

Substantial Neuroprotective and Neurite Outgrowth-Promoting Activities by Bis(propyl)-cognitin via the Activation of Alpha7-nAChR, a Promising Anti-Alzheimer's Dimer

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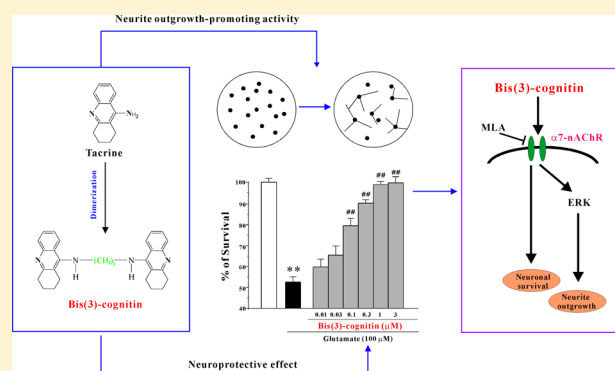
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ABSTRACT: The cause of Alzheimer's disease (AD) could be ascribed to the progressive loss of functional neurons in the brain, and hence, agents with neuroprotection and neurite outgrowth-promoting activities that allow for the replacement of lost neurons may have significant therapeutic value. In the current study, the neuroprotective and the neurite outgrowth-promoting activities and molecular mechanisms of bis(propyl)-cognitin (B3C), a multifunctional anti-AD dimer, were investigated. Briefly, B3C (24 h pretreatment) fully protected against glutamate-induced neuronal death in primary cerebellar granule neurons with an IC_{50} value of $0.08 \mu\text{M}$. The neuroprotection of B3C could be abrogated by methyllycaconitine, a specific antagonist of alpha7-nicotinic acetylcholine receptor ($\alpha 7$ -nAChR). In addition, B3C significantly promoted neurite outgrowth in both PC12 cells and primary cortical neurons, as evidenced by the increase in the percentage of cells with extended neurites as well as the up-regulation of neuronal markers growth-associated protein-43 and β -III-tubulin. Furthermore, B3C rapidly upregulated the phosphorylation of extracellular signal-regulated kinase (ERK), a critical signaling molecule in neurite outgrowth that is downstream of the $\alpha 7$ -nAChR signal pathway. Specific inhibitors of ERK and $\alpha 7$ -nAChR, but not those of p38 mitogen-activated protein kinase and c-Jun NH(2)-terminal kinase, blocked the neurite outgrowth as well as ERK activation in PC12 cells induced by B3C. Most importantly, genetic depletion of $\alpha 7$ -nAChR significantly abolished B3C-induced neurite outgrowth in PC12 cells. Taken together, our results suggest that B3C provided neuroprotection and neurite outgrowth-promoting activities through the activation of $\alpha 7$ -nAChR, which offers a novel molecular insight into the potential application of B3C in AD treatment.

KEYWORDS: Alzheimer's disease, bis(propyl)-cognitin, neuroprotection, neurite outgrowth, alpha7-nicotinic acetylcholine receptor, extracellular signal-regulated kinase



Neuroprotective effect

As the population ages, people are more likely to get Alzheimer's disease (AD), which is the most common neurodegenerative disorder among the elderly in the world. The ultimate cause of AD can be ascribed to a progressive and severe loss of functional neurons in the brain, which correlate with the neurological dysfunctions in the nervous system.^{1,2} As such, neuroprotection against the neurotoxins in combination with induction of neurite outgrowth that allow for the replacement of lost neurons might represent potential strategies for AD treatment.

Although the exact mechanism of AD is far from being fully elucidated, glutamate-induced excitotoxicity is believed to play a vital role in AD pathogenesis.³ Excessive glutamate overstimulates N-methyl-D-aspartate (NMDA) receptor on the membranes of postsynaptic neurons, subsequently causes the rapid influx of Ca^{2+} and eventually results in mitochondrial

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dysfunction and neuronal death.⁴ Actually, the use of uncompetitive NMDA receptor antagonists for neurodegenerative disorders has been realized in the use of memantine in successful treatment of moderate-to-severe AD.⁵ In addition, the magnitude of cognitive impairment observed as AD progresses from mild-to-moderate stages correlates well with the degree of loss of nicotinic acetylcholine receptors (nAChRs),⁶ particularly $\alpha 7$ -nAChR ($\alpha 7$ -nAChR), which is highly expressed in brain regions associated with learning and memory. Furthermore, recent evidence has convincingly shown that activation of $\alpha 7$ -nAChR is another important underlying mechanism in antagonizing excitotoxic events such as damage to cortical neurons and cerebellar granule neurons (CGNs) observed after exposure to glutamate.^{7,8} $\alpha 7$ -nAChR is, therefore, considered as an important target for AD.

Primary cultures of CGNs were established a few decades ago and since have become one of the useful in vitro models to study almost every aspect of developmental or pathological neurobiology.⁹ And more notably, CGNs stably expressing both NMDA receptors and $\alpha 7$ -nAChR are widely applied for the study of molecular mechanisms against a variety of neurotoxins, typically glutamate.^{3,10} It has been reported that neuroprotectants often employ different underlying mechanisms against glutamate, depending on their pretreatment time (acute or chronic pretreatment). For instance, 2 h pretreatment of CGNs with bis(heptyl)-cognitin, a dimeric acetylcholinesterase (AChE) inhibitor synthesized in our lab, protected against glutamate-induced excitotoxicity mainly through its NMDA receptor antagonism.¹¹ On the contrary, 48 h pretreatment with donepezil prevented glutamate-induced neuronal death in cortical neurons involving the stimulation of $\alpha 7$ -nAChR and subsequent internalization of NMDA receptors.⁸ Pretreatment with bis(12)-hupyrindone, a novel dimeric acetylcholinesterase (AChE) inhibitor derived from huperzine A, for 24 h protected CGNs against glutamate-induced excitotoxicity via the activation of $\alpha 7$ -nAChR/Akt pathway.¹²

PC12 cells and primary cortical neurons have been extensively used as in vitro model systems for the study of neurite outgrowth.^{13–15} Exposure of PC12 cells to nerve growth factor (NGF) induces differentiation into neuronal phenotype similar to those found in sympathetic neurons, characterized by long-term and stable neurite outgrowth.¹⁶ Recently, it has been reported that $\alpha 7$ -nAChR plays pivotal roles in neurite outgrowth.^{17,18} Upon $\alpha 7$ -nAChR activation, PC12 cells displayed the extended neurites and differentiation-like formation, as well as sustained activation of extracellular signal-regulated kinase (ERK) pathway,¹⁹ while $\alpha 7$ -nAChR knockout may result in the lack of maturation of dendritic neurons,²⁰ all are indicative of the involvement of $\alpha 7$ -nAChR for neurite outgrowth.

Bis(propyl)-cognitin (B3C), together with homologues in which two tacrine moieties were linked by spacers which varied in the number of methylene ($-\text{CH}_2-$) groups, were originally synthesized by our team as novel bifunctional AChE inhibitors that interacted with dual binding sites within the catalytic cavity of AChE.²¹ We have previously investigated the therapeutic effects of B3C in various in vivo models associated with AD and other related neurodegenerative disorders in which neurogenesis impairment is involved. More specifically, B3C could efficiently reverse the spatial and recognition memory deficits in mice induced by scopolamine.²² B3C was also demonstrated to substantially protect dopaminergic neurons and ameliorate Parkinsonian motor defects in mice induced by 1-methyl-4-

phenyl-1,2,3,6-tetrahydropyridine.²³ Furthermore, B3C was found to effectively protect against middle cerebral artery occlusion (MCAO)-induced brain damage in rats,³ and potentiate rehabilitation of treadmill exercise after MCAO.²⁴ Taken together, these in vivo results strongly suggest that B3C could have great usefulness in the prevention and treatment of neurodegenerative diseases.

Furthermore, to better probe the mechanisms underlying the neuroprotection of B3C observed in these in vivo models, a variety of molecular and cellular studies was conducted in succession. The antineurodegenerative effects of B3C have been proved by us to be associated with its acetylcholinesterase inhibition,²¹ myocyte enhancer factor-2D (MEF2D) enhancement,²³ uncompetitive *N*-methyl-D-aspartate receptor antagonism³ and vascular endothelial growth factor receptor activation abilities.²⁵

In the current study, we extended our effort in evaluating B3C-mediated neuroprotective and neurite outgrowth-promoting activities, in the hope that we could seek more novel molecular insights into the potential application of B3C. Encouragingly, it was shown that B3C provided remarkable neuroprotection against glutamate-induced excitotoxicity in primary CGNs, and promoted substantial neurite outgrowth in both PC12 cells and primary cortical neurons, possibly through the activation of $\alpha 7$ -nAChR.

RESULTS AND DISCUSSION

Accumulating lines of evidence underscore the tight link between the neuronal loss and the pathogenesis of neurodegenerative disease, including AD in particular.^{3,26,27} Compounds that protect neurons and stimulate neurite outgrowth would be of great importance for developing new therapeutics against these brain disorders. In the current study, we investigated the effects and molecular mechanisms by which B3C provided substantial neuroprotective and neurite outgrowth-promoting activities in various cell models.

Pretreatment with B3C for 24 h Protects against Glutamate-Induced Excitotoxicity in CGNs via the Activation $\alpha 7$ -nAChR. We have previously established a primary cell model in which excitotoxicity is induced by glutamate in CGNs.²⁷ Since B3C is a novel dimer derived from tacrine and proved to be a potent NMDA receptor antagonist,³ in the current study, we investigated and compared the neuroprotections of B3C, tacrine (the monomer of B3C) and MK801 (an NMDA receptor antagonist) against glutamate-induced excitotoxicity in terms of different lengths of pretreatment time. At 8 DIV, CGNs were pretreated with B3C, tacrine and MK801 2 or 24 h before 100 μM glutamate insult. The dose–response curves were plotted after incubation with these compounds. As shown in Figure 1A, 2 h pretreatment with B3C and MK801, but not tacrine, almost fully protected CGNs against glutamate-induced excitotoxicity, suggesting that the neuroprotection is primarily dependent on the NMDA receptor blockage.²⁷ Interestingly, after 24 h pretreatment, the dose–response curve of B3C markedly shifted to the left (IC_{50} from 0.45 to 0.08 μM), while that of MK801 remained unchanged (Figure 1B), suggesting B3C may protect CGNs through other targets in addition to NMDA receptors. Under the same condition, 24 h pretreatment with tacrine also mildly prevented the death of CGNs with a IC_{50} value of 2.12 μM , an observation consistent with previous studies.^{28,29}

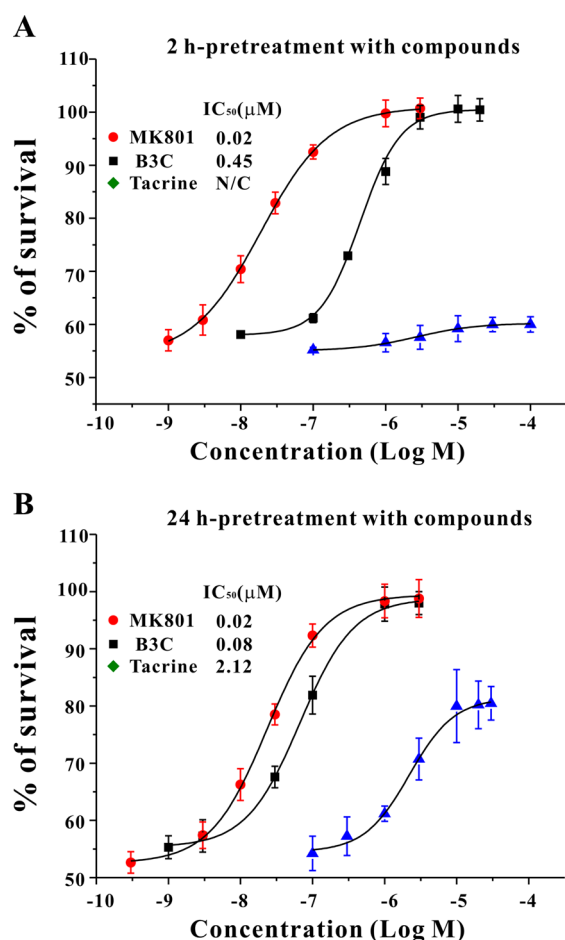


Figure 1. Pretreatment with B3C for 2 and 24 h prevents the death of CGNs induced by glutamate. At 8 DIV, CGNs were pretreated with gradually increasing doses of MK801, B3C, or tacrine 2 h (A) or 24 h (B) before the addition of 100 μM glutamate. Twenty-four hours after glutamate challenge, CGNs were subjected to MTT assay.

B3C is a potent inhibitor of AChE and it is unclear whether the neuroprotections of B3C (24 h pretreatment) were produced by its inhibition on AChE activity and subsequent activation of AChRs. Therefore, atropine (an mAChR antagonist), and mecamylamine and tubocurarine (nAChR antagonists), were used in our model. It was found that 10 μM mecamylamine and tubocurarine, but not atropine, abolished the neuroprotection of B3C (Figure 2A). Moreover, when MLA (an α7-nAChR antagonist) and DHβE (an α4β2-nAChR antagonist) were employed in this model, MLA, but not DHβE, abrogated the neuroprotection induced by B3C (Figure 2B), suggesting that B3C protected against glutamate-induced excitotoxicity via the activation of α7-nAChR. Additionally, the neuroprotection of tacrine was mediated through an α7-nAChR-independent pathway as suggested by earlier studies.²⁸

It is suggested that there are at least two molecular mechanisms underlying neuroprotections, including the reduction of the neurotoxic system and the up-regulation of a defensive system. For the former mechanism, neurotoxicity induced glutamate involves the excessive activation of NMDA receptors and subsequent Ca²⁺ influx.^{4,11} B3C was shown to fully protect against glutamate-induced excitotoxicity through the blockage of NMDA receptors and subsequent proapoptotic pathway.³ For the latter mechanism, α7-nAChR activation would initiate subsequent PI3-K/Akt pathway, or promote

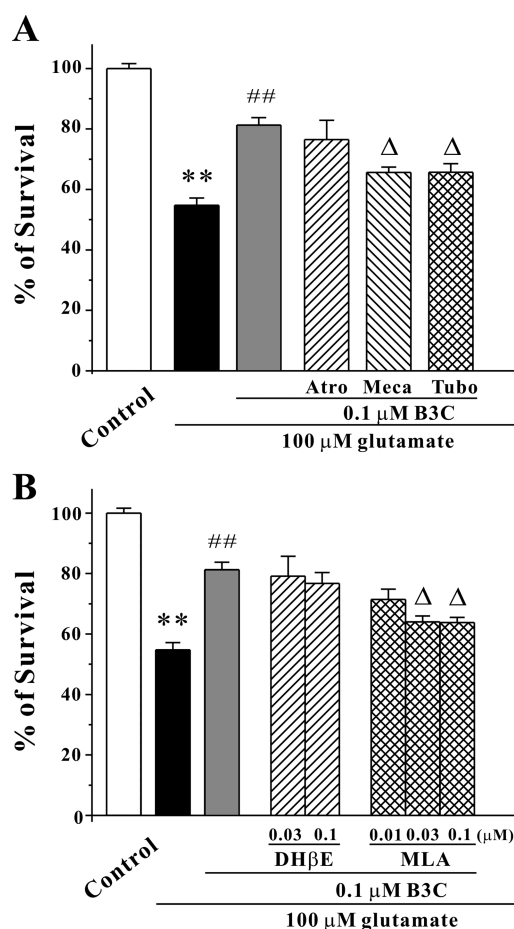


Figure 2. Inhibition of α7-nAChR partially abrogates the neuroprotection of B3C against glutamate-induced neuronal death. (A) At 8 DIV, CGNs were pretreated with atropine (Atr), mecamylamine (Meca), or tubocurarine (Tubo) for 2 h, then incubated with 0.1 μM B3C for 24 h, and finally incubated with 100 μM glutamate. Twenty-four hours after glutamate challenge, CGNs were subjected to MTT assay. (B) CGNs were pretreated with MLA or DHβE for 2 h, then incubated with B3C for 24 h prior to the addition of 100 μM glutamate. Twenty-four hours after glutamate challenge, CGNs were subjected to MTT assay. ***p* < 0.01, compared to control group; ##*p* < 0.01, compared to glutamate group; Δ*p* < 0.05, compared to B3C group.

NMDA receptor internalization via phosphorylation of its core receptor subunit (NR1) suggested by earlier studies.^{8,12} B3C may provide neuroprotection against glutamate through the activation of α7-nAChR and subsequent pro-survival pathway.

B3C Promotes Neurite Outgrowth in PC12 Cells and Primary Cortical Neurons.

In addition to neuroprotective drugs, induction of neurite outgrowth-based therapy also has broad prospects against neurodegenerative disorders.³⁰ Both NGF and dbcAMP that are strong inducers of neurite outgrowth in PC12 cells were served as positive controls in our neuronal model. PC12 cells were incubated with various compounds that were renewed every other day (Figure 3A). 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 (Figure 3B), an observation consistent with several previous studies.^{18,31} B3C, but not tacrine, remarkably promoted neurite outgrowth of PC12 cells in a concentration- and time-dependent manner (Figure 3B and C), with maximum percentage of 63.7 ± 2.0% at the concentration of

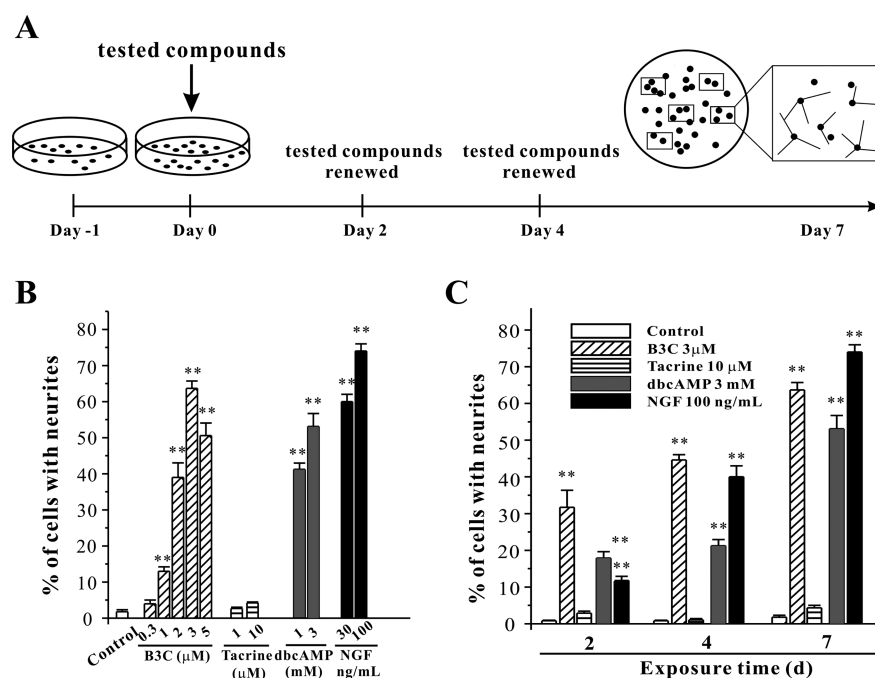


Figure 3. Induction of neurite outgrowth in PC12 cells by B3C. (A) Schematic diagram shows the timeline and general procedures for assessment of the neurite outgrowth-promoting activity of various compounds in PC12 cells. Cells were plated on day -1 , then treated with various compounds that were renewed every 2 days on day 0, and morphologically quantified on day 2, day 4, or day 7. The cells were scored as differentiated if at least one neurite was longer than the diameter of cell body observed in at least six 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 switched to low serum medium containing various compounds 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 3 μ M B3C, 10 μ M tacrine, 3 mM dbcAMP, or 100 ng/mL NGF for different lengths of time, and the percentage of cells with neurites was then measured. $**p < 0.01$, compared to control group.

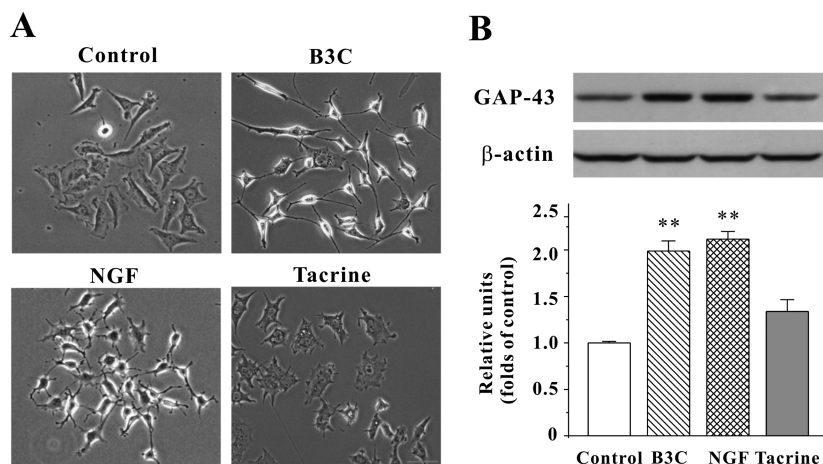


Figure 4. Differentiation of PC12 cells toward a neuronal phenotype induced by B3C. (A) Morphological characteristics of neurite outgrowth in PC12 cells induced by B3C. PC12 cells were incubated with in low serum medium containing 3 μ M B3C, 100 ng/mL NGF or 10 μ M tacrine in low serum medium for 7 days, and cell morphology was observed using phase-contrast microscopy and photographed by the digital camera. Scale bar = 50 μ m. (B) B3C upregulated the protein expression of GAP-43 in PC12 cells. PC12 cells were incubated with various compounds shown as in (A) 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 μ M. It was observed that PC12 cells treated with B3C displayed typical differentiated morphologies of condensed cell bodies and extended neurite outgrowths, in contrast to the flattened cell bodies of undifferentiated cells in the vehicle control (Figure 4A).

Besides morphological observation, biochemical examination is also an effective method for evaluating neurite outgrowth.

GAP-43 is a neuron-specific protein that exhibit elevated synthesis and fast axonal transport during neuronal development. Overexpression of GAP-43 in PC12 cells and transgenic mice was reported to potentiate NGF-induced neurite outgrowth³⁶ and induce neuronal sprouting,³² respectively. On the contrary, depletion of GAP-43 remarkably resulted in shortened neurites, collapsed growth cone and abnormal axonal

pathfinding.³³ Thus, GAP-43 is considered as a major and useful indicators of PC12 cell neurogenesis. In our study, with the assay of Western blotting analysis, the protein expression of GAP-43 in PC12 cells treated by B3C increased by approximately 100%, an observation comparable to that provided by NGF (Figure 2B), again suggesting that B3C has a robust neurite outgrowth-promoting effect.

Most encouragingly, we further confirmed the neurite outgrowth-promoting activity of B3C in primary rat cortical neurons, which is an important primary cell model that has been frequently employed by earlier studies.^{34,35} Two days treatment with B3C (0.1 and 0.3 μM) significantly promoted neurite outgrowth in cortical neurons, as compared with the vehicle control (Figure 5A). Immunostaining-based quantita-

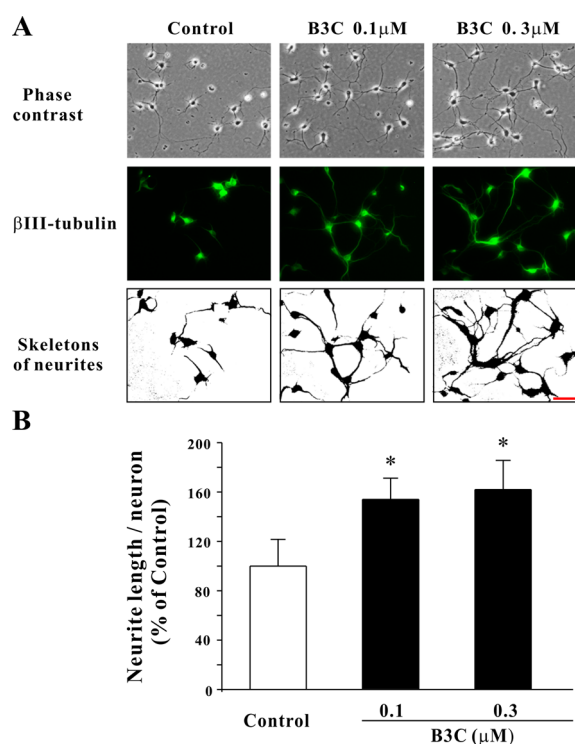


Figure 5. Promotion of neurite outgrowth in rat cortical neurons by B3C. (A) Cortical neurons were treated at 3 DIV in the absence or presence of B3C for 2 days. Upper: Phase-contrast digital images of the cells were taken using a phase-contrast microscope. Middle: Neuronal cultures were stained with anti- β III-tubulin antibody. Lower: β III-tubulin-positive neurites were digitally identified and skeletonized for quantification by NeuriteTracer program. Scale bar = 50 μm . (B) Cortical neurons were treated at 3 DIV in the absence or presence of B3C for 2 days, neurons were then fixed and quantitatively analyzed for the length of β III-tubulin-positive neurites by using NeuriteTracer program. * $p < 0.05$, compared to control group.

tive study also demonstrated that treatment with B3C resulted in a concentration-dependent increase in the length of β III tubulin (another indicator for neurite outgrowth)-positive neurites (Figure 5B).

To further understand the proneurogenesis property of B3C in vivo, neurogenesis within the adult central nervous system is being actively evaluated in our lab by using an exogenous cell tracer, BrdU, in combination with endogenous neuronal markers (e.g., doublecortin).

B3C Promotes Neurite Outgrowth via Activation of ERK Pathway in 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, including neurite outgrowth.^{36,37} To determine whether ERK, p38 mitogen-activated protein kinase and/or c-Jun NH(2)-terminal kinase (JNK) signaling pathways were involved in the neurite outgrowth-promoting activities induced by B3C, the specific inhibitors of ERK (PD98059 and U0126), P38 (SB203580), and JNK (SP600125), were selected to pretreat PC12 cells 2 h before the addition of B3C. It was found that PD98059 and U0126, but not SB203580 or SP600125, partially abolished the outgrowth of neurites induced by B3C (Figure 6A). To further confirm whether

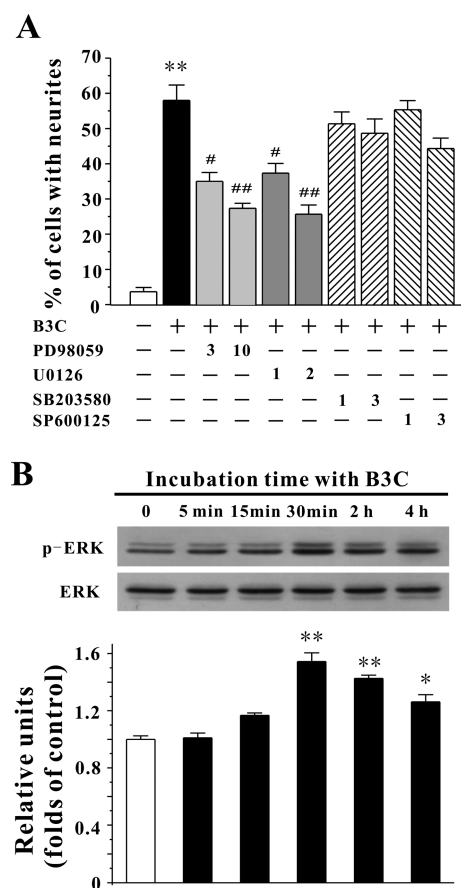


Figure 6. Activation of ERK pathway is involved in B3C-mediated PC12 cell neurite outgrowth (A) MEK inhibitors, but not P38 and JNK inhibitors, partially attenuated the neurite outgrowth induced by B3C in PC12 cells. Cells were pretreated with specific inhibitors (PD98059, U0126, SB203580, and SP600125) 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; # $p < 0.05$, ## $p < 0.01$, 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 lengths of time, and the total proteins were subjected to Western blot analysis using anti-p-ERK and anti-ERK antibodies. * $p < 0.05$ and ** $p < 0.01$, compared to control group.

B3C induced neurite outgrowth of PC12 cells through the activation of ERK pathway, the expression of phospho-ERK was measured by Western blotting analysis. It was evident that B3C upregulated the expression of phospho-ERK, which peaked at 30 min and lasted for 4 h after treatment (Figure 6B).

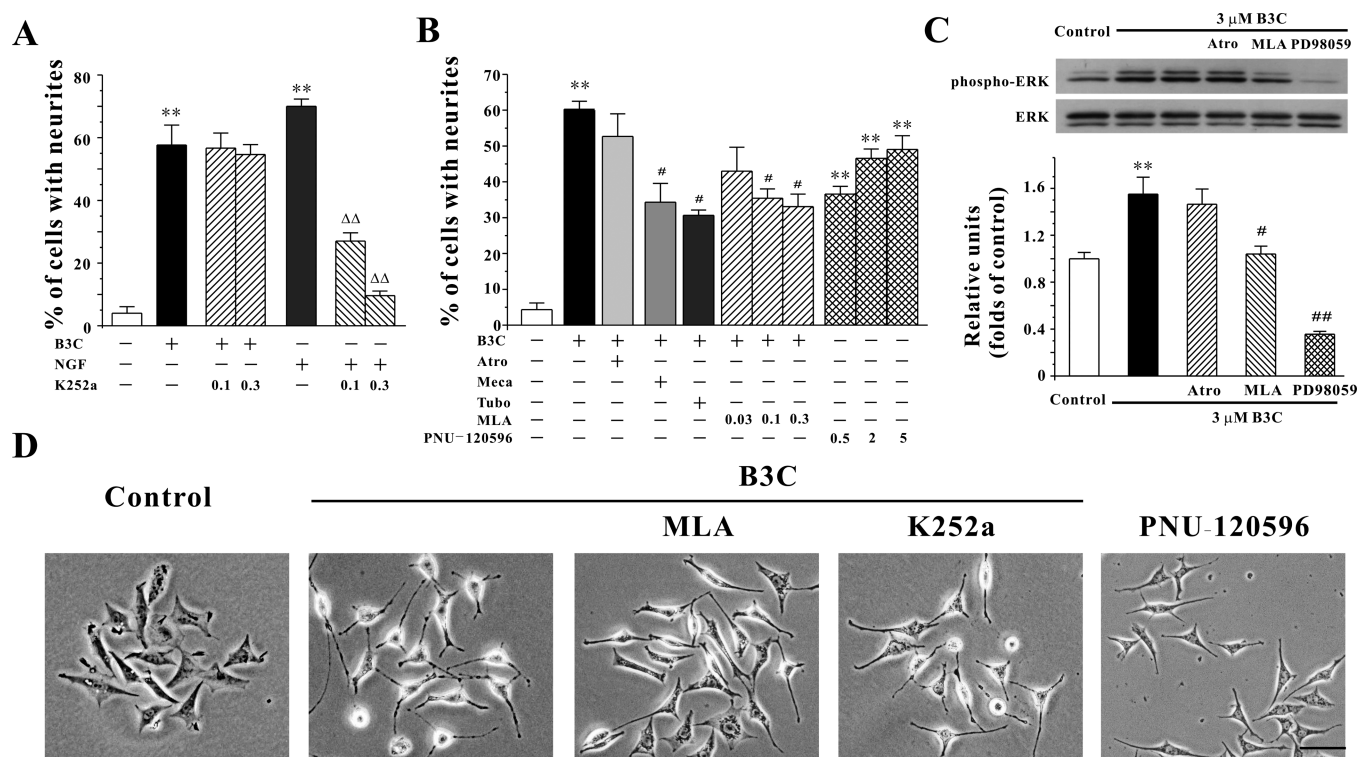


Figure 7. Pharmacological blockage of $\alpha 7$ -nAChR partially abolishes the B3C-induced neurite outgrowth in PC12 cells (A) Trk A specific inhibitor failed to attenuate neurite outgrowth induced by B3C in PC12 cells. Cells were pretreated with K252a (0.1, 0.3 μ M) for 2 h in low serum medium, and then incubated with 3 μ M 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 $\alpha 7$ -nAChR partially abolished neurite outgrowth induced by B3C in PC12 cells. Cells were pretreated with Atro (10 μ M), Meca (10 μ M), Tubo (10 μ M), and MLA (0.03, 0.1, 0.3 μ M) for 2 h before the addition of 3 μ M B3C, or cells were incubated with PNU-120596 (0.5–5 μ M) in low serum medium. 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 $\alpha 7$ -nAChR attenuated the activation of p-ERK induced by B3C in PC12 cells. Cells were pretreated with atropine, mecamylamine and MLA for 2 h in low serum medium, and then incubated with 3 μ M 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; # $p < 0.05$, ## $p < 0.01$, compared to B3C group. (D) Morphological characteristics of neurite outgrowth in PC12 cells treated with specific inhibitors or PAM of $\alpha 7$ -nAChR. Cells were pretreated with PD98059, MLA, and K252a for 2 h before the addition of B3C, or cells were incubated with PNU-120596 (0.5–5 μ M) in low serum medium. Seven days later, cell morphology was then observed using phase-contrast microscopy and photographed by a digital camera. Scale bar = 50 μ m.

Most analyses that use reductionist systems such as PC12 cells to identify pathways governing neurite outgrowth have focused on the ERK pathway.^{36,38} “Sustained” activation of ERK (hours), such as typically seen for fibroblast growth factor (FGF) or NGF, would be the signal that specifies a neuritogenic outcome as opposed to the “transient” (<1 h) ERK induction associated with non-neuritogenic factors such as epidermal growth factor (EGF).^{38,39} Our findings that B3C elicited a sustained ERK activation (4 h) indicate that B3C may act as a neuritogenic inducer. And more notably, the kinetics of ERK phosphorylation of B3C 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,⁴⁰ suggesting that B3C and NGF may employ different mechanisms in enhancing neurite outgrowth.

Pharmacological Blockage of $\alpha 7$ -nAChR Abolishes B3C-Induced Neurite Outgrowth in PC12 Cells. To determine whether B3C-induced neurite outgrowth was through the regulation of NGF/TrkA system, K252a, the specific inhibitor of Trk A, was selected to pretreat PC12 cells 2 h before the addition of B3C. It was observed that K252a at its generally accepted effective concentrations did not block neurite outgrowth induced by B3C, despite it could block the

neurite outgrowth induced by NGF (Figure 7A). It has been revealed that activation of AChRs are implicated in neurite outgrowth.¹⁸ B3C inhibits the activity of AChE and indirectly improves the increase of concentrations of acetylcholine in vitro and in vivo, which may contribute to the therapeutic effects against AD. It has also been reported that PC12 cells stably expresses AChE, synthesizes and secretes acetylcholine.⁴¹ Therefore, to test whether B3C could exert neurite outgrowth-promoting activity via signal transduction of AChRs, atropine, mecamylamine and tubocurarine were selected to treated PC12 cells 2 h before the addition of B3C. It was shown that 10 μ M tubocurarine and 10 μ M mecamylamine, but not 10 μ M atropine partially abolished the outgrowth of neurite induced by B3C (Figure 7B). Moreover, MLA partially inhibited the outgrowth of neurite induced by B3C (Figure 7D). Furthermore, 2 h of pretreatment of PC12 cells with MLA as well as PD98059, but not atropine, significantly inhibited the induced elevated level of phospho-ERK (Figure 7C), suggesting that B3C induced neurite outgrowth via the activation of $\alpha 7$ -nAChR. Tacrine did not affect $\alpha 7$ -nAChR as suggested by earlier studies,²⁸ which may explain why tacrine did not promote neurite outgrowth in PC12 cells (Figure 3B and C).

Genetic Depletion of $\alpha 7$ -nAChR Abrogates the B3C-Induced Neurite Outgrowth in PC12 Cells. To further confirm the neurite outgrowth-promoting activity of B3C through activating $\alpha 7$ -nAChR, short hairpin (ShRNA)-mediated genetic inhibition was used. $\alpha 7$ -nAChR ShRNA (Sh $\alpha 7$ -nAChR) caused a significant reduction in $\alpha 7$ -nAChR protein level, whereas the negative control ShRNA (ShNC) and vector had no effect on $\alpha 7$ -nAChR protein level (Figure 8A). 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 $\alpha 7$ -nAChR knockdown PC12 cells was no longer able to induce neurite outgrowth (Figure 8B).

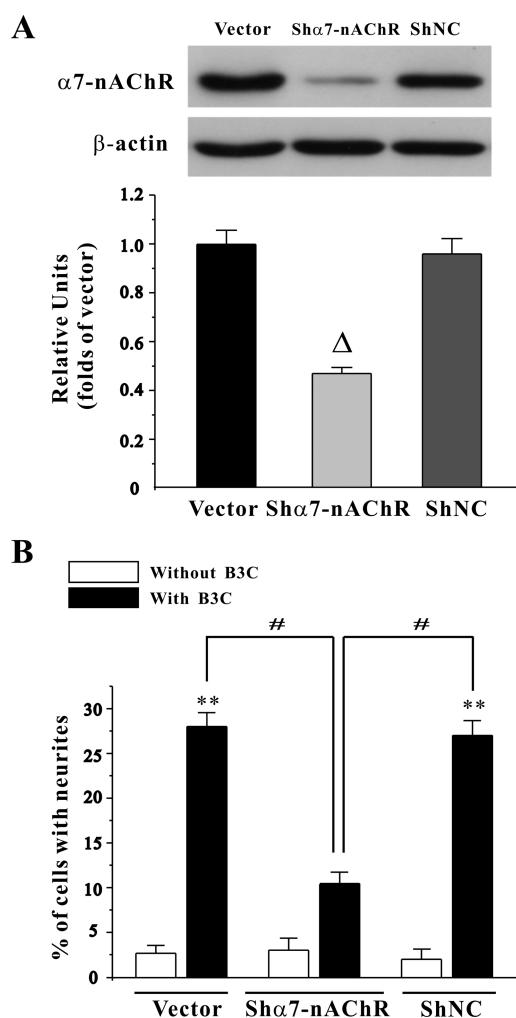


Figure 8. Genetic depletion of $\alpha 7$ -nAChR partially abolishes the B3C-induced neurite outgrowth in PC12 cells. (A) PC12 cells were transiently transfected with pGPU6-green fluorescent protein (GFP) plasmid (vector) and pGPU6-GFP plasmid encoding $\alpha 7$ -nAChR ShRNA (Sh $\alpha 7$ -nAChR ShRNA). GFP was evaluated by fluorescence microscopy to estimate transfection efficiency. The levels of $\alpha 7$ -nAChR and β -actin in the cell lysates were tested by Western blot using specific antibodies. (B) $\alpha 7$ -nAChR depletion abolished the neurite outgrowth-promoting activity of B3C. PC12 cells transfected with vector or Sh $\alpha 7$ -nAChR were incubated with 3 μ M 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 Sh $\alpha 7$ -nAChR group.

Pharmacological inhibition of nicotinic acetylcholine receptors using specific inhibitors (Meca, Tubo, and MLA) and genetic depletion of $\alpha 7$ -nAChR could not completely abolish the neurite outgrowth-promoting effects in PC12 cells produced by B3C, suggesting other possible mechanisms independent of nicotinic acetylcholine receptors may underlie the neurotogenic activity of B3C. For instance, vascular endothelial growth factor (VEGF), initially identified as an angiogenic and vessel-permeability factor, has been extensively reported to be directly neurotrophic to central nervous system neurons,^{42–45} including enhancement of neurite extension in particular. Based on the evidence that B3C is able to exert neurotrophic effects in primary neurons through the activation of VEGF/VEGF receptor 2 system,²⁵ it is reasonably speculated that B3C promotes neurite outgrowth in PC12 cells and primary cortical neurons, possibly via up-regulation of VEGF. In addition, we could not exclude other possible contributing targets in the membrane, cytoplasm or nucleus, such as G-protein coupled receptor or cyclic adenosine monophosphate.

The $\alpha 7$ -nAChR has been implicated in AD and other related neurodegenerative disorders, leading to efforts targeted toward discovering agonists and PAMs of this receptor. The responsiveness of ligand-gated ion channels to orthosteric agonist can be markedly altered by allosteric modulators upon binding of modulator molecules to specific allosteric ligand recognition sites on the receptor.⁴⁶ The allosteric sites are distinct from the orthosteric sites which are the primary sites for binding of conventional agonists (antagonists) responsible for receptor activation (inhibition). Some allosteric ligands exhibit a mixed activity profile as they both enhance the efficacy of orthosteric agonists and directly activate the receptor by binding to specific allosteric sites.⁴⁷ Recent studies highlight that activation of $\alpha 7$ -nAChR plays a vital role in neurite outgrowth. There is clear evidence that $\alpha 7$ -nAChR agonist, such as acetylcholine and nicotine, was able to induce neurite outgrowth in primary cells.⁴⁸ And we have demonstrated that PNU-120596, a PAM of $\alpha 7$ -nAChR effectively induced cultured PC12 cells into typical neuronal phenotype (Figure 7B and D). These findings taken together suggest that conventional agonists and PAMs of $\alpha 7$ -nAChR may display similar effectiveness in neurite outgrowth through the binding of different sites (orthosteric and allosteric sites).

In our study, we provided the evidence that B3C promoted neurite outgrowth in PC12 cells via the activation of $\alpha 7$ -nAChR. However, pharmacological inhibition of $\alpha 7$ -nAChR by using its specific antagonist MLA significantly but not completely abolish the neurotogenic effects of B3C. There are several possible explanations for these findings. First, MLA competitively binds to and occupies the orthosteric site, which hinders the binding of B3C on the orthosteric site of $\alpha 7$ -nAChR. In this case, B3C may act as a conventional orthosteric agonist. Second, MLA binding to $\alpha 7$ -nAChR may induce a conformation change in this receptor, which may interfere with the binding of B3C on the orthosteric site, or allosteric site of $\alpha 7$ -nAChR. Under this circumstance, B3C could possibly be a orthosteric agonist, or an allosteric ligand (such as PAM).

To figure out whether B3C function as an agonist or PAM of $\alpha 7$ -nAChR, more molecular biological assays, including whole cell electrophysiological analysis, receptor–ligand binding assay and molecular dynamics simulation, are being systematically undertaken in our team. The exact mechanism underlying the activation of $\alpha 7$ -nAChR by B3C will be revealed in our future studies.

Though memantine has been approved for the treatment of AD on the basis of its uncompetitive NMDA receptor antagonism (IC_{50} value, $5.1 \mu M$), there is evidence suggesting that memantine more potently blocks $\alpha 7$ -nAChR (IC_{50} value, $0.34 \mu M$) in rat hippocampal neurons.⁴⁹ Memantine-induced $\alpha 7$ -nAChR inhibition could explain why memantine destroyed eye-blink conditioning in healthy, young human subjects, a response that is regulated by the cholinergic system.⁵⁰ Thus, $\alpha 7$ -nAChR inhibition by memantine could be counterproductive to its effectiveness in AD treatment. In the current study, we identified a small molecule B3C as a potent activator of $\alpha 7$ -nAChR. In this regard, B3C may be a better alternative than memantine as it is able to block NMDA receptor and activate $\alpha 7$ -nAChR concurrently.

In conclusion, B3C, a novel multifunctional dimer, protects against glutamate-induced excitotoxicity and promotes neurite outgrowth in PC12 cells via the activation of $\alpha 7$ -nAChR. Combining these and those pharmacological effects demonstrated by us previously, B3C, which concurrently exerts AChE inhibition and NMDA receptor antagonism as well as $\alpha 7$ -nAChR-mediated neuroprotective and neurite outgrowth-promoting activities, might be an ideal candidate drug against AD and other related neurodegenerative disorders.

METHODS

Chemicals and Reagents. B3C was synthesized as previously described.²¹ Unless noted otherwise, all media and supplements used for cell culture were purchased from Gibco (Carlsbad, CA). PD98059, SB203580, and SP600125 were obtained from Calbiochem (San Diego, CA). Atropine (Atr), dibutyryl cAMP (dbcAMP), mecamylamine (Meca), tubocurarine (Tubo), MK801, methyllycaconitine (MLA), dihydro- β -erythroidine (DH β E), K252a, tacrine, and PNU-120596 were obtained from Sigma Chemicals (St. Louis, MO). Recombinant nerve growth factor (NGF) was obtained from R&D Systems (Minneapolis, MN). Antibodies against phospho-ERK, ERK, and β III tubulin were purchased from Cell Signaling Technology (Cell Signaling Technology Inc., MA). Antibodies against growth-associated protein (GAP-43), $\alpha 7$ -nAChR, and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture. PC12 cells, obtained from the American Type Culture Collection (Rockville, MD), were maintained in high glucose DMEM supplemented with 6% fetal bovine serum (FBS), 6% horse serum (HS), 2 mM glutamine, and 100 U/mL penicillin/streptomycin at 37 °C under an atmosphere of water-saturated 5% CO₂.

Primary Culture of CGNs and Drug Treatment. Sprague–Dawley (SD) rats were handled in accordance with the institutional animal experimental ethical guidelines at The Hong Kong Polytechnic University. Primary CGNs obtained using 8 day old SD rats were prepared as previously described by us.²⁶ Briefly, CGNs were seeded onto 96-well plates in basal modified Eagle's medium containing 10% fetal bovine serum (FBS), 25 mM KCl, 2 mM glutamine, and 100 units/mL penicillin/streptomycin. Cytosine arabinonucleoside at 10 μM was added 24 h after seeding to suppress the growth of non-neuronal cells. After 8 days in vitro (8 DIV), CGNs were pre-treated with various compounds (B3C, tacrine, or MK801) for 2 or 24 h, and then incubated with 100 μM glutamate for 24 h.

Primary Culture of Cortical Neurons and Drug Treatment. Primary cortical neurons were prepared from 18-day-old SD rat embryos as previously described.⁵¹ Briefly, cortical neurons were seeded onto 35 mm cell culture dishes in neurobasal medium containing 10% FBS, 0.5 mM glutamine, 100 units/ml penicillin/streptomycin. At 24 h after plating, half-medium was removed and replaced with neurobasal medium containing 1% B-27, 0.25 mM glutamine and 100 U/mL penicillin/streptomycin. After 2 days in culture, cortical neurons were incubated with B3C for 2 days.

Measurement of Cell Viability. Cell viability was assessed using MTT reduction assay as previously described by us.²⁵ Briefly, after

drug treatment, MTT solution was added to CGNs at a final concentration of 0.5 mg/mL and the cells were then incubated at 37 °C for 4 h. The resulted formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and the absorbance at 570 nm was measured using a microplate reader (model 680, BIO-RAD Laboratories, Hercules, CA).

Analysis of Neurite Outgrowth in PC12 Cells. The morphology and the quantification of cells with extended neurite outgrowth was performed as previously described.¹⁸ Briefly, PC12 cells were seeded onto poly-L-lysine-coated 6-well plates. Twenty-four h later, cells were switched to fresh low serum medium (0.5% FBS and 0.5% horse serum) containing various compounds that were renewed every 2 days. After drug treatment, PC12 cells with neurite outgrowth were observed and photographed using an inverted microscope equipped with a phase-contrast condenser and a digital camera. Approximately 300 cells in 6 randomly chosen visual fields were scored as positive for neurite outgrowth if at least one neurite was longer than the diameter of cell body.

Immunocytochemical Staining. After 2 day incubation with B3C, primary cortical neurons were fixed in the fixing buffer (10% sucrose, 4% paraformaldehyde, 15 $\mu g/mL$ Hoechst 33342) at room temperature for 20 min, and then incubated in blocking buffer (0.5% BSA, 5% goat serum, 0.1% Triton X-100) for 1 h, followed by addition of mouse anti- β III tubulin antibody at 4 °C overnight. After two washes with PBS, Alexafluor-488 anti-mouse secondary antibodies were added for 1 h at room temperature. The extended neurites were visualized using a fluorescence microscope (Nikon Instruments Inc., Melville, NY).

$\alpha 7$ -nAChR Short Hairpin RNA (ShRNA). The genetic depletion of $\alpha 7$ -nAChR was carried out using ShRNA assay as previously described.⁵² A pGPU6-GFP-neo shRNA expression vector containing DNA oligonucleotides (21 bp) specially targeting sequences (5'-GCAGTGCAAACTGAAGTTTGG-3') of rat $\alpha 7$ -nAChR (GenePharma, Shanghai, China) was transfected into PC12 cells. Briefly, 24 h after seeding, PC12 cells were transfected with the plasmids in serum-free DMEM for 6 h by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at the ratio of 1:2 (plasmids: liposome). Thereafter, the culture medium was removed and the cells were incubated in DMEM containing 6% FBS and 6% HS for 24 h. B3C was added into cultures for 2 days.

Image Acquisition and Analysis. All images, acquired from Nikon fluorescence microscope, were adjusted as 12-bit TIFF images. Image analysis was carried out using ImageJ Software. Briefly, ultraviolet excitation and emission wavelengths were chosen to obtain images of nuclei labeled with Hoechst-33342, which allowed identification of the correct focal plane for further image acquisition. Second excitation wavelengths were selected to illuminate neurites with Alexa-488 secondary antibody. Neuronal cell bodies were identified as Hoechst-33342-positive objects. Neurites were labeled as β III tubulin-positive structures, analyzed by a NeuriteTracer program. All of the above selection criteria were user-defined, and all subsequent image analysis used the same criteria throughout.

Western Blotting. Western blotting analysis was performed as previously described by us.²⁵ Briefly, after treatment, cells were lysed in lysis buffer. The collected proteins (20 μg) were resolved by 12% SDS-PAGE and then transferred to polyvinylidene fluoride membranes. After blocking for 2 h at room temperature, the membranes were incubated with antiphospho-ERK, anti-ERK, anti-GAP-43, anti- $\alpha 7$ -nAChR or anti- β -actin antibodies overnight at 4 °C. After that, the membranes were washed and then probed with the respective horseradish peroxidase-conjugated secondary antibody, developed with a Super ECL kit (Thermo Scientific, Rockford, IL), and finally exposed to autoradiographic film.

Statistical Analysis. Data were presented as means \pm SEM of at least three separate experiments. Analysis of variance (ANOVA) followed by Bonferroni's post-test was employed for statistical comparisons. $p < 0.05$ or less was considered to be statistically significant.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

alpha7 nicotinic acetylcholine receptor, $\alpha 7$ -nAChR; AChE, acetylcholinesterase; AD, Alzheimer's disease; B3C, bis-(propyl)-cognitin; CGNs, cerebellar granule neurons; dbcAMP, dibutyryl cAMP; DH β E, dihydro- β -erythroidine; ERK, extracellular signal-regulated kinase; GAP-43, growth-associated protein; MLA, methyllycaconitine; NGF, nerve growth factor; NMDA, N-methyl-D-aspartate; ShRNA, short hairpin RNA

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