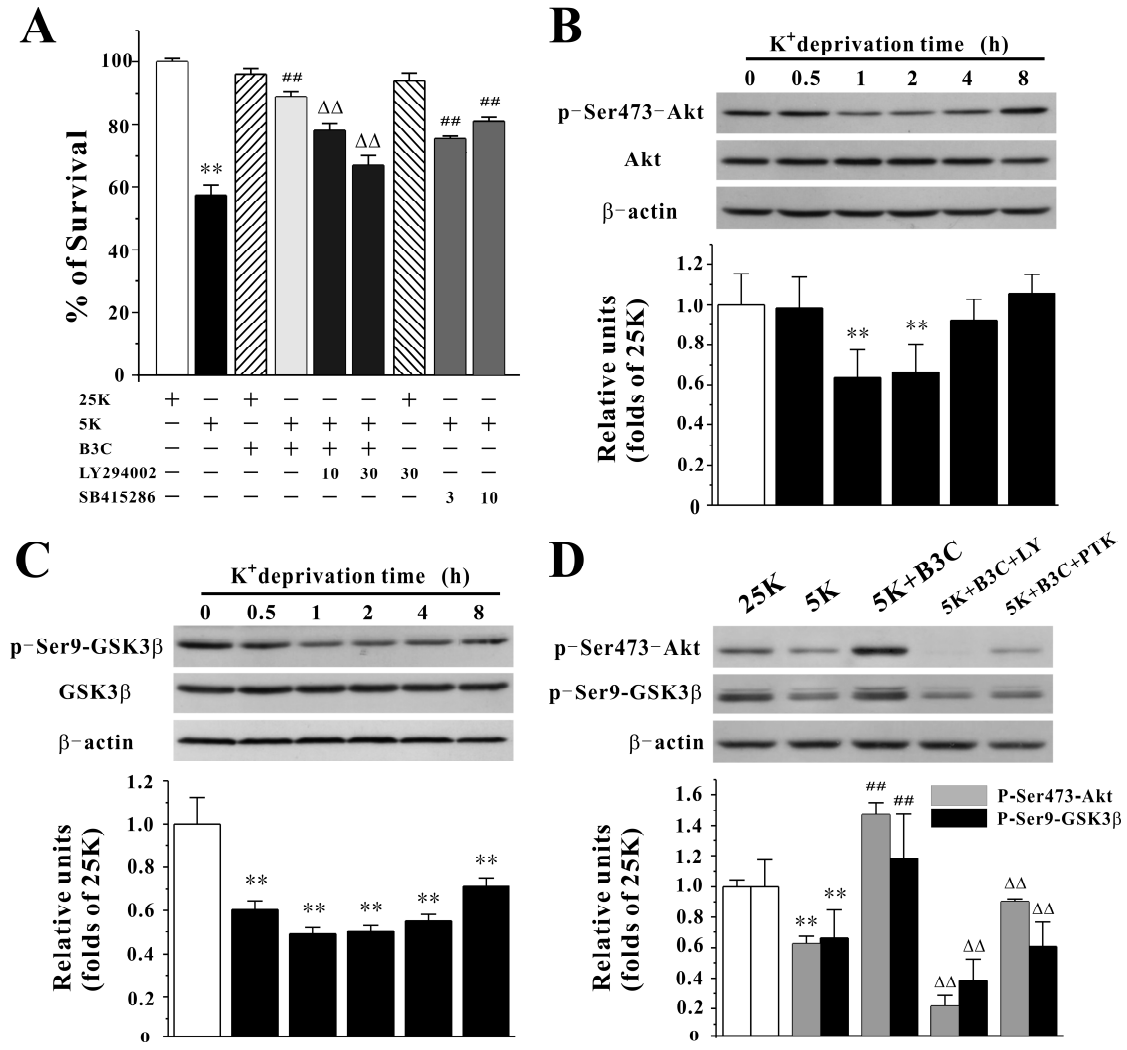
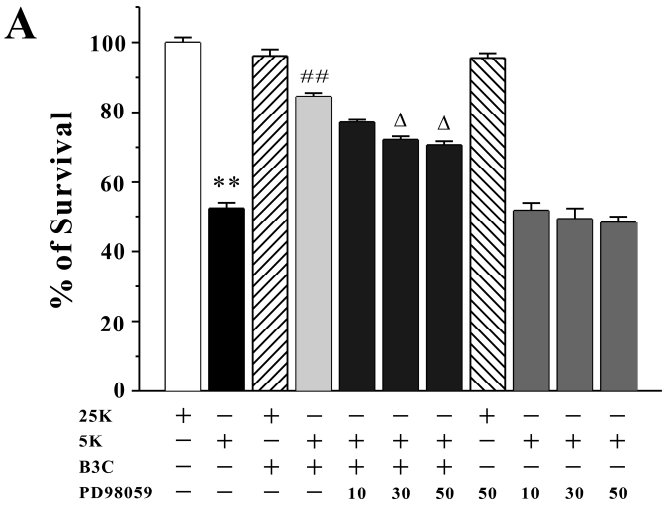
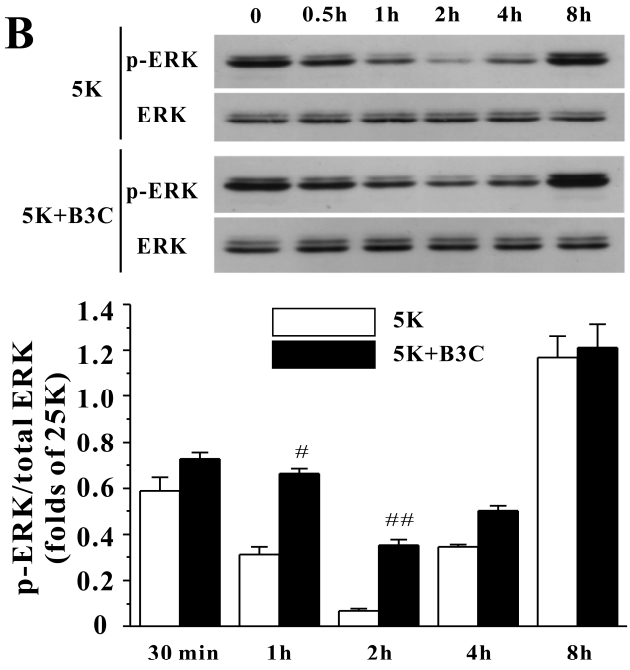


Fig.3.2.5 B3C reverses the inhibition of the PI3-K/Akt/GSK3β pathway caused by K^+ deprivation.

(A) PI3-K specific inhibitor (LY294002) partially abolished the neuroprotective effects of B3C. CGNs were pre-treated with LY294002 for 0.5 h in serum-free medium containing 25 mM KCl, then switched to K^+ deprived medium containing 3 μM B3C, and finally subjected to MTT reduction assay at 24 h after K^+ deprivation. **, $p< 0.01$, compared to control (25K group), $^{\#\#}$, $p< 0.01$, compared to 5K group, $^{\Delta\Delta}$, $p< 0.01$, compared to 5K plus B3C group. (B) K^+ deprivation decreased the protein expression of p-Ser473-Akt in a time-dependent manner. CGNs were switched to K^+ deprived medium for 0.5, 1, 2, 4 and 8 h, and then subjected to Western blot for determining the protein expression of p-Ser473-Akt. **, $p< 0.01$, compared to control (25K group). (C) K^+ deprivation decreased the protein expression of p-Ser9-GSK3β in a time-dependent manner. CGNs were switched to K^+ deprived medium for 0.5, 1, 2, 4 and 8 h, and then subjected to Western blot for determining the protein expression of p-Ser9-GSK3β. **, $p< 0.01$, compared to control (25K group). (D) B3C reversed the decrease of p-Ser473-Akt and p-Ser9-GSK3β caused by K^+ deprivation. CGNs

were switched to K^+ deprived medium in the absence or presence of 3 μM B3C, and then subjected to Western blot at 1 h after treatment. **, $p< 0.01$, compared to control (25K group), $^{\#\#}$, $p< 0.01$, compared to 5K group, $^{\Delta\Delta}$, $p< 0.01$, compared to 5K plus B3C group.


A
% of Survival
100
80
60
40
20
0
**
##
Δ
Δ
25K + − + − − − − + − − −
5K − + − + + + + − + + +
B3C − − + + + + + − − − −
PD98059 − − − − 10 30 50 50 10 30 50


B
0 0.5h 1h 2h 4h 8h
5K
p-ERK
ERK
5K+B3C
p-ERK
ERK
p-ERK/total ERK (folds of 25K)
5K
5K+B3C
#
##
30 min 1h 2h 4h 8h

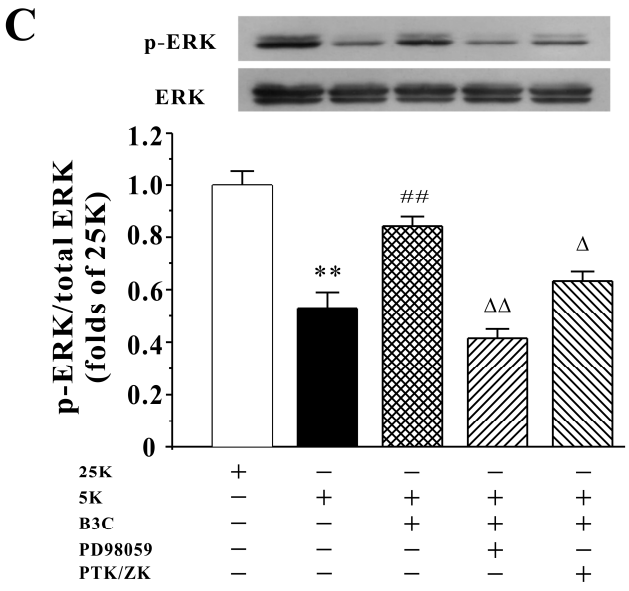

C
p-ERK
ERK
p-ERK/total ERK (folds of 25K)
**
##
ΔΔ
Δ
25K + − − − −
5K − + + + +
B3C − − + + +
PD98059 − − − + −
PTK/ZK − − − − +

Fig.3.2.6 B3C reverses the inhibition of the ERK pathway caused by K^+ deprivation.

(A) Pharmacological inhibition of MEK with specific inhibitor PD98059 (30 and 50 μM) abolished the neuroprotective effects of B3C. CGNs were pre-treated with PD98059 for 0.5 h in serum-free medium containing 25 mM K^+, then switched to K^+ deprived medium containing 3 μM B3C, and finally subjected to MTT reduction assay at 24 h after K^+ deprivation. **, $p< 0.01$, compared to control (25K group); $^{\#\#}$, $p< 0.01$, compared to 5K group; $^{\Delta}$, $p< 0.05$, compared to 5K plus B3C group. (B) B3C treatment induced a significant ERK phosphorylation. CGNs were switched to K^+ deprived medium in the absence or presence of 3 μM B3C, and then subjected to Western blot for determining the protein expression of p-ERK and ERK. $^{\#}$, $p<0.05$, $^{\#\#}$, $p<0.01$, compared to 5K group. (C) B3C reversed the decrease of p-ERK caused by K^+ deprivation. CGNs were switched to K^+ deprived medium in the absence or presence of 3 μM B3C at 1 h after treatment, and then subjected to Western blot analysis with p-ERK and ERK. **, $p< 0.01$, compared to control (25K group); $^{\#\#}$, $p< 0.01$, compared to 5K group; $^{\Delta}$, $p< 0.05$, $^{\Delta\Delta}$, $p< 0.01$, compared to 5K plus B3C group.

____(Hu S, *et al.* 2013, *CNS Neurosci Ther*. 19(10):764-72)

3.3

B3C promotes neurite outgrowth in PC12 cells via the activation of α7-nAChR

3.3.1 Induction of neurite outgrowth in PC12 cells by B3C

NGF and dbcAMP substantially induced neurite outgrowth in PC12 cells, serving as positive controls in our neuronal model. The percentage of cells with neurites for cells treated with 7 days of NGF or dbcAMP reached to 74.0 ± 2.0 % and 53.1 ± 3.6 %, respectively (Fig. 3.3.1), an observation consistent with several previous studies (Daniele et al. 2010; Cui et al. 2011). B3C, but not tacrine and memantine, remarkably stimulated neurite outgrowth of PC12 cells in a concentration- and time-dependent manner (Figs. 3.3.1B and 3.3.1C), with the maximum percentage of 63.7 ± 2.0 % at 3 μM (Fig. 3.3.2A). It is obvious that cells treated with B3C displayed typical differentiated morphologies of condensed cell bodies and extended neurite outgrowths, in contrast to the flattened cell bodies of the undifferentiated cells observed in the vehicle control (Fig. 3.3.2A). To further confirm our morphological observation at the molecular level, the expression of growth-associated protein-43 (GAP-43), a neuron-specific protein which exhibits enhanced synthesis and axonal fast-transport during nerve regeneration, was examined using Western blot analysis. It was found that 7-day treatment of PC12 cells with 3 μM B3C showed extensive expression of GAP-43, which is comparable to those treatment with NGF and dbcAMP (Fig. 3.3.2B).

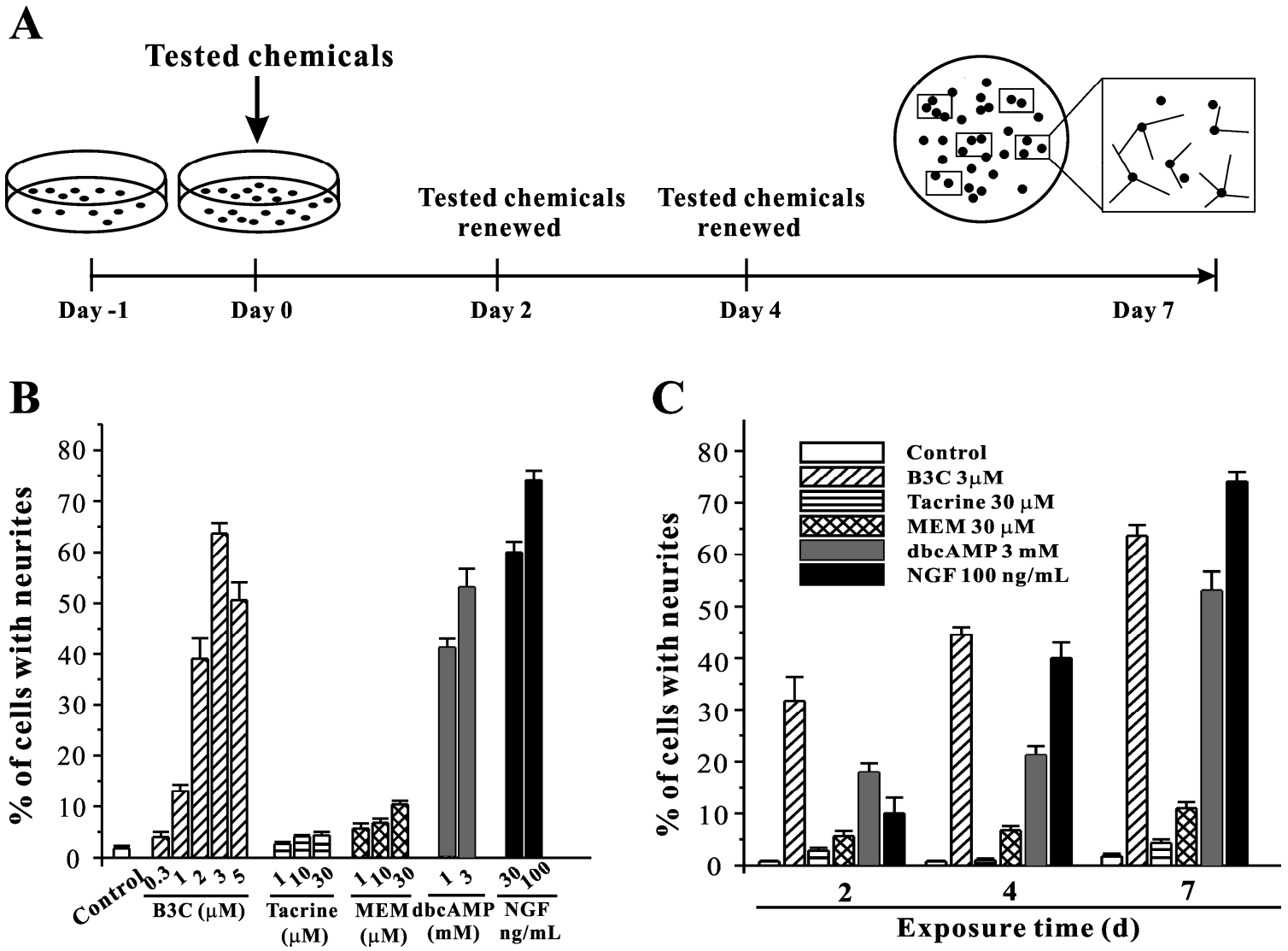

A
Tested chemicals
Tested chemicals renewed
Tested chemicals renewed
Day -1
Day 0
Day 2
Day 4
Day 7
B
% of cells with neurites
0
10
20
30
40
50
60
70
80
Control
0.3
1
2
3
5
1
10
30
1
10
30
1
3
30
100
B3C (μM)
Tacrine (μM)
MEM (μM)
dbcAMP (mM)
NGF ng/mL
C
% of cells with neurites
Control
B3C 3μM
Tacrine 30 μM
MEM 30 μM
dbcAMP 3 mM
NGF 100 ng/mL
2
4
7
Exposure time (d)

Fig. 3.3.1 Induction of neurite outgrowth in PC12 cells by B3C.

(A) Schematic diagram shows the timeline and general procedures for assessment of the neurite outgrowth-promoting activity of various chemicals in PC12 cells. Cells were plated on day -1, then treated with various chemicals on day 0, and morphologically quantified on day 2, day 4 or day 7. When cells had been differentiation for a number of days, experimental chemicals were renewed every other day. The cells were scored as differentiated if one or more neurites were longer than the diameter of cell body observed in at least 5 randomly selected microscopic fields. (B) B3C induced neurite outgrowth in a concentration-dependent manner. PC12 cells were seeded on poly-L-lysine-coated 6-well plates in growth medium for 24 h, then shifted to low serum medium containing various chemicals for 7 days, and the percentage of cells with neurites were counted using phase-contrast microscope. (C) B3C induced neurite outgrowth in a time-dependent manner. PC12 cells were incubated in low serum medium containing B3C (3 μM), tacrine (30 μM), memantine (30 μM), dbcAMP (3 mM) or NGF (100 ng/ml) for different lengths of time, and the percentage of cells with neurites was then analyzed.

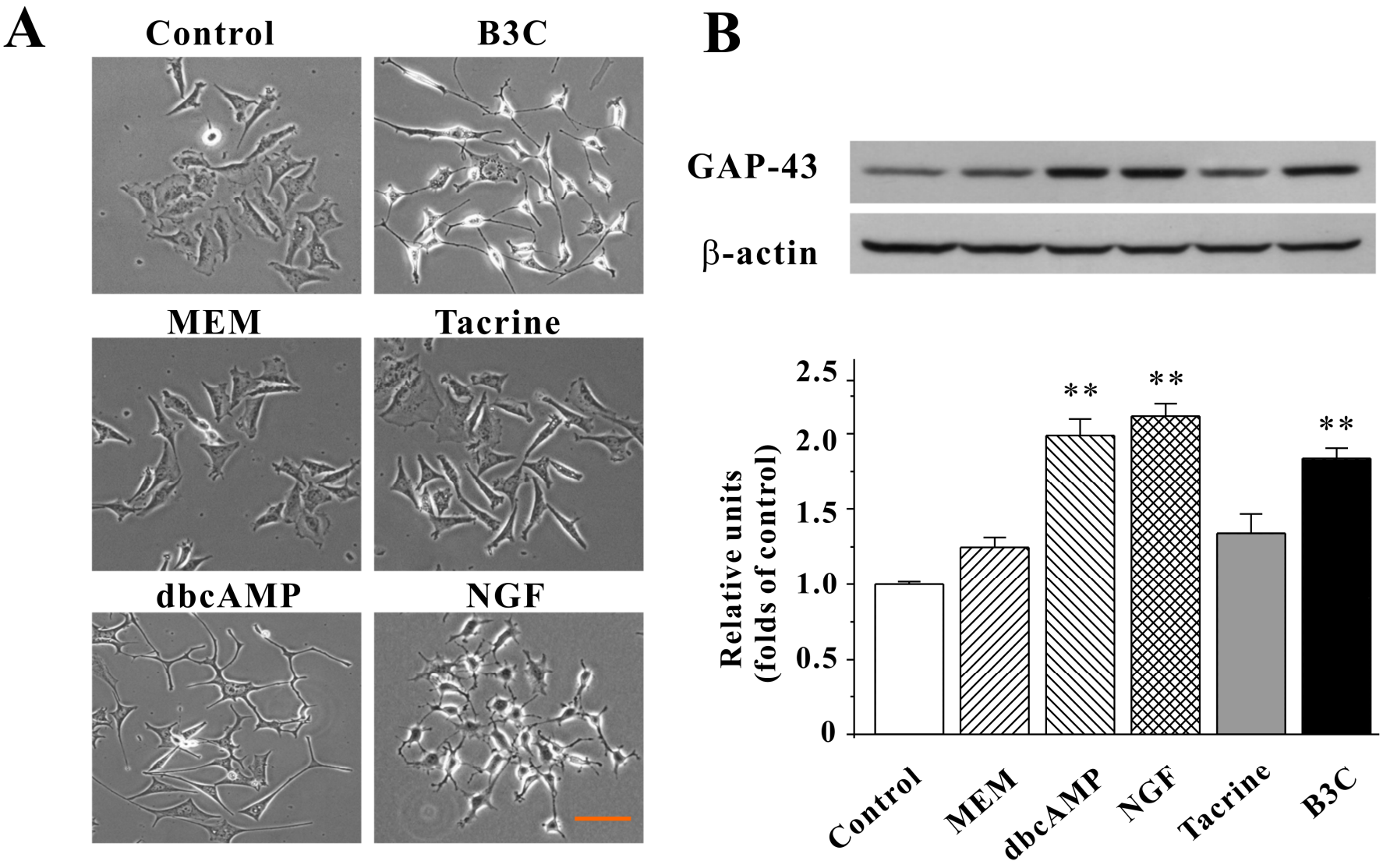

A
Control
B3C
MEM
Tacrine
dbcAMP
NGF
B
GAP-43
β-actin
Relative units
(folds of control)
2.5
2.0
1.5
1.0
0.5
0
**
**
**
Control
MEM
dbcAMP
NGF
Tacrine
B3C

Fig. 3.3.2 Differentiation of PC12 cells toward a neuronal phenotype induced by B3C.

(A) Morphological characteristics of neurite outgrowth in PC12 cells induced by B3C. PC12 cells were incubated with various chemicals in low serum medium for 7 days, and cell morphology was observed using phase-contrast microscopy and photographed by the digital camera. Scale bar = 50 μm. (B) B3C upregulated the protein expression of GAP-43 in PC12 cells. PC12 cells were incubated with various chemicals in low serum medium for 7 days, and the total proteins were subjected to Western blot analysis using anti-GAP-43 and anti-β-actin antibodies. **, $p<0.01$, compared to control group.

3.3.2 Promotion of neurite outgrowth in primary cortical neurons by B3C

To confirm the neurite outgrowth-promoting activity of B3C, we further used dissociated rat cortical neuron cultures as our model system. As shown in Fig. 3.3.3, treatment of cortical neurons with B3C (0.1 and 0.3 μM) for 48 h significantly increased neurite outgrowth, as compared with the vehicle control. Immunostaining-based quantitative study also demonstrated that B3C caused a concentration-dependent increase in the length of βIII tubulin-positive neurites.

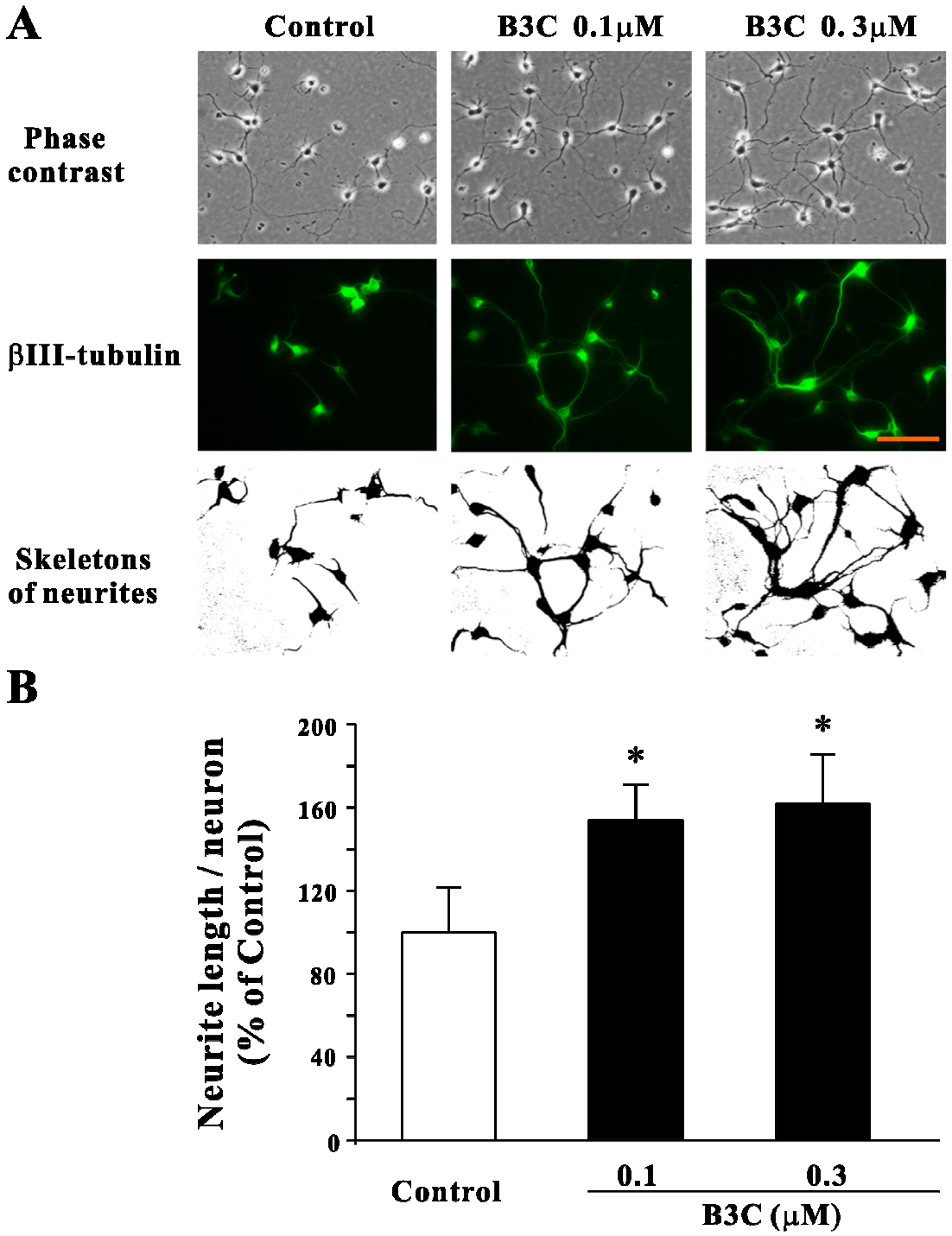


Fig. 3.3.3 Promotion of neurite outgrowth in rat cortical neurons by B3C.

(A) Cortical neurons were treated at 3 DIV in the absence or presence of B3C for 48 h. Upper: Phase-contrast digital images of the cells were taken using a phase-contrast microscope. Middle: Neuronal cultures were anti-βIII-tubulin-positive. Lower: βIII-tubulin-stained neurites were digitally characterized and skeletonized for quantification by NeuriteTracer program. Scale bar = 100 μm. (B) Cortical neurons were treated at 3 DIV in the absence or presence of B3C for 2 days, neurons were then fixed and analysis for the length of βIII-tubulin-positive neurites by using

NeuriteTracer program.

3.3.3 Synergistic neurite outgrowth induced by B3C plus NGF or dbcAMP

To investigate the synergistic effects of B3C plus NGF and dbcAMP, PC12 cells were treated with B3C in combination with either NGF or dbcAMP. It was observed that the effects of 3 ng/ml NGF and 0.3 μM dbcAMP on neurite outgrowth was remarkably potentiated by simultaneous treatment with 0.5 μM B3C (Fig. 3.3.4).

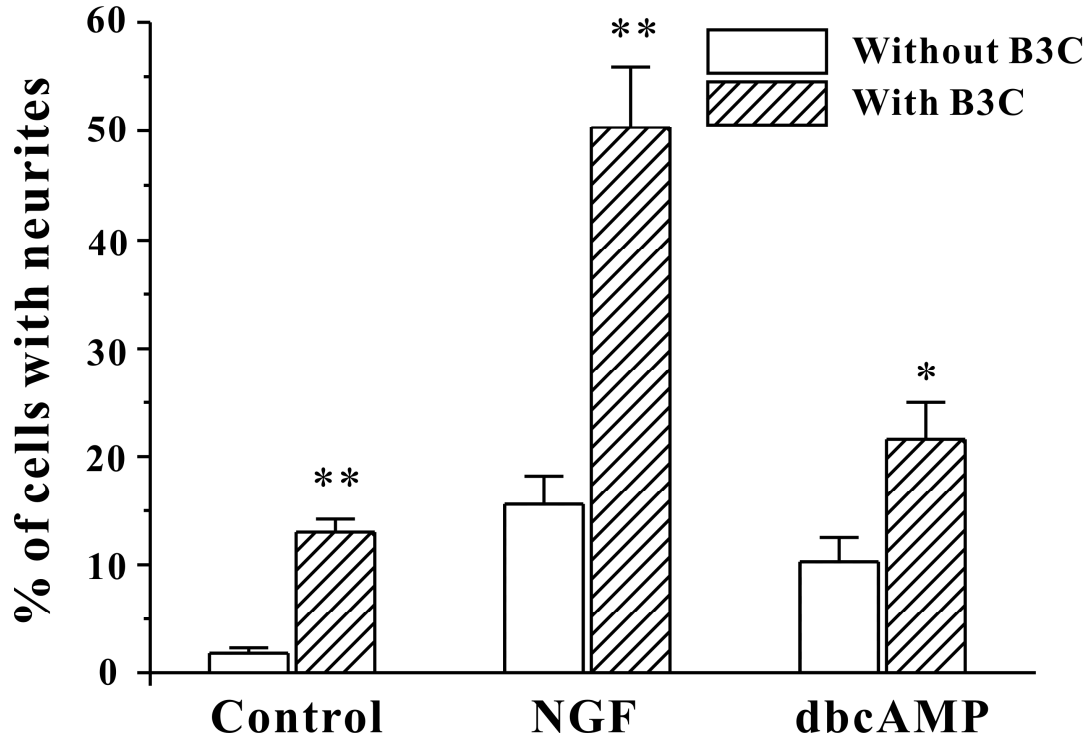


Fig. 3.3.4 Synergistic neurite outgrowth induced by B3C plus NGF or dbcAMP.

PC12 cells were exposed to B3C (1 μM) in the presence or absence of NGF (3 ng/ml) or dbcAMP (0.1 mM) for 7 days. The percentage of cells with neurites was counted using phase-contrast microscopy. *, $p<0.05$, compared to control group, **, $p<0.01$, compared to control group.

3.3.4 Activation of ERK pathway, but not P38 and JNK pathway, is associated with B3C-induced neurite outgrowth in PC12 cells

To determine whether ERK, P38 and JNK signaling pathways were involved in B3C-induced neurite outgrowth, PD98059 and U0126, the specific inhibitors of MEK, SB203580, the specific inhibitor of P38 and SP600125, and the specific inhibitor of JNK, were selected to treat PC12 cells 2 h before the addition of B3C. It was found that PD98059 and U0126, but not SB203580 and SP600125, partially abolished the outgrowth of neurites induced by B3C (Fig. 3.3.5A). To further confirm whether B3C induced neurite outgrowth of PC12 cells through the activation of ERK pathway, the level of p-ERK was measured by Western blot analysis. It was shown that B3C upregulated the expression of p-ERK, which peaked at 1 h and lasted for 4 h after treatment (Fig. 3.3.5B).

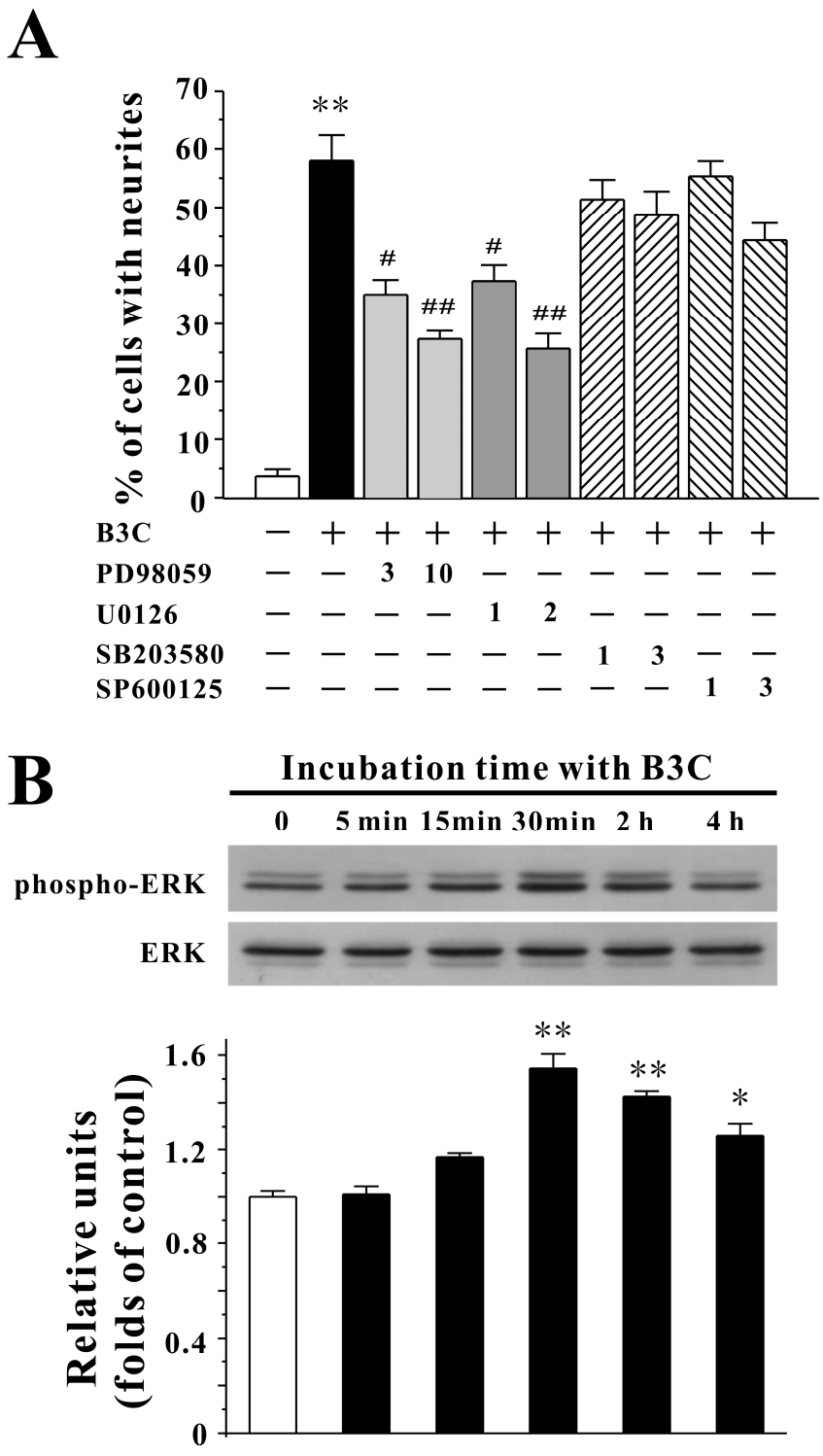


Fig. 3.3.5 Activation of ERK pathway is involved in the neurite outgrowth-promoting activities of B3C.

(A) MEK inhibitors, but not P38 and JNK inhibitors, partially attenuated the neurite outgrowth induced by B3C in PC12 cells. Cells were pre-treated with specific inhibitors (PD98059, U0126, SB203580 and SP600125) in low serum medium for 2 h, and then incubated with 3 μM B3C for 7 days. The percentage of cells with neurites was counted using phase-contrast microscopy. **, $p<0.01$, compared to control group,

[#], $p<0.05$, compared to B3C group, [##], $p<0.01$, compared to B3C group. (B) B3C upregulated p-ERK in PC12 cells. Cells were incubated with 3 μM B3C in low serum medium for different durations, and the total proteins were subjected to Western blot analysis using anti-p-ERK and anti-ERK antibodies. [**], $p<0.01$, compared to control group.

3.3.5 Pharmacological inhibition of alpha7-nAChR partially abolishes the B3C-induced neurite outgrowth in PC12 cells

To determine whether B3C-induced neurite outgrowth through regulation of NGF/TrkA system, K252a, the specific inhibitor of Trk A, was used to treat PC12 cells 2 h before the addition of B3C. It was found that K252a, at its effective concentration, did not block neurite outgrowth induced by B3C, whereas it could block the neurite outgrowth induced by NGF (Fig. 3.3.6A). It has been revealed that acetylcholine receptors (AChRs) were implicated in neuronal differentiation. To test whether B3C could exert neurite outgrowth-promoting activity via regulation of AChRs, atropine, the specific inhibitor of muscarinic AChR (mAChR), and mecamylamine and tubocurarine, the specific inhibitors of nicotinic AChR (nAChR) were selected to treat PC12 cells 2 h before the addition of B3C. It was shown that 10 μM tubocurarine and 10 μM mecamylamine, but not 10 μM atropine partially abolished the outgrowth of neurite induced by B3C (Fig. 3.3.6B). Additionally, methyllycaconitine (MLA), the specific antagonist of α7-nAChR, partially inhibited the outgrowth of neurite induced by B3C (Fig. 3.3.6D). Furthermore, 2 h of pre-treatment of PC12 cells with MLA as well as PD98059, but not atropine, significantly inhibited the elevated level of p-ERK (Fig. 3.3.6C).

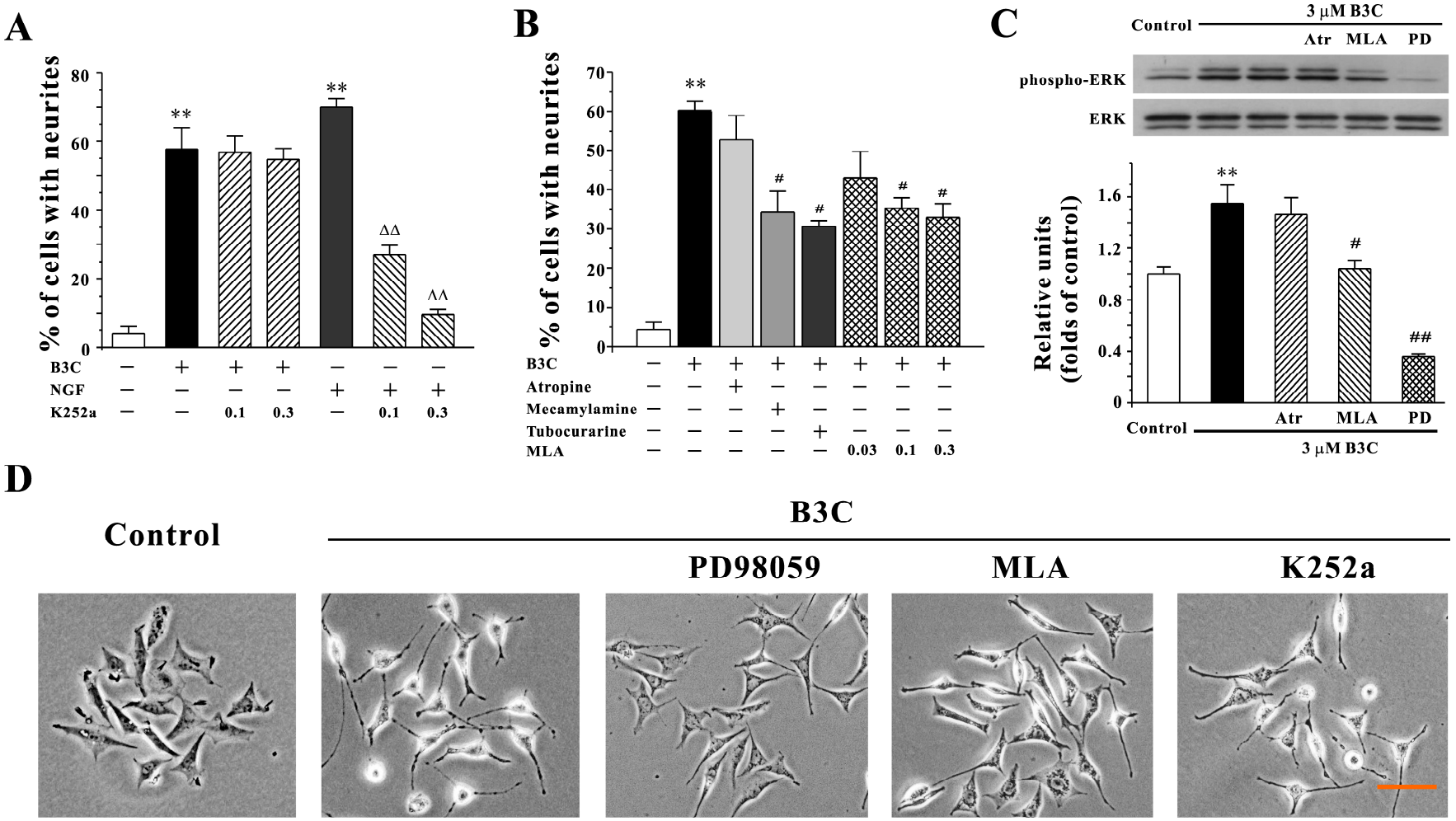

A
% of cells with neurites
80
60
40
20
0
**
**
ΔΔ
ΛΛ
B3C − + + + − − −
NGF − − − − + + +
K252a − − 0.1 0.3 − 0.1 0.3
B
% of cells with neurites
70
60
50
40
30
20
10
0
**
#
#
#
#
B3C − + + + + + + +
Atropine − − + − − − − −
Mecamylamine − − − + − − − −
Tubocurarine − − − − + − − −
MLA − − − − − 0.03 0.1 0.3
C
Control
3 μM B3C
Atr MLA PD
phospho-ERK
ERK
Relative units (folds of control)
1.6
1.2
0.8
0.4
0
**
#
##
Control
Atr MLA PD
3 μM B3C
D
Control
B3C
PD98059
MLA
K252a

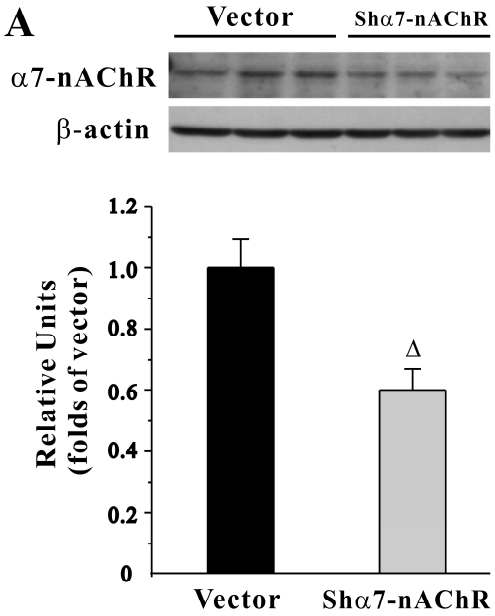
Fig. 3.3.6 Pharmacological inhibition of α7-nAChR partially abrogates the neurite outgrowth-promoting activities of B3C.

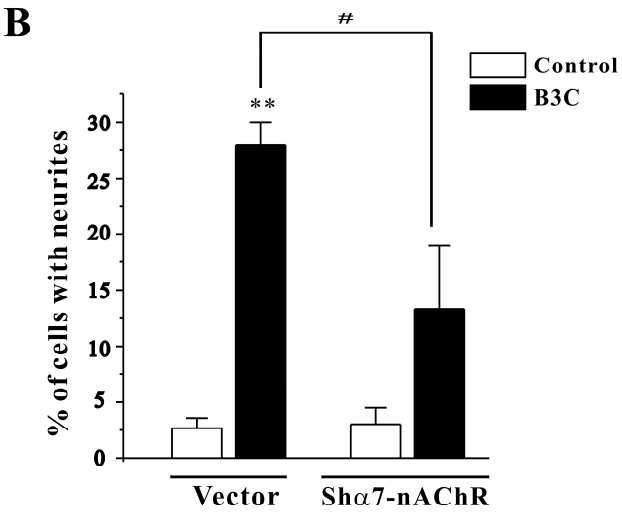
(A) Trk A specific inhibitor was unable to attenuate neurite outgrowth induced by B3C in PC12 cells. Cells were pre-treated with K252a for 2 h, and then incubated with 3 μM B3C or 100 ng/ml NGF. The percentage of cells with neurites were counted using phase-contrast microscopy 7 days after treatment. **, $p<0.01$, compared to control group, $^{\Delta\Delta}$, $p<0.01$, compared to NGF group, (B) The antagonist of α7-nAChR partially abolished neurite outgrowth induced by B3C in PC12 cells. Cells were pre-treated with atropine, mecamylamine, tubocurarine and MLA for 2 h, and then incubated with 3 μM B3C. The percentage of cells with neurites was counted using phase-contrast microscopy 7 days after treatment. **, $p<0.01$, compared to control group, $^{\#}$, $p<0.05$, compared to B3C group. (C) The antagonist of α7-nAChR attenuated the activation of p-ERK induced by B3C in PC12 cells. Cells were pre-treated with atropine, mecamylamine and MLA for 2 h, and then incubated with 3 μM B3C for 30 min. The total proteins were extracted and subjected to Western blot analysis using anti-p-ERK and anti-ERK antibodies. **, $p<0.01$, compared to control group. $^{\#}$, $p<0.05$, compared to B3C group, $^{\#\#}$, $p<0.01$, compared to B3C group. (D) Morphological characteristics of neurite outgrowth in PC12 cells treated with specific inhibitors. Cells were pre-treated with PD98059, MLA and K252a for 2 h, and then incubated with 3 μM B3C for 7 days, and cell morphology was observed using phase-contrast microscopy and photographed by a digital camera. Scale bar = 50 μm.

3.3.6 Gene knockdown of α7-nAChR abolishes the B3C-induced neurite outgrowth in PC12 cells

To investigate whether the neurite outgrowth-promoting activity of B3C mainly act through α7-nAChR, we tested the neurite outgrowth-promoting effects of B3C in ShRNA-mediated α7-nAChR knockdown PC12 cells. With the use of western blot, α7-nAChR ShRNA (Shα7-nAChR) caused a reduction in α7-nAChR protein level, whereas the negative control ShRNA (ShNC) and vector had no effect on α7-nAChR protein level (Fig. 3.3.7A). In addition, in contrast to the neurite outgrowth-promoting activity of B3C observed in the vector or in the ShNC-treated PC12 cells, B3C in α7-nAChR knockdown PC12 cells was no longer able to induce neurite outgrowth

(Fig. 3.3.7 B, C).


A
Vector
Shα7-nAChR
α7-nAChR
β-actin
Relative Units
(folds of vector)
1.2
1.0
0.8
0.6
0.4
0.2
0
Δ
Vector
Shα7-nAChR


B
#
**
Control
B3C
% of cells with neurites
30
25
20
15
10
5
0
Vector
Shα7-nAChR

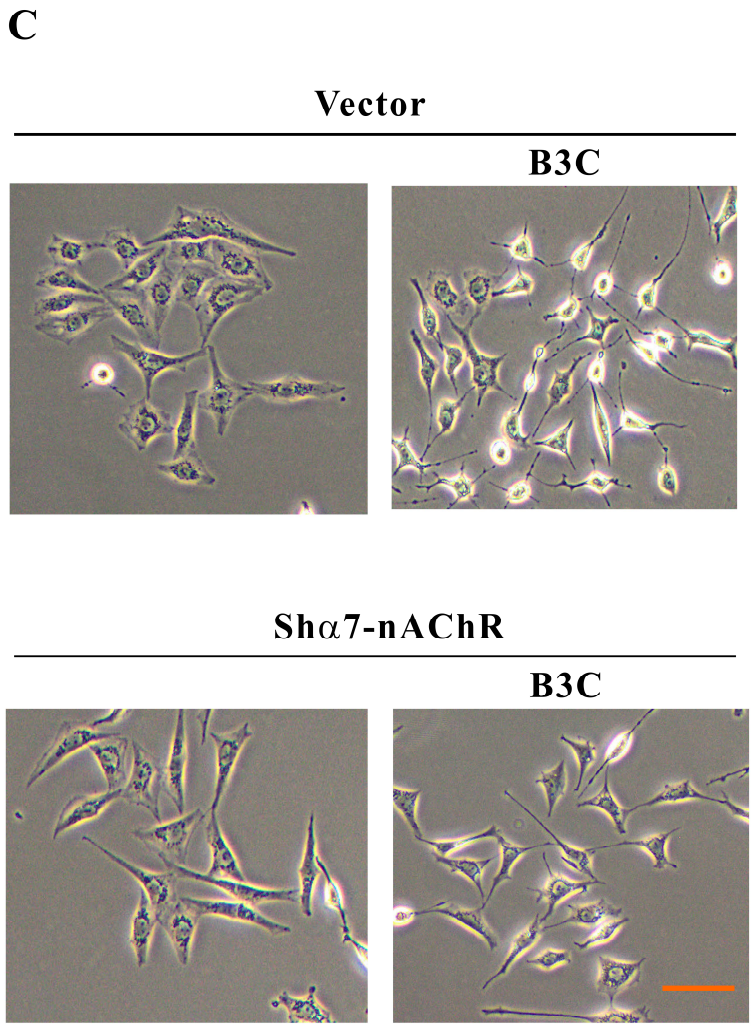

C
Vector
B3C
Shα7-nAChR
B3C

Fig. 3.3.7 α7-nAChR depletion partially abrogates the neurite outgrowth-promoting activities of B3C.

(A) PC12 cells were transiently transfected with pGPU6-green fluorescent protein (GFP) plasmid (vector) and pGPU6-GFP plasmid encoding α7-nAChR ShRNA (Shα7-nAChR ShRNA). GFP was evaluated by fluorescence microscopy to estimate transfection efficiency. The levels of α7-nAChR and β-actin in the cell lysates were tested by western blot using specific antibodies. (B, C) α7-nAChR depletion abolished the neurite outgrowth-promoting activity of B3C. PC12 cells transfected with vector or Shα7-nAChR were incubated with 3 μM B3C in low serum medium for 2 days, and the percentage of cells with neurites was counted using phase-contrast microscope. $^{\Delta}$, $p<0.05$, compared to vector group, **, $p<0.01$, compared to control group, $^{\#}$, $p<0.05$, compared to Shα7-nAChR group. Scale bar = 50 μm.

Chapter 4

Discussion

4.1 B3C protects against glutamate-induced apoptosis more potently than memantine via concurrent regulation of NO, ERK and PI3-K/Akt/GSK3β, downstream of NMDA receptors

Preparation of primary cultures of CGNs were first established 30 years ago (Gallo et al. 1982), and since then have been widely used in the study of neuroscience. Neurotoxicity triggered by small molecules, such as β-amyloid, tau protein, glutamate and oxidative stress, have been well characterized in cultured CGNs (Wigdal et al. 2002; Maycotte et al. 2010; Thellung et al. 2013). These molecules trigger the activation / inactivation of similar signaling pathways in CGNs as those in the hippocampal and cortical neurons (Contestabile, 2002). Moreover, over 95 % of the neurons in cerebellum belong to CGNs, it is much more convenient to obtain homogeneous CGNs from the dissected cerebellum than other homogenous neurons. CGNs were therefore used as a cell model in our research.

Excitatory neurotransmission mediated by the activation of NMDA receptors is of vital importance in the physiological processes in the mammalian CNS, however, over-stimulation of NMDA receptors triggers several pathological processes that underlie the pathogenesis of AD and other neurodegenerative diseases (Chen et al. 2008). As such, uncompetitive NMDA receptor antagonists with UFO property have been demonstrated to be effective and well tolerated for treating acute or chronic neurodegenerative diseases in which the neurotoxicity mediated by over-activation of NMDA receptors are involved (Lipton 2007a, 2007b). B3C (novel dimer synthesized in our team) and memantine (FDA-approved anti-AD drug), two well-characterized

uncompetitive NMDA receptor antagonists, were investigated for their neuroprotective effects and downstream signaling pathways in this research. In Section 3.1, it was found that B3C protected against glutamate-induced excitotoxicity in rat primary CGNs 10 times more potently than memantine, and the full neuroprotections of B3C and memantine were associated with the concurrent modulation of NO, ERK and PI3-K/Akt/GSK3β pathways, three downstream signaling pathways of NMDA receptors.

Although it has been demonstrated that B3C, similar to memantine, moderately antagonize NMDA receptors on the membrane with moderate potency and strong voltage dependence (Luo et al. 2010), little is known about the detailed mechanisms of the neuroprotective effects and the intracellular signaling pathways downstream of NMDA receptor against neurotoxins, such as glutamate. In the current study, by using the primary rat CGNs, it was observed that B3C protects neurons against 100 μM glutamate-induced excitotoxicity in a concentration-and time-dependent manner with an IC_{50} value of 0.45 μM (MTT assay) and 0.46 μM (LDH assay), respectively, an observation 10 times more potent than those produced by memantine (4.58 μM and 4.63 μM, respectively). In addition, B3C at 3 μM, which is equivalent to memantine at 30 μM, almost completely blocked the hallmarks of apoptosis induced by glutamate, as assayed by Hoechst staining and DNA fragmentation. Based on the fact that the most distinctive neuronal loss associated with AD is seen in the CA1 region of hippocampus, and that NMDA receptors show different subunits in various regions of the brain (hippocampus, cortex, cerebellum), it is much more valuable to investigate

the neuroprotective effects of B3C against glutamate-induced excitotoxicity in hippocampal neurons. Our previous study has shown that B3C effectively reverses middle cerebral artery occlusion (MCAO)-induced impairments in rats (Luo et al. 2010). A growing body of evidence has indicated that multiple targets including over-activation of NMDA receptors in hippocampus, striatum and cortex are implicated in MCAO-insulted rats (Luo et al. 2010; Zhao et al. 2012). We therefore speculate B3C may protect hippocampal neurons against glutamate–induced neuronal death. And we will directly analyze the neuroprotective effects of B3C in hippocampal neurons in our future study.

Why did B3C block glutamate-induced apoptosis at such a high potency (10 times more potent than memantine)? Presumably, there are several reasons. First, the neuroprotection ability produced by NMDA receptor antagonists is strongly associated with their ability in inhibiting NMDA-activated currents. It has been reported that B3C inhibits NMDA-activated currents with an IC_{50} value of 0.52 μM, an observation 3 times more potent than memantine (IC_{50}, 1.68 μM), which may be responsible for the better NMDA receptor blockage of B3C. The discrepancy between inhibition against the NMDA-activated current (B3C is 3 times more potent than memantine) and neuroprotection against glutamate excitotoxicity (B3C is 10 times more potent than memantine) may be mainly explained by the different *in vitro* model systems and / or different patterns of treatments with chemicals. Second, since B3C is derived from its monomer tacrine and has comparable AChE inhibition to tacrine, it is reasonably asked whether the powerful neuroprotection of B3C is produced by its

inhibition on AChE activity. We've studied this, and it turns out that the neuroprotection of B3C is independently associated with its AChE inhibitory effect, as two other AChE inhibitors (tacrine and donepezil) failed to block the neuronal death induced by glutamate. However, we could not exclude the possibility that B3C may affect other contributing targets in the membrane, cytoplasm, mitochondria or even nucleus. We have recently shown that bis(7)-tacrine, another tacrine dimer that has methylene linkers instead of 3 in B3C, provides full neuroprotection against glutamate-induced apoptosis through concurrent blockage of both NMDA receptor on the membrane and nNOS in the cytoplasm (Li et al. 2007), suggesting that direct inhibition of nNOS may contribute to the high potency of B3C. Further examinations are being conducted to validate this hypothesis.

It has been demonstrated that over-activation of NMDA receptors by excessive glutamate causes a massive influx of calcium into neurons, and subsequently initiates a series of pathological processes in cytoplasm, mitochondria and nucleus, and eventually destroy cellular components and result in neuronal death (Molinuevo et al. 2005; Hilton et al. 2006; Stanika et al. 2009). Therefore, NMDA receptor antagonists such as B3C and memantine are believed to inhibit this cascade of process through blocking the upstream target on the membrane. On the contrary, the intracellular signaling pathways have been yet unknown. Several lines of evidence have indicated that the activation of NO, MAPK pathways and inhibition of GSK3β pathway represent the main well-characterized downstream pathways of calcium and NMDA receptor and responsible for the intracellular signaling pathways in glutamate-induced

neurotoxicity (Stanciu et al. 2000; Montoliu et al. 2001; Chen et al. 2003; Li et al. 2007; Lee et al. 2008). However, the relationship among these three pathways remains still unknown. In our *in vitro* system, it is found that NOS inhibitor, MEK inhibitor and GSK3β inhibitor, at their efficacy, could only partially prevents against glutamate-induced excitotoxicity in CGNs, which is consistent with our previous reports (Pi et al. 2004; Li et al. 2005). Most importantly, the co-application of these three inhibitors provides full neuroprotection in CGNs, suggesting that these inhibitors of NOS, MEK1/2 and GSK3β might function in a separate parallel signaling pathway rather than in an upstream or downstream of a single cascade pathway to reverse glutamate-induced excitotoxicity. However, we have not excluded any uncharacterized side effects of these inhibitors that may contribute to the full neuroprotection.

The findings from pharmacological inhibition and western blot indicate that pre-treatment with B3C and memantine blocks a series of cellular pathological changes induced by glutamate, such as the abnormal increase of intracellular NO production, the activation of phosphorylated ERK protein, and the down-regulated phosphorylated Akt and GSK3β. These data taken together suggest that the two agents might provide full neuroprotection via concurrent modulation of NO, ERK and GSK3β pathways, the well-characterized downstream signaling pathways of NMDA receptor, to attenuate subsequent caspase cascades and rescue the dying neurons.

4.2 B3C blocks K^+ deprivation-induced apoptosis in CGNs via the activation of VEGFR-2

Apoptotic cell death is of vital importance and can be observed throughout the neural development (Nijhawan et al. 2000; Fu et al. 2008). On the contrary, unregulated excessive apoptosis correlates with the pathogenesis of AD and other neurodegenerative disorders. As such, agents with anti-apoptotic property may have therapeutic significance (Fu et al. 2008). In Section 3.2, it is found that B3C effectively blocks K^+ deprivation-induced apoptosis in rat CGNs in a concentration-dependent manner, and the neuroprotection of B3C is associated with the activation of VEGFR-2/Akt/GSK3β and VEGFR-2/ERK pathways.

With the use of CGNs, a well studied *in vitro* model for the study of neuroscience, it was observed that B3C dose-dependently blocked K^+ deprivation-induced apoptosis in CGNs with an IC_{50} value of 0.37 μM. Since B3C is synthesized through the dimerization of tacrine and has comparable AChE inhibition to tacrine, it is reasonably asked whether the robust neuroprotection of B3C is associated with its AChE inhibition. However, tacrine and donepezil (commonly used AChE inhibitors), failed to block the apoptosis induced by K^+ deprivation. Moreover, it has been reported the activation of cholinergic receptors could attenuate the apoptosis induced by various neurotoxins (Meyer et al. 2002). In our study, neither atropine nor dihydro-β-erythroidine was able to abolish the neuroprotective effect of B3C. Taken together, these results indicate that the inhibition of B3C against K^+ deprivation-induced apoptosis is independently associated with its AChE inhibition

and / or cholinergic transmission. In addition, MK801 and memantine, two commonly used NMDA receptor antagonists, hardly provided any neuroprotection against neuronal apoptosis induced by K^+ deprivation, suggesting that the neuroprotection of B3C is independently associated with its NMDA receptor antagonism.

Under K^+ deprivation condition, dissected CGNs depend on neurotrophic support to differentiate, develop and survive. For instance, VEGF has been recently demonstrated to be directly neurotrophic to cultured CNS neurons *in vitro* through the activation of VEGFR-2 (Keck et al. 1989), (Wick et al. 2002; Rosenstein et al. 2003), the major VEGF receptors widely expressed on neurons, *e.g.* CGNs (Zachary 2005). It has been reported that VEGF could be secreted by CGNs or the small quantity (5 %) of contaminated astrocytes and oligodendrocytes in the culture (Wick et al. 2002). Based on these, we hypothesize that B3C, through the modulation of VEGF/VEGFR-2 system either by enhancing the secretion of VEGF or by acting as a VEGFR-2 agonist, blocks the apoptosis induced by K^+ deprivation. In this current study, it is found that K^+ deprivation down-regulates the protein expression of phosphorylated VEGFR-2 in CGNs, and that B3C effectively reverse the activation of VEGFR-2 inhibited by K^+ deprivation. Moreover, the neuroprotective effect of B3C is partially abolished by PTK787/ZK222584, a specific VEGFR-2 inhibitor, suggesting that the neuroprotective effect is, at least, associated with the activation of VEGFR-2.

To further investigate the involvement of VEGFR-2 in the neuroprotective effect of B3C against K^+ deprivation, Akt and ERK pathways, two well-characterized critical pathways which act downstream of VEGFR-2 and involved in neuronasl

survival (Matsuzaki et al. 2001; Wick et al. 2002), have been examined in succession. For CGNs in particular, growth factors-mediated PI3-K/Akt pathway appears to be the predominant pro-survival pathway (Dudek et al. 1997). It is obvious that K^+ deprivation induces a rapid, reversible and time-dependent decrease in the phosphorylation, rather than protein expression, of Akt and GSK3β, which is consistent with other previous studies (Zhu et al. 2004; Zhong et al. 2007; Yeste-Velasco et al. 2009). However, B3C effectively reversed these activation of phosphorylated proteins inhibited by K^+ deprivation. Furthermore, both VEGFR inhibitor PTK/ZK and PI3-K inhibitor LY294002, partially abolished the neuroprotective effect and activation of VEGFR-2/Akt pathway induced by B3C, suggesting the neuroprotective effect of B3C is dependent on the VEGFR-2/Akt/GSK3β pathway.

Several studies have indicated that an early increase in the activation of ERK protein is related to the neuroprotective effect provided by extracellular factors to CGNs subjected to K^+ deprived medium (Bonni et al. 1999). On the other hand, some reports have argued that the delayed activation of ERK is associated with CGNs death in K^+ deprived medium (Subramaniam et al. 2003). As such, such neuroprotection against K^+ deprivation-induced apoptosis would probably depend on an early positive and a delayed negative modulation of ERK phosphorylation. In accordance with these, it is observed that treatment with B3C induced an early activation of ERK as demonstrated by the increase in the phosphorylation of ERK. Although it remains to be further elucidated whether B3C could inhibit the delayed increase in ERK

phosphorylation caused by K^+ deprivation, the fact that pharmacological inhibition of MEK1/2 significantly inhibited the increase in neuronal survival and phosphorylated ERK mediated by B3C clearly indicates that the neuroprotection of B3C against apoptosis induced by K^+ deprivation in CGNs is dependent on a MEK/ERK signaling pathway. Most importantly, the activation of ERK pathway mediated by B3C is obviously from the activation of VEGFR-2, as the VEGFR-2 specific inhibitor PTK/ZK partially abolishes the increased phosphorylated ERK caused by B3C.

Though the exact target or the interaction still remains to be further studied, results form our study has demonstrated that B3C effectively blocks K^+ deprivation-induced apoptosis in CGNs via the activation of VEGFR-2/Akt and VEGFR-2/ERK pathways, rather than the inhibition of AChE and the blockage of NMDA receptor. Based on the fact that activation of VEGFR-2 is mainly modulated by receptor dimerization and autophosphorylation after specific ligand binding (Holmes et al. 2007), we speculate that B3C provides neuroprotection through either directly interacting with VEGFR-2 as a potential agonist or indirectly facilitating the activation of VEGFR-2. B3C might stabilize the dimerization or increase the endogeneous 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. To confirm that B3C exerts neuroprotection through enhancing the endocrine secretion of VEGF, several experiments may needed as follows: a) evaluating the amount of secreted VEGF using ELISA; b) investigating the effects of

B3C on VEGF transcription using RT-PCR; c) examining the effects of B3C on VEGF translation using Western blot. The precise mechanisms of the neuroprotective effect of B3C would be revealed by our further studies.

Because of its neuroprotective property, VEGF has been recently considered to be a therapeutic agent against AD and other neurodegenerative diseases (Storkebaum et al. 2005). However, side effects such as pro-inflammation, pro-angiogenesis and pro-carcinogenesis limit its further development of anti-AD drugs (Takahashi and Shibuya 2005). Our findings that B3C offers robust neuroprotection via the activation of VEGFR-2/Akt and VEGFR-2/ERK pathways provide a new molecular insight into the therapeutic potential of B3C for preventing and treating neurodegenerative disorders.

4.3 B3C induces neuronal differentiation via the activation of α7-nAChR

Many lines of evidence underscore the tight link between neuronal loss and the pathogenesis of neurodegenerative disease, including in four of serious diseases Alzheimer's, Parkinson's, ALS and Huntington's (Rossler et al. 2002; Zilkova et al. 2006; Golde 2009; Marinova-Mutafchieva et al. 2009). Compounds that stimulate neuronal differentiation and regeneration would be of great importance for developing new therapeutics against these brain disorders. In Section 3.3, we investigated the effects of B3C on neurite outgrowth in PC12 cells and cortical neurons, and came up with several novel observations: 1), B3C, but not tacrine and memantine, induced neurite outgrowth in PC12 cells and cortical neurons in a concentration- and time-dependent manner. 2), the neurite outgrowth-promoting activity of B3C is associated with the sustained activation of ERK pathway possibly through the activation of α7-nAChR.

GAP-43 is a nervous tissue-specific cytoplasmic protein that displays elevated synthesis and axonal fast-transport during nerve regeneration and therefore, serve as a useful indicator of PC12 cell differentiation (Schimmelpfeng et al. 2004; Lai et al. 2011). In this research, in addition to the morphological changes, B3C induced neurite outgrowth with significant higher expression of GAP-43 protein after incubation for 7 days, which is comparable to those treatment with NGF or dbcAMP. Since B3C is a novel AChE inhibitor derived from tacrine and exerts uncompetitive NMDA receptor antagonism, it is reasonable to investigate whether B3C exerts its effects through

inhibition of AChE activity or NMDA receptors. However, tacrine (an AChE inhibitor) and memantine (an uncompetitive NMDA receptor antagonist), at their effective concentration, were unable to induce outgrowth of neurite, suggesting the AChE- and NMDA receptor-independent pathway in B3C-induced neurite outgrowth of PC12 cells.

It has been well revealed that mitogen-activated protein kinase (MAPKs) phosphorylation may trigger several protein signaling cascades and affect various cellular processes, such as neurite outgrowth. We therefore considered the involvement of MAPKs in B3C-induced neurite outgrowth of PC12 cells. It was found that ERK but not P38 and JNK specific inhibitors attenuated the percentage of cells with neurites induced by B3C, suggesting that ERK but not P38 and JNK pathways may be involved in B3C-induced neurite outgrowth in our model. Consistent with the morphological changes observed by microscope, B3C induced a rapid and reversible upregulation of ERK phosphorylation, peaking at 30 min, similar to that reported for NGF. However, the kinetics of ERK phosphorylation is slightly different because NGF causes a maximal increase in ERK phosphorylation at 5-30 min, and then returns to its basal level 2 h after the treatment (Santos et al. 2007), whereas B3C shows a more sustained activation of ERK phosphorylation that lasted for 4 h after induction. These differences may contribute to the synergistic effects on the neurite outgrowth of PC12 cells treated with the combination of B3C and NGF. It may also sustain and extend the induction time longer than NGF.

Since treatment of PC12 cells with a selective TrkA inhibitor, K252a, could not

abolished the neurite outgrowth-promoting activity induced by B3C, TrkA activation seems not to be involved in the response of cells to B3C. Several lines of evidence have demonstrated the involvement of α7-nAChR in the neuronal differentiation in PC12 cells (Resende et al. 2008; Nery et al. 2010). Upregulation of α7-nAChR activity allows the influx of Ca^{2+} which further activates the downstream signaling pathways, such as ERK. It was observed in our research that α7-nAChR specific inhibitors, MLA, partially abolished neurite outgrowth in parallel to ERK activation induced by B3C, which confirms the importance of α7-nAChR in the neurite outgrowth of PC12 cells and support ERK as a pro-differentiation pathway in PC12 cells. In parallel, gene knockdown of α7-nAChR abolished neurite outgrowth-promoting activity of B3C. However, we do not rule out other contributing targets in the membrane, cytoplasm and nucleus, such as G-protein coupled receptor or cAMP. To validate the hypothesis that B3C exerts neurite outgrowth activity via the direct activation of α7-nAChR, more molecule biology experiments, such as whole cell electrophysiological analysis, receptor-ligand binding assay, are being undertaken in our laboratory. The precise mechanism of these effects of B3C will be revealed by further studies.

Research has shown that the brains of people with Alzheimer's disease (AD) show a progressive loss of neurons that use acetylcholine (ACh) and glutamate as chemical messenger. The loss of these neurons is related to the severity of memory dysfunction that AD patients experience. Actually, the use of acetylcholinesterase (AChE) inhibitors and NMDA receptor antagonists for AD have been realized in the

use of donepezil, rivastigmine, galantamine and memantine in successful treatment of mild to moderate and moderate to severe AD, respectively.

Our previous studies have demonstrated that B3C, a novel dimer derived from tacrine, protects neurons against scopolamine (mAChR antagonist) and middle cerebral artery occlusion (the excitotoxicity is strongly associated with the over-activation of NMDA receptor)-induced brain damage in AD-related animal models (Luo et al. 2010; Han et al. 2012). More encouragingly, under NMDA receptor-mediated physiological conditions, B3C is able to enhance hippocampal long-term potentiation, a molecular basis of memory formation. These results, taken together, suggest that neuroprotection via multiple targets may represent the primary drug actions of B3C in AD patients and normal individual.

Neural stem cells (NSC) proliferate in the subventricular zone (SGZ) and subventricular zone (SVZ) of adult mammals. However, the number of endogenous neural stem cells is insufficient to prevent the insult of neurotoxins associated with AD. Transplantation of exogenous NSC may migrate into brain damaged areas and has thus been proposed as a promising therapeutic strategy in AD. Under this condition, agents that can promote or enhance neuronal differentiation might induce NSC differentiate into fully mature neurons in the micro-environments of the AD patients brain with amelioration of the memory deficits. It is therefore conjectured that neuronal differentiation might represent the primary drug actions of B3C when AD patients is in the therapy of transplantation of NSC.

4.4 Summary and conclusions

In the current study, B3C, a promising anti-Alzheimer's dimer, was found to provide multiple neuroprotections against glutamate and K^+ deprivation-induced apoptosis, as well as neuronal differentiation (Fig. 4.1).

(1) B3C (IC_{50}, 0.45 μM) prevented glutamate-induced excitotoxicity 10 times more potently than memantine (IC_{50}, 4.58 μM) in CGNs, as evidenced by cell viability and lactate dehydrogenase release assays. Additionally, B3C pretreatment could inhibit the increase of intracellular NO and the activation of p-ERK, and reverse the suppression of p-Ser-Akt and p-Ser9-GSK3β caused by glutamate. Furthermore, the neuroprotection of B3C was abolished by PI3-K inhibitor LY294002. Meanwhile, pharmacological inhibition showed that neither the single specific inhibitors of NOS (L-NMMA), MEK1/2 (U0126) and GSK3β (SB415286 and LiCl) nor the combinations of any two of them could fully protect against glutamate-induced apoptosis. However, the co-application of these three inhibitors produced nearly 100% inhibition of glutamate-induced apoptosis. Combining these and our previous studies, it is conjectured that B3C elicits neuroprotection against glutamate-induced neurotoxicity in CGNs through the blockage of NMDA receptor and subsequently concurrent inhibition of NO, ERK pathways and activation of PI3-K/Akt/GSK3β pathway.

(2) B3C remarkably blocked K^+ deprivation-induced apoptosis in CGNs with an IC_{50} value of 0.37 μM. However, the existing anti-Alzheimer's drugs, Aricept (donepezil) and ebixa (memantine), were unable to attenuate the apoptosis under the

same condition, indicating that the neuroprotection of B3C is independent of its AChE inhibition and NMDA receptor antagonism. A rapid decrease in p-Tyr1054-VEGFR-2 was observed after the switch of CGNs to K^+ deprived medium. B3C significantly reversed the inhibition of p-Tyr1054-VEGFR-2 in our model. Moreover, the Akt and ERK pathways, two critical pathways downstream of VEGFR-2, were also recovered from K^+ deprivation-induced inhibition by B3C. VEGFR-2 specific inhibitor PTK/ZK, as well as PI3-K inhibitor LY294002 and MEK inhibitor PD98059, each abolished the neuroprotection of B3C. Taken together, these results suggest that B3C blocks K^+ deprivation-induced apoptosis in CGNs possibly through regulating VEGFR-2/Akt/GSK3β and VEGFR-2/ERK signaling pathways but independent of AChE inhibition and NMDA receptor blockage.

(3) B3C substantially induced neurite outgrowth in a concentration- and time-dependent manner in PC12 cells, as evidenced by the increase in the percentage of cells with condensed cell bodies and extended neurites as well as expression of neuronal marker GAP-43. The neurite outgrowth-promoting activities were also observed in primary cultured cortical neurons, as evidenced by the increase in the length of βIII tubulin-positive neurites. In addition, B3C synergistically induced neurite outgrowth with NGF or dbcAMP. Furthermore, B3C rapidly and reversibly upregulated phosphorylation of ERK, a critical signaling molecule in neuronal differentiation. Specific inhibitors of MEK and α7-nAChR, but not those of P38 and JNK, blocked the neurite outgrowth as well as the activation of ERK induced by B3C. Concordantly, gene knockdown of α7-nAChR using short hairpin RNA (ShRNA)

abolished the neurite outgrowth-promoting activity of B3C. These results taken together suggest that B3C induces neurite outgrowth possibly through activation of α7-nAChR and subsequent ERK pathway.

(4) In conclusion, B3C provided multiple neuroprotections through a) the blockage of NMDA receptor and subsequently concurrent regulation of NO, ERK pathways and PI3-K/Akt/GSK3β pathway; b) the activation of VEGFR-2/Akt/GSK3β and VEGFR-2/ERK signaling pathways. In addition, B3C promoted neurite outgrowth in PC12 cells via the activation of α7-nAChR. Combining these novel findings with our previous studies, we propose that B3C could provide great therapeutic efficacy by concurrently targeting different sites in the brain of patients with neurodegenerative diseases. To be specific, as the synergism between anti-AChE, anti-$GABA_A$ receptor, anti-NMDA receptor, anti-apoptosis and pro-neuronal differentiation might serve as the most effective therapeutic strategy for prevention and treatment and even modification of pathological processes of neurodegenerative diseases, our findings not only provide the molecular insights and detailed mechanisms by which B3C may have therapeutic significance, but also offer novel insights into the molecular basis for the development of potent therapeutic strategies for neurodegenerative diseases.

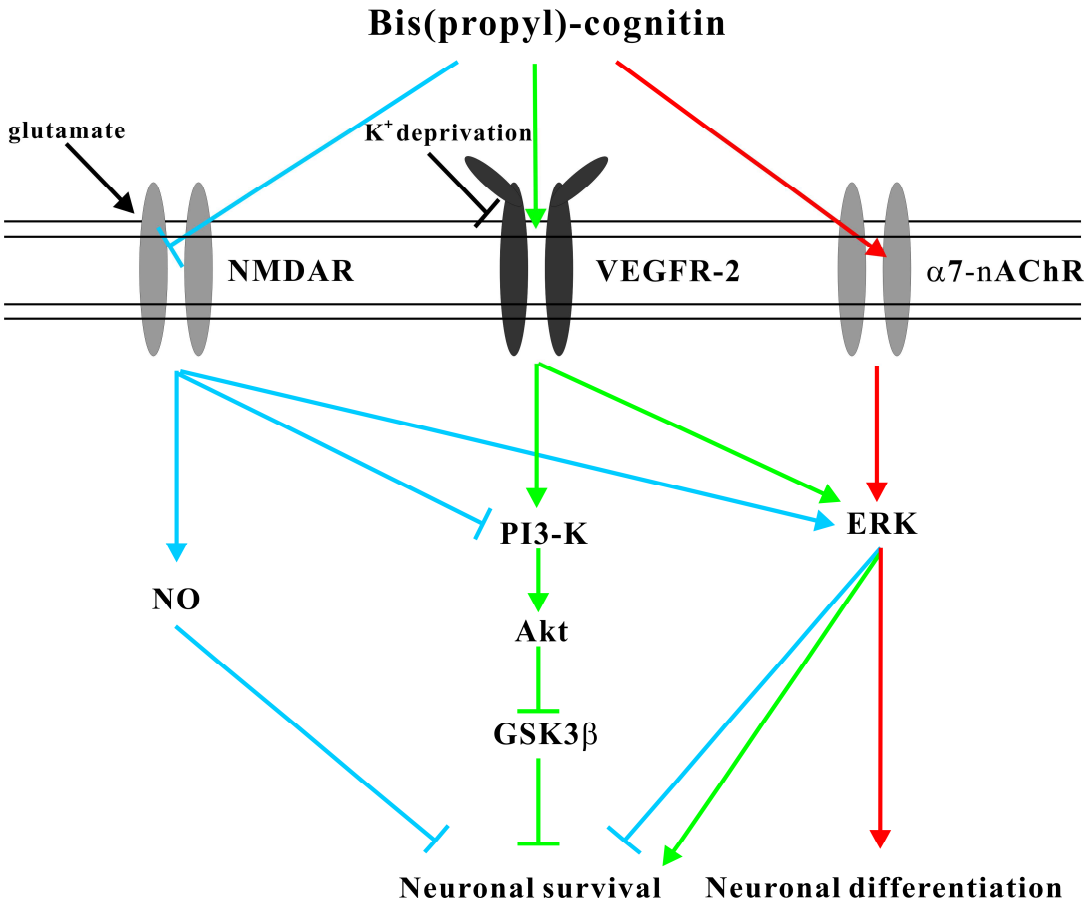


Fig. 4.1 The molecular mechanisms underlying the multiple activities of B3C.

Signaling transduction pathways involved in glutamate-induced excitotoxicity are depicted by blue lines, those involved in K^+ deprivation-induced apoptosis are indicated by the green lines, and those involved in B3C-mediated neuronal differentiation are represented by the red lines.

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