Robust Neuritogenesis-Promoting Activity by Bis(heptyl)-Cognitin Through the Activation of $\alpha 7$ -Nicotinic Acetylcholine Receptor/ERK Pathway

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Keywords

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SUMMARY

Aims: Neurodegenerative disorders are caused by progressive neuronal loss in the brain, and hence, compounds that could promote neuritogenesis may have therapeutic values. In this study, the effects of bis(heptyl)-cognitin (B7C), a multifunctional dimer, on neurite outgrowth were investigated in both PC12 cells and primary cortical neurons. **Methods:** Immunocytochemical staining was used to evaluate the proneuritogenesis effects, and Western blot and short hairpin RNA assays were applied to explore the underlying mechanisms. **Results:** B7C (0.1–0.5 μ M) induced robust neurite outgrowth in PC12 cells, as evidenced by the neurite-bearing morphology and upregulation of growth-associated protein-43 expression. In addition, B7C markedly promoted neurite outgrowth in primary cortical neurons as shown by the increase in the length of β -III-tubulin-positive neurites. Furthermore, B7C rapidly increased ERK phosphorylation. Specific inhibitors of $\alpha 7$ -nicotinic acetylcholine receptor ($\alpha 7$ -nAChR) and MEK, but not those of p38 or JNK, blocked the neurite outgrowth as well as ERK phosphorylation induced by B7C. Most importantly, genetic depletion of $\alpha 7$ -nAChR significantly abolished B7C-induced neurite outgrowth in PC12 cells. **Conclusion:** B7C promoted neurite outgrowth through the activation of $\alpha 7$ -nAChR/ERK pathway, which offers novel insight into the potential application of B7C in the treatment of neurodegenerative disorders.

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Introduction

The progressive neuronal loss in specific regions of the brain is regarded as the main cause of neurodegenerative disorders [1,2], including Alzheimer's, Parkinson's, and Huntington's diseases. Among these, Alzheimer's disease (AD) is the most common form characterized by amyloid plaques and neurofibrillary tangles in the brain. Neuroprotection against neurotoxins in combination with neuritogenesis induced by neurotrophic factors that allow for the replacement of lost neurons have been proposed as a potential therapeutic strategy for these neurodegenerative diseases. However, therapeutic application of neurotrophic factors (e.g. nerve growth factor, NGF) is severely restricted by their poor penetration through the blood-brain barrier (BBB) and the

undesirable apoptotic effect through interaction with the p75^{NTR} receptor. The identification of small molecules that mimic the neurotrophic action to promote neuritogenesis at the impaired sites may have therapeutic values against these devastating neurodegenerative diseases.

The rat pheochromocytoma cell line PC12 is widely used as an *in vitro* model that differentiates into a neuronal type with extended outgrowth of neurites in response to NGF. The binding of NGF to tyrosine kinase A (TrkA) receptor triggers a canonical signaling cascade: Raf→mitogen-activated protein kinase kinase (MAPKK)→extracellular signal-regulated kinase (ERK) [3]. Recent studies have also indicated the involvement of $\alpha 7$ -nicotinic acetylcholine receptor ($\alpha 7$ -nAChR) in neuritogenesis [4,5]. Upon $\alpha 7$ -nAChR activation, PC12 cells display a neuron-like

morphology and upregulate ERK phosphorylation [4,6], while $\alpha 7$ -nAChR knockout may result in a lack of maturation of dendritic neurons in hippocampus [7]. Therefore, $\alpha 7$ -nAChR is essential for neuritogenesis and is considered as an important therapeutic target for neurodegenerative disorders.

Cortical neurons constitute the brain's largest region, including the two hemispheres of the cerebral cortex that are commonly affected by AD and other related neurodegenerative diseases. Most of the complex activities of the brain that enable thinking, perception, and voluntary movement are connected to the activity of these neurons. The primary cortical neurons have therefore been used extensively to study developmental or pathological neurobiology, particularly regarding neurite outgrowth [8–10]. Hence, primary cortical neurons are utilized as the paradigm in our study.

Bis(heptyl)-cognitin (B7C) (Figure 1A), a dimeric tacrine analog linked by 7 methylene groups in our laboratory, has been demonstrated as a promising neuroprotectant against neurodegenerative disorders on the basis of its inhibitory effect on acetylcholinesterase (AChE) [11], neuronal nitric oxygen synthase (nNOS), and N-methyl-D-aspartate (NMDA) receptor [12]. Moreover, B7C was reported to reduce scopolamine and middle cerebral artery occlusion-induced brain damage in rats [13]. Most encouragingly, B7C is highly lipophilic and can readily cross the BBB [14], suggesting that B7C has the potential to be developed as a central nervous system drug. However, there is no knowledge about its neuritogenesis activity. In this study, we extended our effort in evaluating the effects and underlying mechanisms of B7C on neuritogenesis in both PC12 cells and primary cortical neurons, and examining whether B7C could prevent the reduction of neurite length in differentiated PC12 cells when induced by A β , a neurotoxin associated with AD. Our results provided novel molecular insight into the potential of B7C in the treatment of AD and other related neurodegenerative disorders.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), horse serum (HS), and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA, USA). PD98059, U0126, SP600125, atropine, mecamylamine, methyllycaconitine (MLA), K252a, and SB203580 were purchased from Calbiochem (San Diego, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), PNU-120596, tacrine, and tubocurarine were from Sigma Chemicals (St Louis, MO, USA). Purified synthetic A β_{1-42} was from GL Biochem (Shanghai, China). Recombinant nerve growth factor (NGF) was from R&D Systems (Minneapolis, MN, USA). Antibodies against phospho-ERK, ERK, β III-tubulin were from Cell Signaling Technology (Cell Signaling Technology Inc, Beverly, MA, USA). Antibodies against growth-associated protein (GAP-43), $\alpha 7$ -nAChR, and β -actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of A β_{1-42}

A β_{1-42} peptide preparation was carried out as we previously reported [15]. Briefly, the lyophilized A β was dissolved in 100%

1,1,1,3,3,3,-hexafluoro-2-propanol (HFIP) to produce monomeric A β , sonicated in a water bath for 10 min, and subsequently stored in small aliquots at -80°C . The HFIP-treated monomeric A β was then dried in a fume hood, dissolved in sterilized distilled water, and aged by incubating at 37°C for 6 days.

PC12 Cell Culture

PC12 cells were maintained in DMEM containing 6% FBS, 6% HS, and 2 mM glutamine at 37°C under an atmosphere of water-saturated 5% CO $_2$.

PC12 Cell Viability

PC12 cell viability was examined by MTT reduction assay as we previously described [16]. Briefly, after various treatments, MTT solution was added to cells in each well in 96-well plates at a final concentration of 5 mg/mL, and the plates were then incubated at 37°C for 4 h. All culture medium was then removed from the cells, and the formazan produced by live cells was resuspended in 150 μL DMSO. Cell viability was evaluated by observing colorimetric changes using a Bio-Rad Microplate Reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) at a test wavelength of 570 nm with 655 nm as a reference wavelength. Data were expressed as a percentage of untreated control cultures.

Assay of Neurite Outgrowth in PC12 Cells

The quantification of neurite-bearing cells was carried out as previously described [17] with minor modifications. For the study of neuritogenesis-promoting activity of various compounds, PC12 cells were seeded onto 6-well plates at a density of 1×10^4 cells/well. 24 h after incubation, cells were switched to low-serum medium (DMEM supplemented with 0.5% FBS and 0.5% HS) containing various compounds that were renewed every 2 days. For the study of neuroprotective effects of B7C, PC12 cells were differentiated with 100 ng/mL NGF in low-serum medium for 7 days. Cells were then washed twice with phosphate-buffered saline to remove NGF and replaced with 10 μM aged A β with or without B7C for 2 days. Pre-incubation with B7C (0.1, 0.3, 0.5 μM) was conducted 2 h before A β addition. After treatment, neurite-bearing PC12 cells were observed and photographed using a light microscope equipped with a phase-contrast condenser, a 10 $\times$ objective lens and a digital camera. Cells were scored as positive for neurite outgrowth if at least one neurite was longer than the diameter of the cell body. The neurite length was measured for all identified neurite-bearing cells in a field by tracing the longest length of neurite per cell using Image J software. Approximately 300 cells in 6 randomly chosen visual fields were counted.

Primary Culture of Cortical Neurons and Drug Treatment

Primary rat cortical neurons were prepared from 18-day-old SD rat embryos as we previously described [18]. Briefly, the freshly dissociated cortical neurons were seeded onto 35-mm petri dishes

Figure 1 B7C robustly induces neurite outgrowth in PC12 cells. **(A)** The structure of B7C. **(B, D)** B7C dose-dependently induced a robust neurite outgrowth in PC12 cells. 24 h after seeding, cells were incubated in low-serum medium containing various compounds for 7 days, and then the percentage of cells with neurites as well as the neurite length for identified neurite-bearing cells was quantified using Image J software. $**P < 0.01$, compared to control group. **(C, E)** B7C time-dependently induced neurite outgrowth in PC12 cells. 24 h after seeding, cells were incubated in low-serum medium containing 0.3 μM B7C, 10 μM tacrine, 10 μM memantine, or 100 ng/mL NGF for different lengths of time (2, 4, or 7 days), and the percentage of cells with neurites was then calculated and the neurite length measured. $**P < 0.01$, compared to control group.

at a density of 150 cells/ mm^2 in neurobasal medium supplemented with 10% FBS and 0.5 mM glutamine. 24 h after plating, half-medium was removed and replaced with neurobasal medium containing 1% B27 and 0.25 mM glutamine. At 3 days *in vitro* (DIV), cortical neurons were incubated with B7C for 2 days.

Immunocytochemical Staining

After incubation for 2 days, cortical neurons were fixed in 4% paraformaldehyde containing 10% sucrose and 15 $\mu\text{g/mL}$ Hoechst 33342 for 20 min. After blocking at room temperature in blocking

buffer (0.5% bovine serum albumin, 0.1% Triton X-100, and 5% goat serum) for 1 h, neurons were exposed to mouse anti- β III-tubulin antibody (Cell Signaling Technology) at 4°C overnight, followed by addition of anti-mouse secondary antibody for 1 h. The extended neurites were visualized using a fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA).

α 7-nAChR Knockdown

To reduce endogenous α 7-nAChR expression, short hairpin RNA (ShRNA) was performed as we previously reported [19]. A pGPU6-GFP-neo ShRNA expression vector containing DNA oligonucleotides (21 bp) specially targeting sequences (5'-GCAGTGCAAAGTTTGG-3') of rat α 7-nAChR (GenePharma, Shanghai, China) was transiently transfected into PC12 cells. Briefly, 24 h after seeding, PC12 cells were transfected with the plasmids in serum-free DMEM for 6 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at the ratio of 1:2–2.5 (plasmids: liposome). Thereafter, the culture medium was removed and the cells were incubated in DMEM containing 6% FBS, 6% HS, and 100 μ g/mL G418 for 24 h. B7C was then added into cultures for 2 days.

Neuronal Imaging and Analysis

Images were captured by a Nikon ECLIPSE Ti-U microscope (Nikon Instruments Inc) at $\times 200$ magnifications. The ratio of neurite length to the number of neuron was measured using NeuriteTracer, a neurite tracing plugin for the image-processing program Image J as previously described [20]. Briefly, given a directory containing pairs of images corresponding to nuclear (Hoechst 33342) and neurite marker (β III-tubulin) images, the plugin opened the images in a nuclear and neuronal stack. Images were first preprocessed to optimize uniformity of illumination and contrast in the input images. These preprocessing steps were performed on a small subset of images to allow user to choose the threshold to be applied to the neuronal images. The plugin then cued the user to input the threshold and image scale, and the remaining images were processed. Following preprocessing, the images were processed by thresholding. Neuronal nuclei were selected using "Image Calculator" command to identify pixels that were present in both the nuclear and neuronal marker images. The neuronal stack was then skeletonized. Six or more randomly captured images were analyzed for each group.

Western Blotting

Western blotting assay was carried out as we previously described [16]. Briefly, after treatment, cells were harvested and lysed. The resultant proteins collected by centrifugation were separated on a 12% SDS-PAGE gel and then transferred to polyvinylidene fluoride membranes. The membranes were immersed in blocking buffer (5% nonfat milk in Tris-buffer saline/0.1% Tween-20) for 2 h, followed by addition of anti-phospho-ERK, anti-ERK, anti-GAP-43, anti- α 7-nAChR, and β -actin antibodies overnight at 4°C. After 3 washes with PBS, the membranes were probed with secondary antibodies, developed with an ECL plus kit (Thermo Scientific, Rockford, IL, USA), and finally exposed to autoradiographic film.

Statistical Methods

Data were shown as mean $\pm$ SEM of three independent experiments. Analysis of variance with Dunnett's test was employed to test for the differences. $P < 0.05$ or less was considered to be statistically significant.

Results

B7C, but not Tacrine or Memantine, Induces Robust Neurite Outgrowth in PC12 Cells

To examine the proneurogenesis effect, PC12 cells were incubated for different lengths of time with various compounds that were renewed every 2 days. PC12 cells exposed to NGF differentiated into neurite-bearing morphology (Figure 2A), and the percentage of cells with neurites after 7 days of incubation with NGF was up to $74.0 \pm 2.2\%$ (Figure 1B and C), which is in consistent with findings of several previous studies [6,21]. B7C induced robust neurite outgrowth in PC12 cells at concentrations of 0.1–0.5 μ M that did not affect cell viability (Figure 4A), and with an efficacy similar to that of NGF (Figure 1B and C), while tacrine (an AChE inhibitor) and memantine (an NMDA receptor antagonist) did not show any effect on neurite outgrowth. The percentages of neurite-bearing cells after 2 days, 4 days, and 7 days of incubation with 0.3 μ M B7C markedly reached to $37.3 \pm 2.3\%$, $44.6 \pm 0.9\%$, and $68.9 \pm 2.0\%$, respectively (Figure 1C). In addition, the maximal neurite length also markedly increased to $71.6 \pm 5.5 \mu$ m in cells treated with B7C (Figure 1D and E), which was compatible with those treated with 100 ng/mL NGF ($73.6 \pm 4.2 \mu$ m) and significantly longer than those of the control ($14.4 \pm 1.9 \mu$ m).

In addition to morphological evaluation, neuritogenesis can also be measured by a biochemical method in determining the expression of the neuronal marker GAP-43, which is found almost exclusively in neurons. The levels of GAP-43 in PC12 cells after 7 days of incubation with 0.3 μ M B7C and 100 ng/mL NGF reached approximately 2.2- and 2.3-fold higher than that observed in the control group, respectively (Figure 2B).

B7C Markedly Promotes Neurite Outgrowth in Primary Cortical Neurons

To further confirm the neuritogenesis of B7C, the primary cortical neurons were used in our study. It was found that 2 days of incubation with B7C (0.01 and 0.03 μ M) remarkably promoted the outgrowth of neurites in primary cortical neurons, as evidenced by the increase in the length of neurites stained by β III-tubulin (Figure 2C and D).

B7C Effectively Blocks Neuronal Loss in PC12 Cells Induced by A β

To better understand the potential application of B7C in the treatment of AD, we introduced a cell model in which cytotoxicity was induced in differentiated PC12 cells by A β . As shown in Figure 3A, B7C efficiently reversed the morphological changes caused by 2 days of A β challenge, including unhealthy cell bodies

Figure 2 Morphological analysis of neurite outgrowth in PC12 cells and primary cortical neurons induced by B7C. **(A)** B7C induced PC12 cells differentiation toward a neuronal phenotype. 24 h after seeding, PC12 cells were incubated in low-serum medium containing 0.3 μM B7C or 100 ng/mL NGF for 7 days, and then, cell morphology was observed using a phase-contrast microscopy. **(B)** B7C upregulated the levels of GAP-43 in PC12 cells. PC12 cells were incubated with compounds shown in **(A)**, and the extracted proteins were subjected to Western blotting analysis using anti-GAP-43 and anti-β-actin antibodies. ** $P < 0.01$, compared to control group. **(C)** At 3 DIV, cortical neurons were incubated in the absence or presence of B7C for 2 days. Upper: Phase-contrast pictures of cortical neurons were obtained using a phase-contrast microscope. Middle: Neurons were stained with neuronal marker βIII-tubulin. Lower: βIII-tubulin-positive neurites were digitally identified and skeletonized for examination by NeuriteTracer program. ** $P < 0.01$, compared to control group. **(D)** The calculation of neurite length/neuron stained by βIII-tubulin as in **(C)**. * $P < 0.05$, compared to control group.

and broken neuritic network. Moreover, B7C (0.1–0.5 μM) markedly prevented the decrease in cell viability and reduction of neurite length in PC12 cells (Figure 3B and C).

B7C Promotes Neurite Outgrowth by Activating ERK Pathway in PC12 Cells

It is widely accepted that activation of mitogen-activated protein kinase family (ERK, p38, JNK) is a critical underlying mechanism in the mediating of neuritogenesis. To examine whether these pathways are involved in B7C-induced neurite outgrowth, PC12 cells were pretreated for 2 h with specific inhibitors including MEK1/2 inhibitors (PD98059 and U0126), p38 inhibitor (SB203580), and JNK inhibitor (SP600125) and then incubated with 0.3 μM B7C for 7 days. The selected specific inhibitors, at their effective concentrations, did not affect PC12 cell viability (Figure 4A). It was evident that PD98059 (3, 10 μM) and U0126 (1, 3 μM), but not SB203580 or SP600125, significantly blocked the neurite outgrowth-promoting activity induced by B7C in PC12 cells (Figure 4B).

To further confirm that B7C promoted neuritogenesis by activating ERK signaling pathway, the expression of phospho-ERK was evaluated by Western blotting analysis. It was observed that the elevated level of phospho-ERK occurred at 15 min, peaked at 30 min and returned to the basal level at 4 h after B7C treatment (Figure 4C).

Pharmacological Blockage of α7-nAChR Blocks the Neurite Outgrowth and ERK Activation Induced by B7C in PC12 Cells

It has been demonstrated that agonists of TrkA and acetylcholine receptors (AChRs) could promote neuritogenesis by activating ERK pathway [3,5]. To examine which receptor was involved in B7C-induced neurite outgrowth, PC12 cells were pretreated for 2 h with specific inhibitors of TrkA (K252a, 0.1 and 0.3 μM), muscarinic AChR (mAChR) (atropine, 10 μM), and nicotinic AChR (nAChR) (tubocurarine and mecamylamine, 10 μM) and then incubated with 0.3 μM B7C. The selected specific inhibitors, at their effective concentrations, did not affect PC12 cell viability

Figure 3 B7C provides neuroprotection against A β in differentiated PC12 cells. **(A)** B7C reversed the morphological changes in PC12 cells induced by A β . Differentiated PC12 cells were pretreated with B7C (0.3 μ M) for 2 h and then incubated with aged A β_{1-42} for 2 days, and cell morphology was then observed using a phase-contrast microscope. **(B)** B7C markedly increased PC12 cell viability. Differentiated PC12 cells were pretreated with B7C (0.3 μ M) for 2 h, incubated with aged A β_{1-42} for 2 days, and then subjected to MTT assay. $^{**}P < 0.01$, compared to control group. $^{\#}P < 0.05$; $^{##}P < 0.01$, compared to A β_{1-42} group. **(C)** B7C reversed the shortening of neurite length caused by A β_{1-42} . Differentiated PC12 cells were pretreated with B7C (0.3 μ M) for 2 h, then incubated with aged A β_{1-42} for 2 days. The neurite length was measured using Image J software. $^{**}P < 0.01$, compared to control group. $^{\#}P < 0.05$; $^{##}P < 0.01$, compared to A β_{1-42} group.

(Figure 5C). It was found that tubocurarine and mecamylamine, but not K252a or atropine, significantly attenuated the neuriteogenesis-promoting activity induced by B7C (Figure 5A and B). Furthermore, MLA, which is a specific $\alpha 7$ -nAChR antagonist, partially abolished B7C-induced neurite outgrowth in PC12 cells

(Figure 5B and D). Consistently, pretreatment with MLA and PD98059 abrogated the elevated phospho-ERK induced by B7C (Figure 5E). To confirm the role of activation of $\alpha 7$ -nAChR in neurite outgrowth, PNU-120596, a potent positive allosteric modulator (PAM) of $\alpha 7$ -nAChR, was used in our cell model. It was

Figure 4 B7C induces neurite outgrowth in PC12 cells by activating ERK pathway. **(A)** B7C and the selected specific inhibitors, at their effective concentrations, did not affect PC12 cell viability. PC12 cells were incubated with B7C and various inhibitors for 7 days, then subjected to MTT assay. **(B)** ERK inhibitors, but not p38 or JNK inhibitors, markedly attenuated the neurite outgrowth in PC12 cells induced by B7C. 24 h after plating, cells were pretreated with PD98059 (3, 10 μ M), U0126 (1, 3 μ M), SB203580 (1, 3 μ M) or SP600125 (1, 3 μ M) for 2 h, and then exposed to 0.3 μ M B7C for 7 days. The percentage of cells with neurites was calculated. $^{**}P < 0.01$, compared to control group, $^{##}P < 0.01$, compared to B7C group. **(C)** B7C remarkably upregulated the levels of p-ERK in PC12 cells. Cells were incubated with 0.3 μ M B7C in low-serum medium for different lengths of time, and the extracted proteins were subjected to Western blotting analysis using anti-p-ERK and anti-ERK antibodies. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to control group.

Figure 5 Pharmacological blockage of $\alpha 7$ -nAChR partially abolishes the neurite outgrowth as well as ERK activation in PC12 cells induced by B7C. **(A)** B7C exerted proneuritogenesis activity in PC12 cells through a TrkA-independent pathway. Cells were pretreated with K252a (0.1, 0.3 μ M) for 2 h before the addition of 0.3 μ M B7C or 100 ng/mL NGF. 7 days after treatment, the percentage of cells with neurites was calculated. $**P < 0.01$, compared to control group; $\Delta\Delta P < 0.01$, compared to NGF group. **(B)** Effects of $\alpha 7$ -nAChR antagonist and PAM on neurite outgrowth in PC12 cells. For the study of the effects of $\alpha 7$ -nAChR antagonist on the neurite outgrowth induced by B7C, cells were pretreated with atropine (10 μ M), mecamylamine (10 μ M), tubocurarine (10 μ M), or MLA (0.03, 0.1, 0.3 μ M) for 2 h before the addition of 0.3 μ M B7C. 7 days after treatment, the percentage of cells with neurites was calculated. For the study of the neuritogenesis-promoting activities of $\alpha 7$ -nAChR PAM, cells were incubated in low-serum medium containing PNU-120596 (1 or 10 μ M) for 7 days, and then, the percentage of cells with neurites was quantified using Image J software. $**P < 0.01$, compared to control group. **(C)** The selected inhibitors and $\alpha 7$ -nAChR PAM, at their effective concentrations, did not affect PC12 cell viability. PC12 cells were incubated with various inhibitors or PNU-120596 for 7 days and then subjected to MTT assay. **(D)** Morphological characteristics of neurite outgrowth in PC12 cells treated with $\alpha 7$ -nAChR antagonist and PAM. Cells were pretreated with MLA for 2 h in low-serum medium and then incubated with 0.3 μ M B7C for 7 days. Or cells were incubated with 10 μ M PNU-120596 for 7 days. After drug treatment, cell morphology was observed using a phase-contrast microscopy. **(E)** $\alpha 7$ -nAChR antagonist significantly attenuated ERK activation induced by B7C in PC12 cells. Cells were pretreated with atropine (10 μ M), MLA (0.3 μ M) and PD98059 (10 μ M) for 2 h before the addition of 0.3 μ M B7C. 30 min after treatment, the extracted proteins were subjected to Western blotting analysis using anti-p-ERK and anti-ERK antibodies. $**P < 0.01$, compared to control group. $##P < 0.01$, compared to B7C group.

found that PNU-120596 effectively promoted neurite outgrowth in PC12 cells (Figure 5B and D).

Genetic Depletion of $\alpha 7$ -nAChR Abolishes the Neurite Outgrowth in PC12 Cells Induced by B7C

To confirm the involvement of $\alpha 7$ -nAChR in the proneuritogenesis activity of B7C, short hairpin (ShRNA)-mediated $\alpha 7$ -nAChR depletion was performed. Inhibition of $\alpha 7$ -nAChR by ShRNA (Sh $\alpha 7$ -nAChR) caused an approximate 50% decrease in the expression of endogenous $\alpha 7$ -nAChR in PC12 cells. B7C was no longer able to induce neurite outgrowth in PC12 cells where endogenous $\alpha 7$ -nAChR was inhibited, in contrast to the

unchanged neuritogenesis-promoting activity observed in the negative control ShRNA (ShNC) or the vector-treated PC12 cells (Figure 6).

Discussion

Progressive neuronal loss might be the fundamental mechanism underlying AD and other related progressive neurodegenerative disorders [22,23]. The identification of proneuritogenesis molecules would therefore be a major breakthrough in the treatment of these diseases. Herein, we provided evidence that B7C is a robust neuritogenesis inducer with therapeutic potential.

Figure 6 Genetic depletion of $\alpha 7$ -nAChR partially abolishes the neurite outgrowth in PC12 cells induced by B7C. **(A)** PC12 cells were transiently transfected with pGPU6-GFP plasmid (vector), pGPU6-GFP plasmid encoding $\alpha 7$ -nAChR ShRNA (Sh $\alpha 7$ -nAChR), or pGPU6-GFP plasmid encoding negative control ShRNA (ShNC). The extracted proteins were subjected to Western blotting analysis using anti- $\alpha 7$ -nAChR and anti- β -actin antibodies. **(B)** $\alpha 7$ -nAChR depletion abolished the neuritogenesis-promoting activity in PC12 cells induced by B7C. PC12 cells transfected with vector or Sh $\alpha 7$ -nAChR were incubated with 0.3 μ M B7C for 2 days, and the percentage of cells with neurites was calculated. $\Delta\Delta P < 0.05$, compared to vector group; $**P < 0.01$, compared to control group; $\#P < 0.05$, compared to Sh $\alpha 7$ -nAChR group.

PC12 cells are widely accepted as a useful model system for the study of neuritogenesis [24]. In response to NGF, PC12 cells differentiate into a neuron-like phenotype, extend long neurites and express neuronal markers including GAP-43 and β III-tubulin. Under a condition of low-serum medium that is to induce transition from a proliferative phase to differentiation stage, PC12 cells themselves did not sprout neurites. B7C (0.1–05 μ M) provided robust proneuritogenesis activity in PC12 cells with an efficacy similar to NGF, as evidenced by the condensed cell body and long and branching neurites (Figure 2A).

In addition to morphological observation, biochemical examination is also an effective method for evaluating neurite outgrowth. GAP-43 is a neuron-specific protein that exhibits 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 outgrowth [25–27] and induce neuronal sprouting [28], respectively. On the contrary, depletion of GAP-43 remarkably resulted in shortened neurites, collapsed growth cone, and abnormal axonal pathfinding [29,30]. Thus, GAP-43 is considered a major and useful indicator of PC12 cell neuritogenesis. In our study, with the assay of Western blotting analysis, the protein expression of GAP-43 in PC12 cells treated by B7C increased by approximately 100%, an observation comparable to that provided by NGF (Figure 2B), again suggesting that B7C has a robust proneurogenesis effect. Most encouragingly, we further confirmed the neuritogenesis-promoting activity of B7C in primary cortical neurons, which is an important primary cell model frequently employed in earlier neuritogenesis studies [8,31]. We have selected β III-tubulin as our primary marker for cortical neuronal morphology, because it is expressed throughout axons, dendrites, and the cell body and delivers consistently high contrast images that are easily analyzed by ImageJ software. In addition, neurite outgrowth in primary cortical neurons has been extensively evaluated through examination of morphological changes and by immunocytochemistry for β III-tubulin [20,32–34]. Using this primary culture model, we found that B7C markedly accelerated the outgrowth of neurites as shown by the increase in length of β III-tubulin-positive neurites (Figure 2C and D). As suggested by earlier studies [35,36], neurite outgrowth and guidance to the proper target require the coordination of filamentous actin (F-actin) and microtubules, the dynamic cytoskeletal polymers that promote shape change and locomotion. Developing neurites that are not yet synaptically connected have highly dynamic, motile structures at their leading edge. These structures are called growth cones that contain F-actin bundles. It is therefore important to evaluate the length of neurites with the stain of F-actin bundles. Experiments such as confocal microscopic analysis of phalloidin-stained F-actin bundles will be carried out in future studies.

In particular, to better understand the potential application of B7C in the treatment of AD, we introduced a widely used cell model in which cytotoxicity was induced by A β in differentiated PC12 cells [37,38]. Using this model, we found that B7C provided neuroprotection against A β insult, as evidenced by the reversal of the decrease in cell viability and the shortening of neurite length caused by A β (Figure 3). The neuroprotective and neuritogenesis-promoting effects described herein, together with the inhibitory activities of nNOS and NMDA receptor, may enable B7C to become a promising agent for the treatment of AD, by concurrently acting through multiple mechanisms.

We then explored the mechanisms underlying the proneuritogenesis activity of B7C. As B7C is an NMDA receptor antagonist, it is reasonable to ask whether B7C promotes neurite outgrowth through inhibiting NMDA receptor. It turned out that memantine, another NMDA receptor antagonist, failed to induce neurite outgrowth in PC12 cells even when the concentration was up to 10 μ M (Figure 1B and C), suggesting that the proneuritogenesis effect of B7C may be independent of its inhibition of NMDA receptor.

Instead, our study indicated that B7C promoted neurite outgrowth in PC12 cells through activating ERK pathway, a conclusion supported by the evidence that MEK1/2 inhibitors (PD98059 and U0126) markedly blocked the neurite outgrowth-promoting

activity as well as the observed sustained ERK activation (Figure 4B and C). A noticeable increase in phospho-ERK occurred 15 min after B7C administration, and a maximal response was observed 30 min after B7C administration, which is analogous with that reported by NGF [39]. However, the activation kinetics of ERK phosphorylation was slightly different. NGF caused a maximal effect at 5–30 min and then returned to its basal level 2 h after treatment. However, B7C showed a more sustained activation of ERK phosphorylation that lasted for 4 h after induction. The different activation kinetics suggested that the intracellular activation of ERK pathway induced by B7C and that by NGF might be from the modulation of different receptors on the membrane. We then examined which receptors were involved in the B7C-induced neuritogenesis. It turned out that B7C exerted its neuritogenic activity via a TrkA-independent pathway as the TrkA-specific inhibitor failed to block the outgrowth of neurites in PC12 cells induced by B7C.

Recently, increasing lines of evidence strongly suggested that activation of AChRs, $\alpha 7$ -nAChR in particular, is an important underlying mechanism in mediating various cellular responses including neuritogenesis [40,41]. $\alpha 7$ -nAChR activation allows the influx of Ca^{2+} , which stimulates the downstream signaling pathways including ERK pathway. As B7C is a superior AChE inhibitor and can indirectly enhance the concentration of ACh in synaptic cleft, we analyzed whether the activation of AChRs, including mAChR and nAChR, was involved in the proneuritogenesis activity of B7C using specific inhibitors. It was evident that nAChR antagonists, but not mAChR antagonist, abrogated the neurite outgrowth induced by B7C. Our findings that pharmacological blockage and genetic depletion of $\alpha 7$ -nAChR partially abolished the neurite outgrowth as well as ERK activation induced by B7C not only confirm the primary importance of $\alpha 7$ -nAChR in mediating neuritogenesis, but also provide novel insights into the potential application of B7C against neurodegenerative diseases. Although tacrine is also an AChE inhibitor and may mimic the function of ACh, most of its effects on neuronal activity were independent of $\alpha 7$ -nAChR as suggested by earlier studies [42,43], which may explain its limited effects on neuritogenesis.

The development of nicotinic acetylcholine receptor activator pharmacophore started in 1970 when it was proposed that the binding of ligands to the receptor was dependent on a positively charged nitrogen atom on the ligands. Molecular docking studies revealed that the hydrogen bond, formed between the cationic center (positively charged nitrogen atom) of the ligands and the main-chain carbonyl oxygen atom of the highly conserved Trp-149 or the side-chain hydroxyl oxygen atom of Tyr-123 in the receptors, is a hallmark of nicotinic ligands binding to $\alpha 7$ -nAChRs [44]. Besides, π – π interactions between π rings located within the benzene rings of ligands and the aromatic side chain of the receptors also contribute to the binding of ligands and $\alpha 7$ -nAChRs. B7C (Figure 1A), as well as other $\alpha 7$ -nAChR activators (agonists and PAMs) including acetylcholine, nicotine, PNU-282987, and PNU-120596, contain one or more nitrogen atoms that are likely to be positively charged. Moreover, B7C possesses a benzene ring in each of its arm. The chemical structure of B7C suggests that B7C may act as a ligand of $\alpha 7$ -nAChRs. In addition to the similarity

in chemical structure, there are also some interesting parallels between the biological effects, such as neuroprotection, induced by B7C and other $\alpha 7$ -nAChR activators. For instance, B7C [45,46] as well as PNU-120596 and PNU-282987 [47] prevented loss of retinal ganglion cells in a glaucoma model.

More noticeably, pharmacological inhibition (Figure 5B, D, and E) and genetic depletion (Figure 6) of $\alpha 7$ -nAChRs could not completely abolish the neuritogenesis-promoting effects in PC12 cells produced by B7C. There could be several reasons for this phenomenon: First, potent PAMs of $\alpha 7$ -nAChR could be an inducer of neurite outgrowth, a conclusion supported by the evidence that PNU-120596 (a potent PAM of $\alpha 7$ -nAChR) promoted effective neuritogenesis activity in our PC12 cell model (Figure 5B). B7C might act as a potent PAM of $\alpha 7$ -nAChR more than conventional $\alpha 7$ -nAChR agonists. PAM might differentially modify the biophysical properties of $\alpha 7$ -nAChR via an allosteric transmembrane site without interacting at the orthosteric binding site at which conventional $\alpha 7$ -nAChR agonists target as suggested by earlier studies [48,49]. MLA, being a $\alpha 7$ -nAChR antagonist and acting competitively with conventional $\alpha 7$ -nAChR agonist, might therefore partially abolish the neurite outgrowth-promoting activity of B7C. Second, we could not rule out other possible contributing targets in the membrane, cytoplasm, or nucleus, such as G-protein-coupled receptor or cyclic adenosine monophosphate. To verify the hypothesis that B7C directly acts on $\alpha 7$ -nAChR and elucidates the detailed binding sites between B7C and $\alpha 7$ -nAChR, more molecular biological assays, including whole-cell electrophysiological analysis, receptor-ligand binding assay, and molecular dynamics simulation, are being undertaken by our team. The exact mechanism underlying the activation of $\alpha 7$ -nAChR by B7C will be revealed in future studies.

In conclusion, B7C promotes robust neurite outgrowth in PC12 cells and primary cortical neurons through activation of the $\alpha 7$ -nAChR/ERK signaling pathway. Given that considerable literature has revealed that multifunctional compounds might provide greater therapeutic efficacy against neurodegenerative disorders by concurrently and synergistically acting on different targets in the brain, the results described herein and published previously may lead us to conjecture that B7C, which possesses anti-AChE, anti-NMDA receptor, anti-nNOS, and proneuritogenesis activities, may have great potential for treatment of the pathological processes of neurodegenerative disorders.

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Conflict of Interest

The authors declare no conflict of interests.

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