Neurochemistry International 124 (2019) 94–105

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

L-NBP, a multiple growth factor activator, attenuates ischemic neuronal impairments possibly through promoting neuritogenesis

Yuming Zhao[a,b,*], Dongmei Liu[c], Jiang Li[c], XiaoJie Zhang[a,d], Xiaoliang Wang[b,c,**]

[a] Department of Pharmacology, School of Basic Medical Sciences, Capital Medical University, Beijing, 100069, PR China
[b] Beijing Institute of Brain Disorders & Research Center for Brain Disorders, Capital Medical University, Beijing, 100069, PR China
[c] State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100050, PR China
[d] State Key Laboratory for Structural Chemistry of Unstable and Stable Species, Center for Molecular Science, Institute of Chemistry, Chinese Academy of Sciences, Beijing, 100190, PR China

ARTICLE INFO

Keywords:
L-NBP
Neuritogenesis
Stroke
EGF receptor
BDNF
NGF

ABSTRACT

In China, L-3-n-butylphthalide (L-NBP) showed promising pharmacological actions in stroke treatment. Analyzing the characteristics of L-NBP might provide valuable hints for new drug design. The current study is aimed to determine the effects of L-NBP on neuritogenesis and further to elucidate the neuronal protection against stroke impairment *in vitro*. L-NBP was applied to rat pheochromocytoma PC12 cells and cultured rat cortical neurons under the normoxic condition and the oxygen-glucose deprivation/reoxygenation (OGD/R) insults, respectively. Immunofluorescence staining, western blot analysis, Sholl analysis, lactate dehydrogenase (LDH) release assay, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay and enzyme-linked immunosorbent assay (ELISA) were performed. L-NBP could concentration-dependently stimulate the development of growth cones, enhance the neuritic branches and synapse formation. It indicated that L-NBP possibly promoted the neuritogenic activity in a stage-dependent manner. Further research proved that L-NBP could promptly activate epidermal growth factor (EGF) receptor, up-regulate the expressions of extracellular signal-regulated kinase1/2 (ERK1/2), cAMP response element-binding protein (CREB) and E−26-like protein 1 (ELK-1). In addition, L-NBP enhanced the sustained expressions of brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). The inhibition to the receptors of EGF, NGF,BDNF could attenuate L-NBP induced neuritogenic and neuronal survival after the OGD/R toxicity. Basing on these investigations, we concluded that L-NBP might reconstruct the impaired neuronal network and improved the neuronal complexity after the ischemic insults through multiple pathways which at least were via the activations of EGF receptor, BDNF and NGF related signals.

1. Introduction

Neuritogenesis is a crucial stage in neuronal development, emerging the axons and dendrite. The cells display three distinct phases during neuritogenesis consisting of neurite initiation, rapid neurite elongation, and a maturation process characterized by the thickening of neurites and synapse maturation (Xiao and Liu, 2003). Loss of neurite has been proven to be one of the key characteristics of neurodegenerative diseases and stroke injury (Polleux and Snider, 2010). Hence, drugs which promote neuritogenic activities might provide therapeutic effects to the functional recovery in stroke insults (More et al., 2012; Rodrigues et al., 2016; Tang et al., 2016).

L-NBP demonstrated a series of promising pharmacological properties in stroke intervention and neurodegenerative disease therapy (Huang et al., 2010; Lei et al., 2014; Lu et al., 2012; Peng et al., 2007, 2012; Qin et al., 2018; Wang et al., 2018; Xiong et al., 2012; Xu and Feng, 2000; Xu et al., 2012; Yang et al., 2015, 2018; Zhao et al., 2016; Zhu et al., 2018). Until now, most researchers focus on how L-NBP direct inhibited the neuronal damage, except for the study by Yang et al. (2015). In the study (Yang et al., 2015), we first demonstrated that L-NBP exert rehabilitation effects through enhancing endo-neurogenesis in the subgranular zone (SGZ), which induced neuronal stem cells

* Corresponding author. Department of Pharmacology, School of Basic Medical Sciences, Capital Medical University, Beijing, 100069, PR China.
** Corresponding author. State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100050, PR China.
E-mail addresses: yumingzhao@ccmu.edu.cn (Y. Zhao), wangxl@imm.ac.cn (X. Wang).

https://doi.org/10.1016/j.neuint.2019.01.002
Received 26 September 2018; Received in revised form 27 December 2018; Accepted 2 January 2019
Available online 07 January 2019
0197-0186/

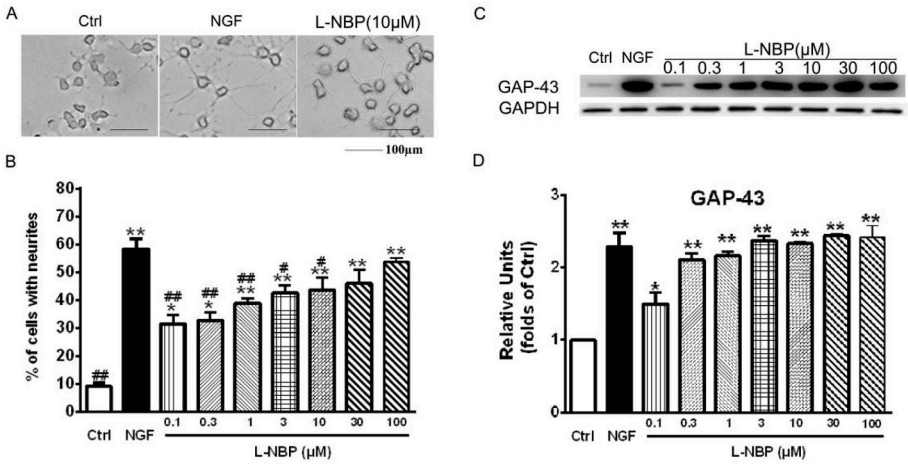


Fig. 1. L-NBP induces neurite extension in PC12 cells in a concentration-dependent manner. PC12 cells were cultured for 7 days and were treated with L-NBP (0.10–100 μM) or NGF 50 ng/ml. (A) Representative images of neurite extension by (a) Ctrl, (b) NGF 50 ng/ml and (c) L-NBP 10 μM. Scale Bar: 100 μm. (B) Statistic analysis of percentage of neurite-bearing cells treated by L-NBP or NGF. The effects of L-NBP and NGF on neurite extension were further demonstrated by the expression of GAP-43 in western blot analysis. (C) Representative bands of western blot analysis; (D) statistical analysis of the expression of GAP-43. Ctrl: Control. Values were shown as mean ± S.E.M. of three independent experiments, with $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group; with $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus NGF group.

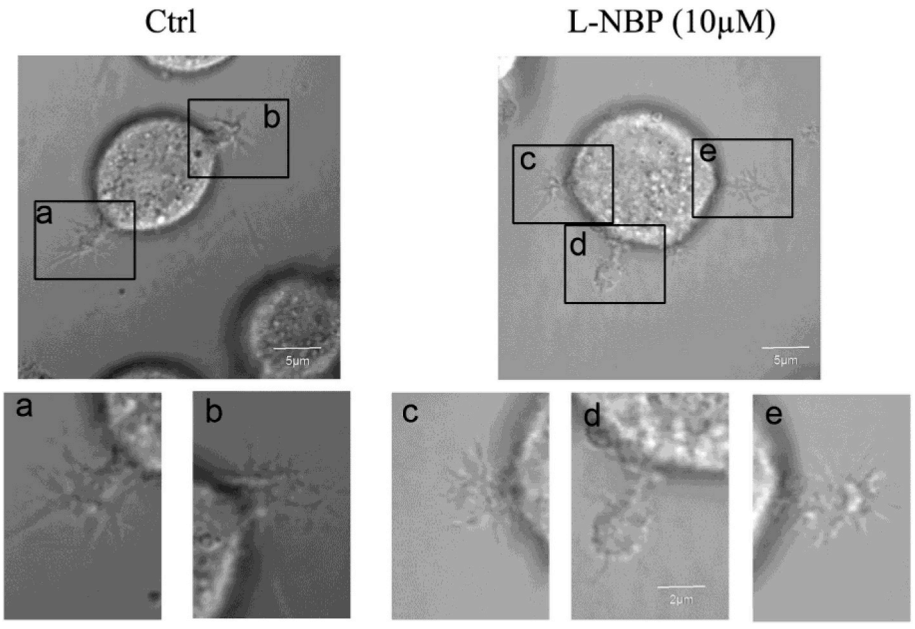


Fig. 2. L-NBP accelerates the growth cone development. PC12 cells were cultured and treated with L-NBP (10 μM) for 2 days. Images in upper panel: representative images of cells in control and L-NBP group. Scale bar: 5 μm; Lower panel: rectangular regions of (a)–(e) in upper panel are magnified. Scale bar: 2 μm. Statistic analysis of the numbers of growth cone per soma and the growth cone area were shown in Table 1. Ctrl: control. Values were shown as mean ± S.E.M., with $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group.

to differentiate into neurons (Yang et al., 2015). However, it remains unknown how L-NBP protects the brain regions that lack of endogenous neurogenesis, in particular the injured cerebral cortex, during the course of stroke treatment.

Based on the *in vivo* findings that L-NBP induces expression of the neurite biomarker GAP-43 (Yang et al., 2015), it is proposed that L-NBP might promote neuritogenesis as well as neurogenesis, which might contribute to the repair of impaired neuronal network in those brain regions outside of hippocampus in stroke. The present study investigated the neuritogenic capacity of L-NBP in pheochromocytoma PC12 cells and primary cultured rat cortical neurons under normoxia. Based on these observations, the effects of L-NBP on the impaired neuronal complex and the neuronal survival were further determined using the oxygen-glucose deprivation/reoxygenation (OGD/R) cell model which mimicked the stroke insults. To understand the protective mechanisms of L-NBP, the signals of growth factors, including the Epidermal Growth Factor (EGF) receptor-, Brain Derived Neurotrophic Factor (BDNF)- and Nerve Growth Factor (NGF)-mediated pathways, were particularly focused on. Results showed that L-NBP stimulated the neurites outgrowth, the development of neuronal complex and the neuronal survival under the OGD/R insults. These effects were at least partially mediated by the promptly activated EGF-receptor and via the sustained enhancements of the neurotrophic factors of BDNF and NGF.

2. Material and methods

2.1. Chemicals and reagents

L-NBP (purity > 99.5%) was obtained from Institute of Materia Medica, Chinese Academy of Medical Sciences. Primary antibodies in the study were listed in the table of Supplement table 1. The media and supplements used for cell cultures were purchased from Invitrogen (Carlsbad, CA, USA). Other reagents were purchased from Sigma Chemicals (St Louis, MO, USA).

2.2. Drug treatment

Recombinant rat beta-NGF was applied as positive control at a concentration of 50 ng/ml (Zhao et al., 2015a). L-NBP was incubated at the indicated concentrations ranging from 0.1 to 100 μM. To observe the roles of the related pathway in the neuritogenesis effects of L-NBP, the inhibitors of Trk receptors (K252a), EGF receptor (AG1478) and MEK (PD95059), and the antibodies of BDNF and NGF were applied 30 min before L-NBP treatment at the concentrations of 0.3 μM, 300 nM,10 μM, 5 μg/ml and 2 μg/ml, respectively.

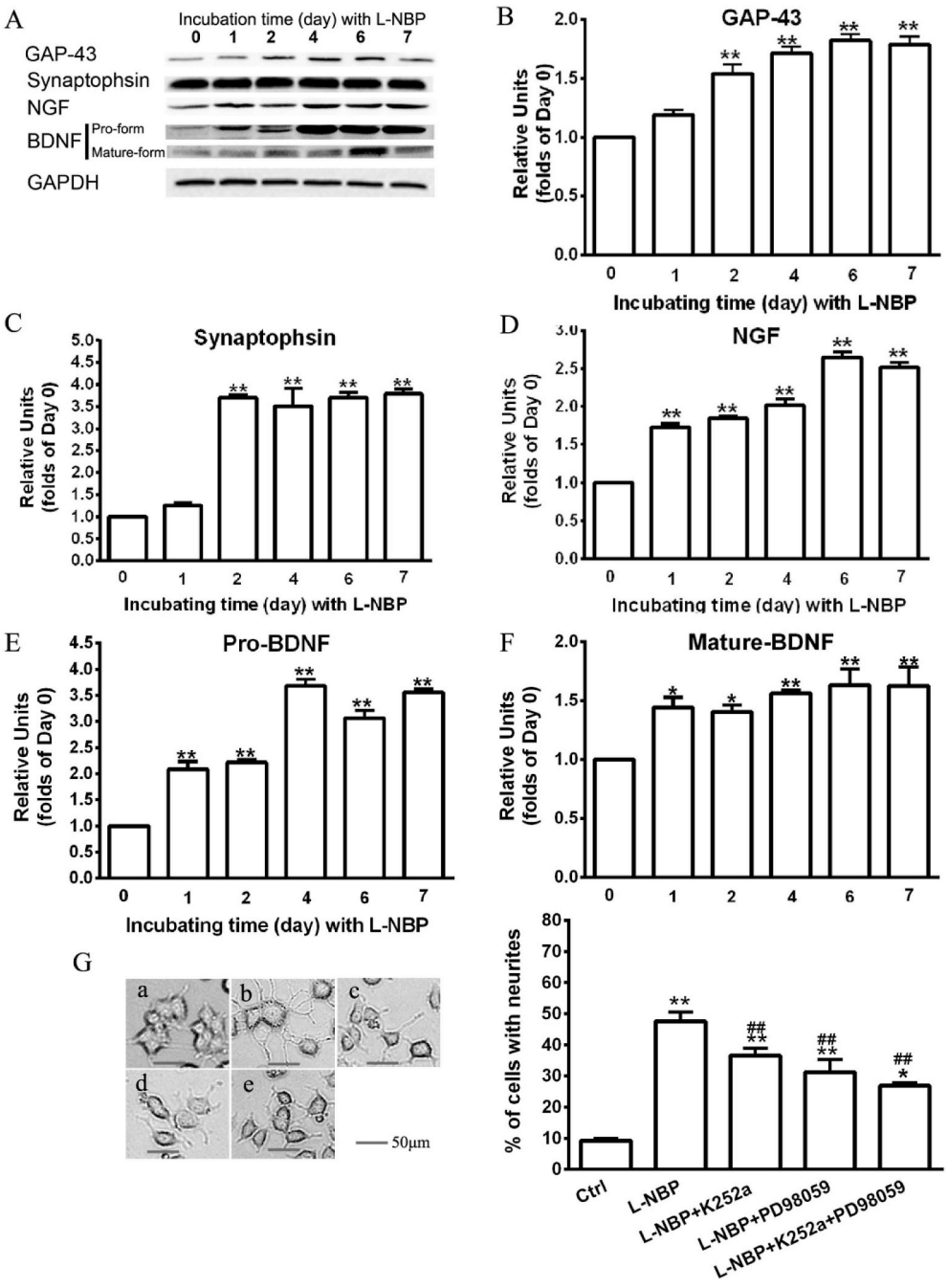


Fig. 3. L-NBP time-dependently promotes the neurites outgrowth and synapse formation by up-regulating the expressions of BDNF and NGF in PC12 cells. The expressions of GAP-43, synaptophsin, NGF and BDNF (pro-form (36KD) and mature form (15kD)) were determined by western blot analysis. Representative bands of the western blot analysis were shown in (A). Statistical analysis of the expressions of GAP-43, Synaptophsin, NGF and BDNF were shown in (B)–(F), respectively. Note that the alteration of the expressions of BDNF and NGF occurred on day 1 of L-NBP treatment was prior to the alteration of Synaptophsin and GAP-43. At 7 days after L-NBP incubation, effects of the inhibitors to TrkB receptor and ERK1/2 on L-NBP induced neurites-bearing cells were observed in (G). Representative images of neurites bearing cells were capture in groups of (a) Control, (b) L-NBP, (c) L-NBP with K252a, (d)L-NBP with PD98059 and (e) combination of L-NBP, PD98059 and K252a after 7 days of treatment. Ctrl: control. Data were shown as mean ± S.E.M. of three separate experiments. The significance was indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group.

2.3. PC12 cell culture

PC12 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), 10% horse serum, 2 mM glutamine, and penicillin (100U/ml)/streptomycin (100 μg/ml) as previously described (Cui et al., 2011b; Zhao et al., 2015a, 2015b).

2.4. Culture of embryonic cortical neurons

Cortical neurons were prepared from the embryonic E18 SD rats (Zhao et al., 2006). Dissociated cells were seeded in DMEM/F12 medium with 10% FBS and 2% B27. L-NBP was incubated every two days.

2.5. OGD/R insults

OGD/R insults were induced as previously described (Hasan et al., 2013; Zhong et al., 2015). Briefly, the culture medium was replaced on DIV 3 with a glucose-free, serum free DMEM/F12 medium and PC12 cells or cortical neurons were kept in an anaerobic incubator (Thermo Biotech, USA) with an atmosphere of 95% N2, 5% CO2 at 37 °C for 2 h. OGD was then terminated and re-oxygenation was followed by changing back to the regular culture medium and the culture condition.

2.6. Neuritogenic activities in PC12 cells

PC12 cells were plated in growth medium at 5×10^4 cells per well in poly-L-lysine-coated 6-well plates as previously described (Cui et al.,

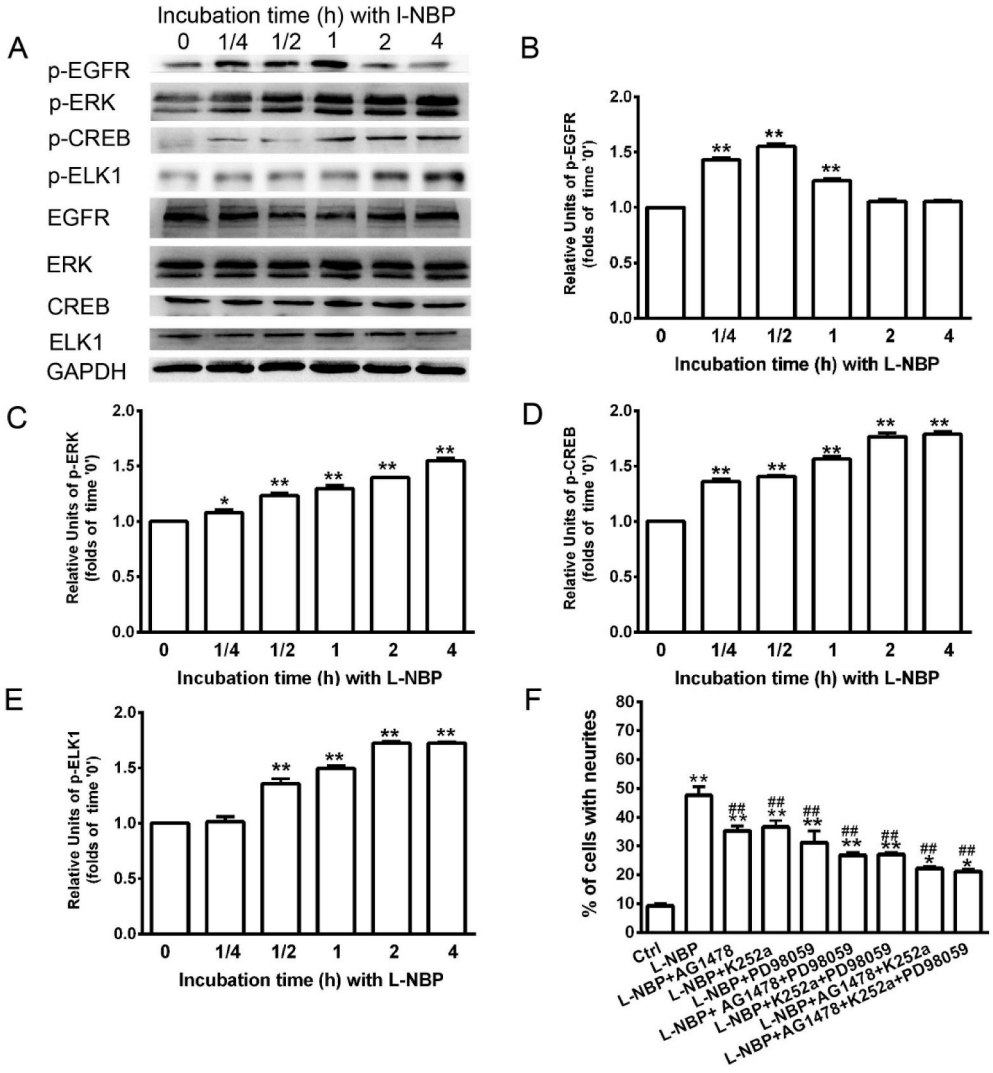


Fig. 4. L-NBP induces neurite outgrowth by the prompt activation of ERK1/2 mediated EGFR pathway in PC12 cells. (A) L-NBP at 10 μM was applied to PC12 cells and cells were collected at different time points as indicated. Expressions of Phospho-EGRF (p-EGFR), Phospho-ERK1/2 (p-ERK), phospho-CREB (p-CREB), phospho-ELK1 (p-ELK1) and their non-phosphorylated forms were examined by western blot analysis. Statistical analysis of the expressions of p-EGFR, p-ERK, p-CREB and p-ELK1 were shown in B, C D and E, respectively. (F) Cells were pretreated with AG 1478, PD98059 and K252a for 30 min and then incubated with L-NBP. After seven days after drug treatment, statistical analysis of the cells with neurites was counted. Single dose or the combinations of these inhibitors showed significant blockage of the effects of L-NBP. The data were shown as mean ± S.E.M. of three separate experiments. The significance was indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group, with $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus L-NBP group.

2011a; Zhao et al., 2015a, 2015b). On the second day, cells were washed and incubated in DMEM supplemented with 1% FBS, 1% horse serum and various concentrations of experimental compounds. L-NBP was incubated every two days.

2.6.1. Observation of growth cone development

After two days of drug incubation, growth cones were captured by Leica TCS SP8 confocal microscope. Numbers of growth cones of each cell were counted. Growth cone area were measured using Image J software (National Institutes of Health, Bethesda, MA, USA) as previous described (Myers and Gomez, 2011).

2.6.2. Neurites outgrowth evaluation

Neurites only processes longer than 20 μm were considered as neurites outgrowth. A cell is regarded as a 'positive' cell with neurites extension if the neurites reach at least one fold of the length of the cell soma. Neurite outgrowth was quantified as the percentage of the 'positive' cells bearing neurite length. GAP-43 expressions were further detected by western blot analysis to observe the neurites extension activity and the further synapse activity.

2.6.3. Synapse formation detection

As well as GAP-43, synapse formation was observed by the expressions of the synapse biomarker, synaptophysin. The expressions of GAP-43 and synaptophysin were detected by western blot analysis at day 0, 1, 2, 4, 6 and 7 after drug treatment. In addition, the immunofluorescent staining of synaptophysin was performed at day 5 after the OGD/R insults.

2.7. Western blot analysis

Cells were harvested in the lysis buffer RIPA. The protein was isolated on SDS-polyacrylamide gel and transferred onto polyvinyldifluoride membranes. Following membrane blocking, proteins were detected by primary antibodies (Supplement table 1) overnight at 4 °C. Then the membranes were incubated with chemiluminescent secondary antibodies. Blots were developed by the ECL Plus kit (Amersham Bioscience, Aylesbury, UK) and images were captured by Fuji Imaging Device. All data obtained from three independent experiments were expressed as the ratio to optical density (OD) values of the corresponding controls for statistical analyses.

2.8. Immunofluorescence staining

Cells were fixed in 4% formaldehyde, membrane-permeabilized in 0.1% Triton X-100 and blocked in 5% bovine serum albumin (BSA). Cells were then exposed to the primary antibody (Supplement table 1) overnight at 4 °C followed by incubation with an Alexa Fluor 488-

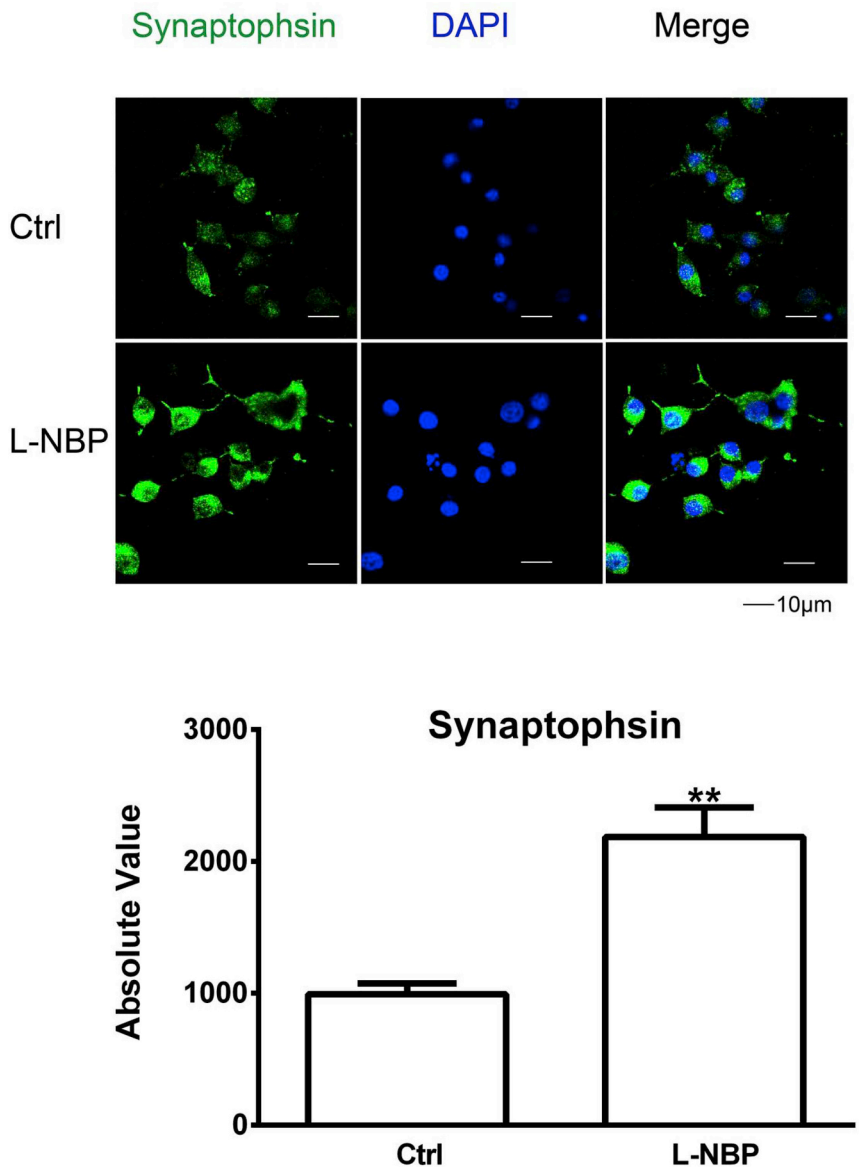


Fig. 5. L-NBP attenuates the OGD/R induced the impaired synapse function in PC12 cells. PC12 cells were incubated with L-NBP at 10 μM during and after the OGD insults. Synaptophsin expressions were detected by immunofluorescent staining after 5 days of OGD insults. Upper panel: Representative images of cells which were stained by synaptophsin antibody were shown. Lower panel: Statistical analysis of the expressions of synaptophsin was shown. Ctrl: Control. The data, expressed as mean ± S.E.M., were shown as three separate experiments, with $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group.

conjugated secondary antibody at room temperature, respectively. Images were captured by Leica TCS SP8 confocal microscope.

2.9. Sholl analysis in cultured neurons

βIII-tubulin expression was detected by immunofluorescent staining in cortical neurons. Immunofluorescent images were acquired by fluorescence microscopy. Neurites morphology was observed through βIII-tubulin staining and was further investigated by Sholl analysis (Day et al., 2014). Images were analyzed by the plugin NeuronN in the Image J software (NIH, US). A calibrated image of concentric circles at 10 μm distances (up to 300 μm) was superimposed onto the cell soma. Primary neurites, neuritic branches and neurite termination points were counted. Primary neurites were classified as those directly stemming from the cell body within circle number 1, while a branch was counted if a neurite clearly divides in two for at least 5 μm. The longest neurite was utilized as the neuritic length.

2.10. Measurement of neurotoxicity

2.10.1. Assessment of cell viability by MTT reduction assay

Cell viability was detected by MTT reduction assay. The absorbance of the samples was measured at a wavelength of 570 nm with 655 nm as a reference wavelength. All experiments were performed in triplicate.

2.10.2. LDH release assay

Culture medium of the neurons was collected and analyzed for LDH activity using the LDH assay kit (Nanjing Jian Cheng Biotechnology, Nanjing, Jiangsu, China) as indicated in the manual. The absorbance was measured at a wavelength of 450 nm using a microplate reader. All experiments were performed in triplicate.

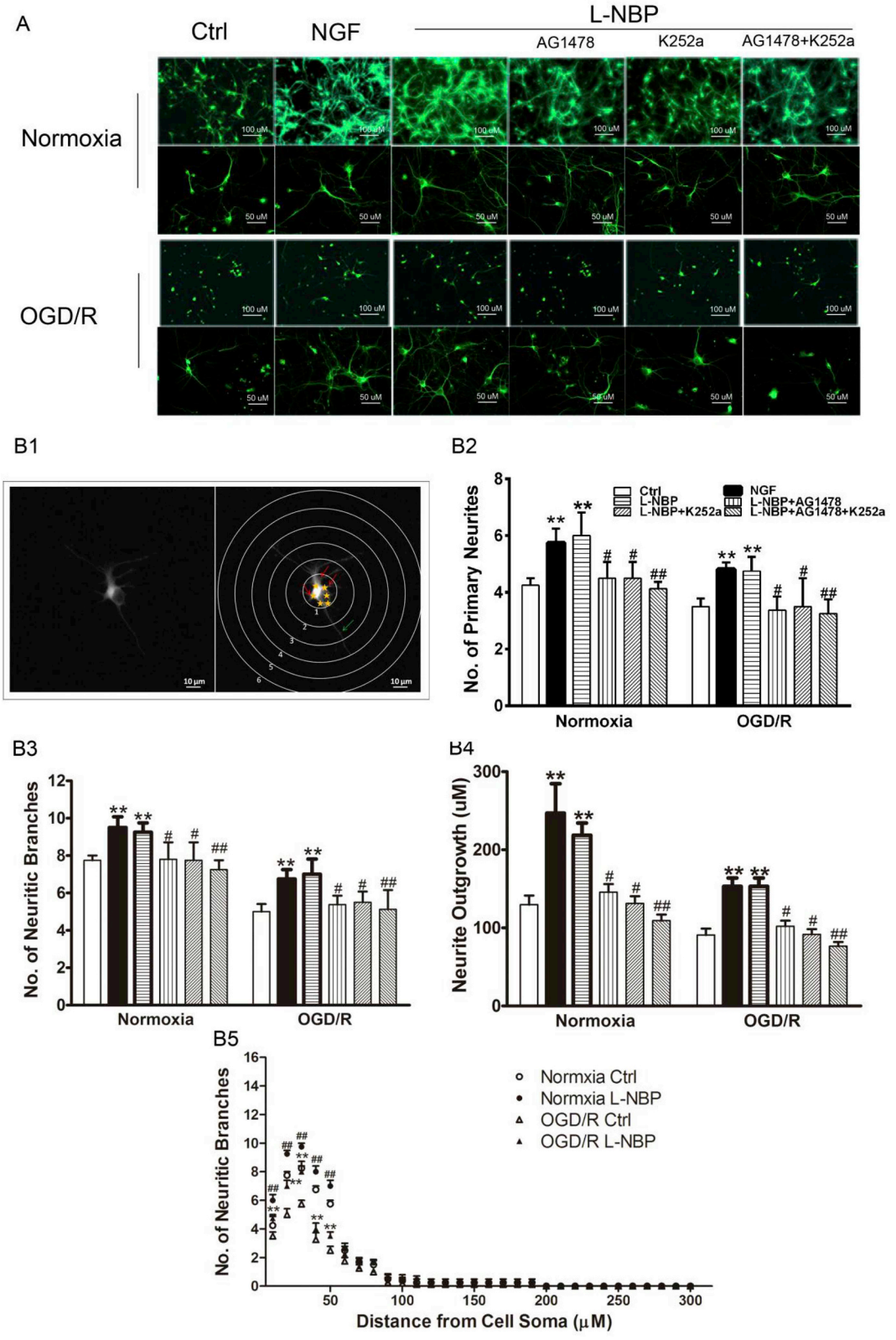
2.10.3. Analysis of chromatin condensation

Chromatin condensation was detected by nucleus staining with Hoechst 33342 (Zhao et al., 2006). Cells were stained with

Hoechst33342 (5 μg/ml) for 5 min at 4 °C. The nuclei were visualized using a fluorescence microscope at 400 × magnification. The apoptotic cells showed condensed or fragmented fluorescent nuclei. Numbers of apoptotic nuclei were scored by counting at least 500 cells for each group in triplicate.

2.11. ELISA assay

Culture medium of cortical neurons was collected. BDNF and NGF immunoassay kits were purchased from Boster (Wuhan, Hubei, China) and applied according to the manufacturer's manual. Absorbance was



(caption on next page)

Fig. 6. L-NBP increases the development of the neuronal complexity of primary cultured cortical neurons under the basal normoxia condition and the OGD/R insults. Neurons were subjected to βIII-tubulin immunofluorescent staining. (A) Representative images of cortical neurons which were treated with NGF 50 ng/ml or L-NBP 10 μM for 7 days were shown. (B) Sholl analysis showed that L-NBP increased the measures of the neuronal complexity. In the Sholl analysis, a calibrated image of concentric circles at 10 μm distances (up to 300 μm) was superimposed onto the cell soma (B_1). A neuron was fluorescently stained with neuronal structural protein βIII-tubulin. The neuron was considered to have six primary neurites (indicated with yellow stars), with three bifurcations between circles 1 and 2 (red arrows), one bifurcations between 2 and 3 and one bifurcations between 3 and 4. The longest neurite terminated between circles 4 and 5 (green arrow). Scale bar: 10 μm. L-NBP significantly increased (B_2) the number of primary neurites, (B_3) the number of neuritic branches and (B_4) the neuritic length. Blockage of Trk receptors and EGFR receptor could significantly attenuated neuronal complexity promoted by L-NBP. (B_5) L-NBP significantly promoted branches extension within 50 μm of the cell soma. The data, expressed as mean ± S.E.M., were shown as three separate experiments, with $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group, with $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus L-NBP group.

determined at a wavelength of 450 nm using a microplate reader.

2.12. Statistical analysis

The data are expressed as mean ± S.E.M. All analyses of data were performed with GraphPad Prism version 6.0 (GraphPad Software, San Diego CA). Analysis of variance (ANOVA) followed by a Dunnett's test was used for statistical comparisons between two groups. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. L-NBP promotes the neuritogenic activities in PC12 cells in a concentration- and time-dependent manner

PC12 cells were incubated with L-NBP for seven days to explore the effects of L-NBP on the neuritogenic activities. L-NBP ranging between 0.1 μM and 100 μM concentration-dependently enhanced the neurites elongation (Fig. 1). Morphological observation showed that the neurites bearing cells were dramatically increased by L-NBP (Fig.1A to B). On the other hand, expressions of GAP-43 confirmed that L-NBP enhanced the neurites outgrowth (Fig. 1C and .D). As reflected by the GAP-43 expression, L-NBP at 30 and 100 μM displayed similar efficacy as NGF at 50 ng/ml.

Further investigations revealed that L-NBP stage- and time-dependently promoted the neuritogenic developments. For the neurite initiation effects, growth cone developments were observed after two days of drug treatment (Fig. 2). On day 2, growth cones could be obviously observed and neurites tips projected outside of the growth cones. Compared with control group, L-NBP (10 μM) significantly enhanced the numbers of growth cone per soma and the average area of the growth cones (Fig. 2). To observe the effects of L-NBP on the neurite extension, GAP-43 expressions were detected (Fig. 3). Compared with control group, L-NBP promoted the GAP-43 expressions over 50% after the two days of drug treatment. And GAP-43 expression was enhanced over 80% by L-NBP after the seven days of drug treatment. To observe the effects of L-NBP on the synapse formation and cell maturation, synaptophsin expression was evaluated (Fig. 3A and C). Similar to the expressions of GAP-43, synaptophsin expression was enhanced by L-NBP over 2.5 folds from day 2 to day 7 after drug treatment.

3.2. Up-regulations of BDNF and NGF play roles in L-NBP induced neurites extension in PC12 cells

The expressions of BDNF and NGF were up-regulated by L-NBP at 10 μM from day 1 of treatment (Fig. 3). Interestingly, the expressions of GAP-43 and synaptophsin were enhanced from day 2 of L-NBP treatment which implicated that BDNF and NGF might be involved in the neuritogenic effects of L-NBP. K252a, the antagonist of tyrosine phosphorylation receptor, could significantly block the effects of L-NBP that supported our deduction (Fig. 3G). PD98059, the specific inhibitor of MEK, could significantly abolish the neurites growth induced by L-NBP (Fig. 3G), indicating that the ERK1/2-mediated pathways might participate in L-NBP induced neurite extension. The combination of PD98059 and K252a could not completely inhibit the neuritogenic actions caused by L-NBP, indicating that other signals might play roles in the effects of L-NBP.

3.3. Rapid activations of EGFR and its downstream factors attribute to L-NBP induced neuritogenesis in PC12 cells

Effects of L-NBP on the EGFR related pathway were detected. Within 15 min, L-NBP promptly activated EGFR and its downstream factors, ERK1/2 and CREB, by enhancing the levels of their phosphorylation forms in PC12 cells (Fig. 4A to Fig. 4D). Within 30 min, L-NBP triggered the activation of ELK1 (Fig. 4E). Activation of EGFR was maintained for 2 h after L-NBP treatment, while other activated signals were sustained for 4 h after drug treatments (Fig. 4A–E). L-NBP did not alter the expressions of the non-activated forms of the proteins investigated. AG1478, the specific EGFR inhibitor, partially attenuated L-NBP-induced neurites extension (Fig. 3F). Compare with AG1478, combination of AG1478, PD98059 and k252a caused more substantial inhibition of L-NBP. However, this combination did not completely suppress the promotion of L-NBP on neurite outgrowth, indicating that L-NBP is a multiple target regulator that some other pathways might also be involved besides the current reported EGFR-, BDNF- and NGF-related signals.

3.4. L-NBP rescued the synapse loss in PC12 cells after OGD/R insults

After five days of OGD insults, neurites were extensively broken. Cell shrinkage, chromatin condensation and fragmented occurred (Fig. 5). Synaptophsin expressions were dramatically decreased by OGD impairment, implying that synapse function was impaired. L-NBP could significantly up-regulated the expressions of synaptophsin and the cells demonstrated a profile of extended neurites around soma. It might indicate that L-NBP probably effectively rescued the OGD induced retrograde synapse degeneration.

3.5. L-NBP enhanced neuronal complexity in primary cultured cortical neurons under normoxia and OGD/R conditions

Based on the observations of PC12 cells, induction of neuritogenesis by L-NBP was further investigated in primary cultured cortical neurons. Immunocytochemical staining for βIII-tubulin was performed in neurons under basal normoxia conditions and OGD/R insults (Fig. 6 and Fig. 7). No matter under the normoxia or OGD/R impairment, morphology observation showed that L-NBP at 10 μM significantly enhanced the neuronal complexity of the cultured neurons (Fig. 6A). Facilitated by Sholl analysis, the effects of L-NBP on the neuronal complexity, including the number of primary neurites extending from the cell soma, the number of neuritic branches and the neuritic lengths, were examined (Fig. 6B). L-NBP significantly improved the Sholl profile by increasing the number of the primary neurites (Fig. 6B_2), the number of the neuritic branches (Fig. 6B_3 and Fig. 6B_5) and the neurite outgrowth (Fig. 6B_4). AG1478 and K252a significantly reduced the effects of L-NBP (Fig. 6). Compared with single usage of AG1478 and K252a, the combination of the two agents significantly blocked the neurite outgrowth enhanced by L-NBP (Fig. 6B_4).

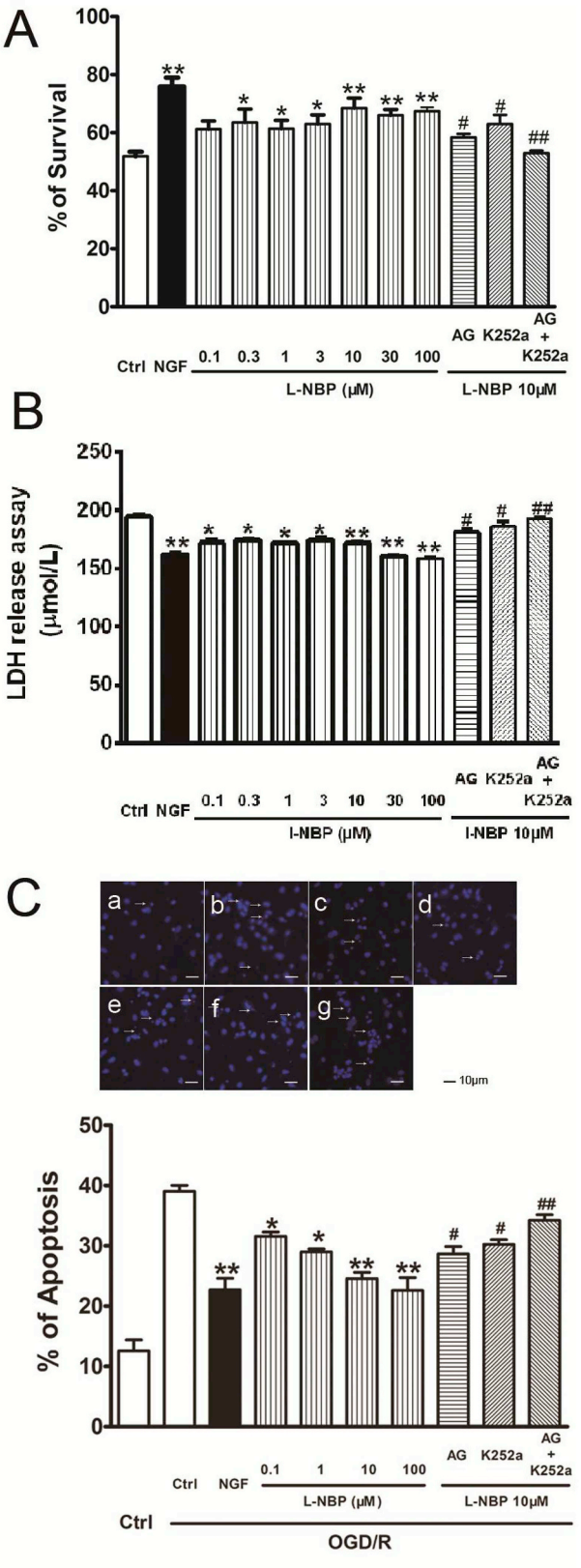


Fig. 7. L-NBP reduces the OGD/R induced neurotoxicity through BDNF, NGF and EGFR related pathways. (A) MTT reduction assay showed that L-NBP blocked the neuron death by the OGD/R insults. In line with MTT reduction assay, (B) LDH release assay proved that L-NBP attenuates the neuron damage irrigated by the OGD/R insults. (C) Hoechst33342 staining demonstrated that L-NBP rescued OGD/R-induced chromatin condensation. Blockage of Trk receptors and EGFR receptor could significantly attenuates the protection of L-NBP against the neurotoxicity. The data, expressed as mean ± S.E.M., were shown as three separate experiments, with $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group, with $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus L-NBP group.

3.6. L-NBP reduced neurotoxicity induced by OGD/R through BDNF-, NGF- and EGFR-related pathways

In our study, OGD/R caused a significant cell death in cortical neurons. And OGD/R increased the ratio of cells with the profile of cell shrinkage, chromatin condensation and fragmented fluorescent nuclei. MTT reduction assay proved that the cell survival rate was up to 51.8% after the OGD/R insults (Fig. 7A). NGF rescued the cell survival to 75.9%. L-NBP attenuated neuronal loss in a concentration dependent manner and the survival rate was up to 68.5%. LDH release was also dramatically inhibited by L-NBP (Fig. 7B). In addition, L-NBP markedly reversed the morphological changes, including the shrinkage of the cell bodies, the broken neuritic network (Figs. 6A and 8C) and the condensed or fragmented nuclei (Fig. 7C). In line with the results from PC12 cell models, L-NBP could up-regulate the expressions of BDNF and NGF in a dose-dependent manner (Fig. 8). Antibodies of BDNF and NGF (Fig. 8) as well as K252a (Fig. 7) could significantly suppress the protection of L-NBP on the OGD insults. Single usage of AG1478, K252a and the combination of the two drugs blocked the neuroprotective effects of L-NBP (Fig. 7). Compared with AG1478 and K252a, the combination of the two agents induced more intensive inhibition of the protection of L-NBP.

4. Discussion

As well as the enhanced endo-neurogenesis activities (Chen et al., 2015), the up-regulated neuritogenic activities play crucial roles in the repair of the impaired neuronal complex (Ip et al., 2016; More et al., 2012; Polleux and Snider, 2010). A recent study by our group demonstrated that L-NBP potentiates neurogenesis which stimulated the stem cells to differentiate into newborn neurons in the CA3 hippocampus region and ameliorated neuronal loss after ischemic insults (Yang et al., 2015). However, the effect of L-NBP on the functional recovery of injured neuronal networks in the other brain regions remains unknown. Hence,it is hypothesized that L-NBP might enhance the neuritogenic actions which rescue the declined brain function in these regions when stroke occurs. The study illuminated that: 1) L-NBP promoted the outgrowth of neurites and neuronal complexity in a concentration and time dependent manner under the normoxia condition, 2) L-NBP contributed to the reconstruction of neuronal network by stimulating neuritogenesis in the damaged neurons under the OGD/R condition, and 3) the underlying mechanism of the neuritogenic and neuronal survival activities of L-NBP was mediated by multiple targets, particularly through the activations of the growth factors/neurotrophic factors related signals.

As reported, several cell models were applied in the investigations of neurite outgrowth and neuronal network ((Cheung et al., 2007; Day et al., 2014; Frimat et al., 2010; Hensel et al., 2016; Loers et al., 2017; Zhao et al., 2015a)). Among them, PC12 cell line is a simple and stable model in the neuritogensis study by which canonical signals,

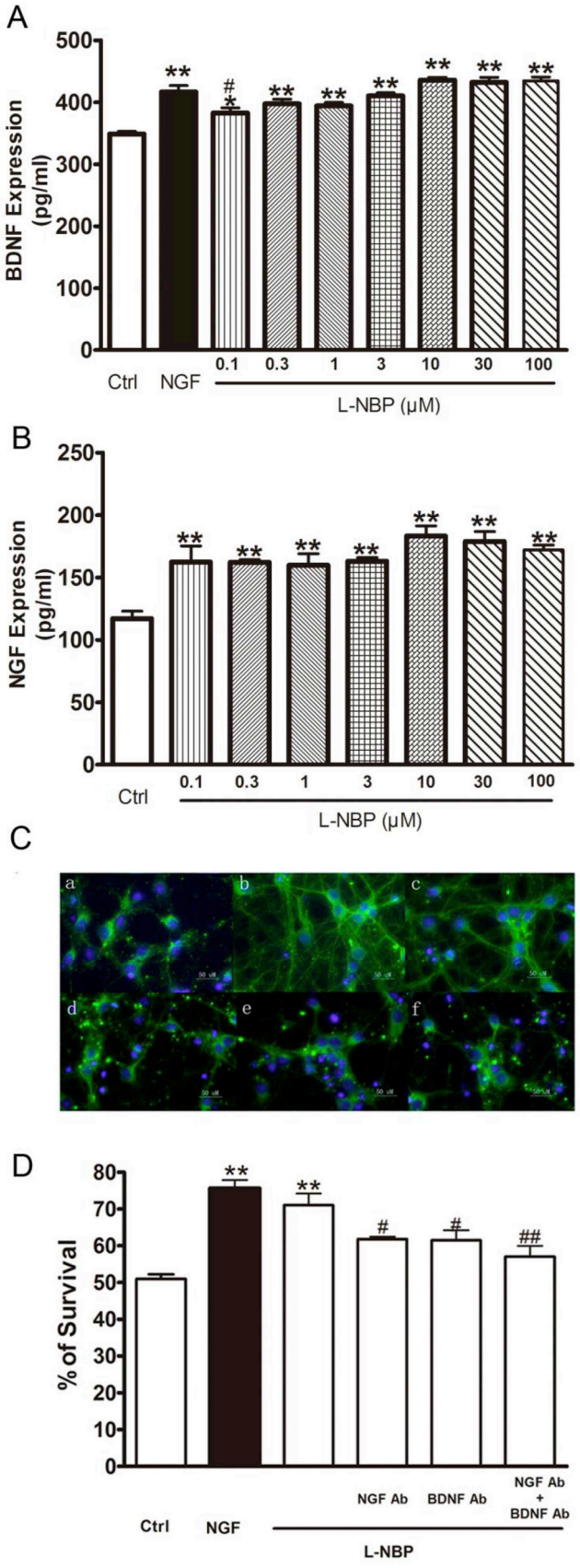


Fig. 8. Blockage of the receptors of BDNF and NGF by their relative antibodies abolishes the neuroprotection of L-NBP in OGD insults. After 7 days of treatment, L-NBP promoted the expressions of BDNF (A) and NGF (B) which were detected by ELISA assay. (C) Cortical neurons were stained by βIII-tubulin antibody under normoxia. (a) Control, (b) NGF 50 ng/ml (c) L-NBP 10 μM; (d) L-NBP + BDNF antibody; (e) L-NBP + NGF antibody; and (f) L-NBP + antibodies of BDNF and NGF. Note that neuronal complexity was obviously impaired by BDNF antibody (Millipore, Ab1778,5 μg/ml), NGF antibody (Abcam, ab6199,2 μg/ml) and their combination. (D) MTT reduction assay were performed to show the survival rates of neurons after 2 h OGD/24 h reperfusion. The data, expressed as mean ± S.E.M., were shown as three separate experiments $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group, with $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus NGF group in (A), and with $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus L-NBP group in (D).

particularly the MAPK/ERK pathway, have been well demonstrated (Cui et al., 2011a; Zhao et al., 2015a, 2015b). In the current study, the neuritogenic effects of L-NBP were first explored in PC12 models. Results revealed that L-NBP promoted the growth cone development, the elongation of the neurites and the further synapse formation in a stage-dependent manner. In particular, L-NBP contributed to the maturation process of the cells which were reflected by the enhanced expressions of the axon biomarker (GAP43) and the synapse biomarker (synaptophsin). Based on its neuritogenic property, L-NBP potentially rescued the synapse loss and improved the neuronal survival under the OGD/R insults.

Besides the PC12 model, the neuritogenesis activity of L-NBP in primary cultured cortical neurons were evaluated. The numbers of primary regenerating neurites were counted by Sholl analysis, which indicated the status of neurite initiation (phase I of neuritogenesis). Meanwhile, the neurites outgrowth and the neuritic branches were observed upon the addition of L-NBP, which reflected the effects of the neuritogenesis Phase II & III. Facilitated by β III-tubulin immunostaining, a biomarker of the special neuronal tubulin, the strengthened synapses formation and neuronal complex by L-NBP were observed.

To detect the cell viability after the OGD insults, MTT reduction assay, LDH release assay and Hoechst staining were investigated. The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells present (Stockert et al., 2018). These enzymes are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple color. Therefore, MTT reduction assays can be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials. Lactate dehydrogenase (LDH) is an essential enzyme for the cell metabolism. Herein, we detect the leaked LDH in the cell culture medium which could indicate the impairment extent of the cells after the insults (Song et al., 2017; Xie et al., 2015). And the variations of chromatin condensation and fragmented nuclei could be observed by Hoechst staining analysis. Through these methods, L-NBP demonstrated significant protections against the OGD insults.

Our previous study demonstrated that L-NBP enhanced the expressions of BDNF even 28 days after the stroke insults (Yang et al., 2015). It is known that BDNF belongs to the neurotrophin family which decide cell fate, axon growth, dendrite pruning, patterning of innervations and the expression of proteins responsible for normal physiological function (Huang and Reichardt, 2001). Neurotrophins could activate two different types of receptors (the Trk receptor family and p75NTR) to induce neurotrophic effects (Botella et al., 2003; Cohen-Cory et al., 2010; Sarma et al., 2015; Zhao et al., 2015b). And NGF, the first identified neurotrophin, plays critical roles in the neurites outgrowth and is a

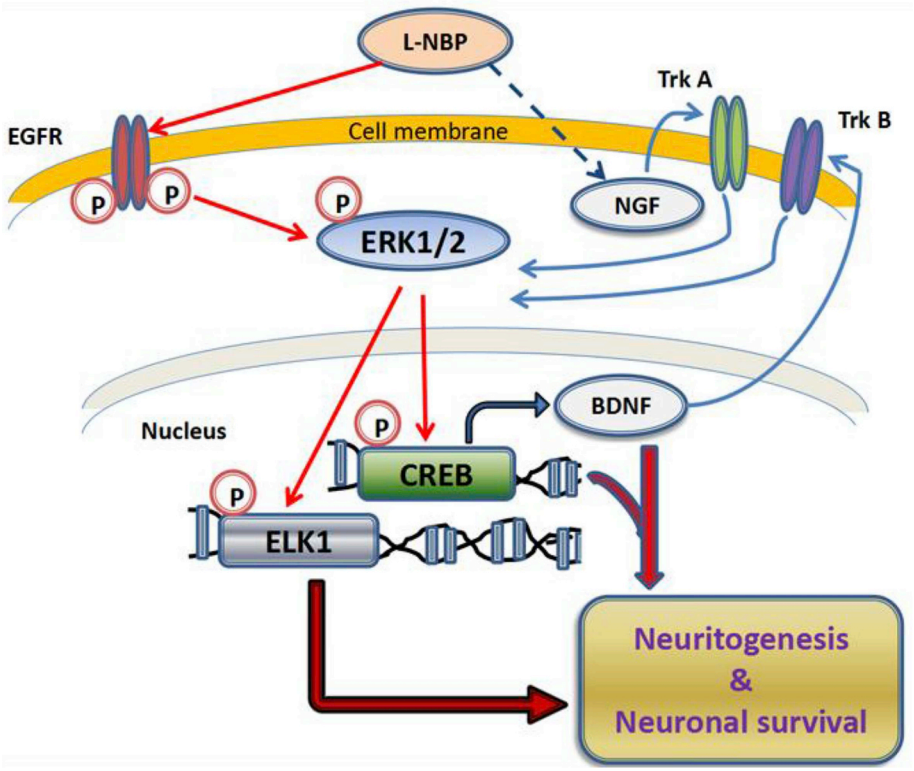


Fig. 9. Illustration summarizes the underlying mechanism of the effects of L-NBP on neuritogenesis and neuronal survival. L-NBP promptly activated ERK1/2 mediated EGFR pathway, raising the activation of the transcriptions of CREB and ELK1 and then the expressions of BDNF. Besides promoting the delayed expression of BDNF, L-NBP enhanced the expression of NGF. The two neurotrophins might activate their Trk receptor and then further activated ERK1/2 and its downstream effectors. Therefore, centered by ERK1/2, a positive feedback loop might be initiated and the effect of L-NBP might be amplified.

promising target for the study of neuritogenesis (Liao et al., 2012; Ling-Sing Seow et al., 2013; Lu et al., 2014; Nagahara et al., 2012; Nardicchi et al., 2014; Phan et al., 2014). Therefore, the effects of targeting BDNF- and NGF-mediated pathways, and the related mechanism of L-NBP on neuritogenesis were investigated. Results demonstrated that L-NBP potentiated neurite outgrowth and synapse formation from day 2 of treatment (Figs. 2 and 3). L-NBP caused the sustained up-regulations of BDNF and NGF from day 1 after its application, which is prior to the events of the neurites extension (Fig. 3). K252a and antibodies of BDNF and NGF partially abolished the neuritogenic effects of L-NBP, indicating that BDNF and NGF were involved in the actions of L-NBP (Figs. 7 and 8).

Since the Trk receptor inhibitor and the antibodies of BDNF and NGF could only partially suppress L-NBP-induced neuritogenesis, it was conjectured that other targets were involved in the effects of L-NBP. In particular, how L-NBP initiated the neuritogenic signals right after the ischemic insults still were unknown. EGFR is a trans-membrane growth receptor that triggers a rich network of signaling pathways to control cell survival and neurites growth (Lu et al., 2014; Xiang et al., 2006; Xu et al., 2014). Binding of the agonist with the receptor results in the receptor dimerizations which activates the intrinsic intracellular protein-tyrosine kinase activity, leading to the autophosphorylation of tyrosine (Y) residues (Y992, Y1045, Y1068, Y1148 and Y1173) in the C-terminal domain of EGFR (Tyson et al., 2003). The activated receptors simultaneously trigger downstream signaling cascades, transfer the signals into the nucleus and manipulate the subsequent transcription processes (e.g. the ERK1/2 mediated pathway). In the current study, it was found that L-NBP promptly activated EGFR (Y1068) within 15min of application, with subsequent activating of its downstream target ERK1/2 and the transcription factors CREB and ELK1 (Fig. 4). AG1478, the EGFR antagonist, partially reduced the effect of L-NBP, suggesting that EGFR contributed to L-NBP-induced neurogenesis. Compared with AG1478 alone, the combination of AG1478 with K252a significantly reduced the neuritogenic effect of L-NBP, indicating that L-NBP potentiated neuritogenesis through the EGFR pathway as well as BDNF and NGF signals.

There might be cross-talk between signaling pathways and ERK1/2 might serve as an important pathway node in the network which triggers the neurites development (Hasan et al., 2013; Hu et al., 2015; Manecka et al., 2013; Zhao et al., 2015b). In signaling cascades activated by NGF, BDNF and EGF, binding of agonists to the Trk receptors result in the activation of these receptors and their downstream effectors. In the current study, L-NBP enhanced the prompt activations of EGFR, ERK1/2, CREB and ELK1, and the sustained expressions of NGF and BDNF. Interestingly, the synthesis of BDNF is controlled by ERK1/2/CREB pathway. We conjecture that the prompt activation of EGFR by L-NBP probably facilitate the activation of ERK1/2 and the synthesis of BDNF. Hence, a positive feedback loop that regulates neuritogenesis might be developed under the induction of L-NBP.

In summary, L-NBP promoted neuritogensis and neuronal survival under OGD/R insult through the activation of multiple growth factor-mediated signaling pathways. The proposed mechanism is illustrated in Fig. 9. This indicated that L-NBP potentially stimulated the synapse formation through the up-regulated neuritogenic activities which compensated the declined brain function after stroke. In addition, these activities might extensively occur in the whole damaged brain regions, including the hippocampus. The regenerative properties of L-NBP, including promoting neurogenesis (Yang et al., 2015), neuritogensis and angiogenesis (Cheung et al., 2007), could be of benefit to the treatment of aging-related neurodegenerative diseases and cognitive impairment.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

We sincerely thank Ms Jun Deng, Ms Yan-yan Jin and Mr. Zhong-xin Xiao from the Laser scanning confocal microscopy laboratory, Core facilities center of Capital Medical University for the instruction and assistance of the imaging capturing. We sincerely thank Ms. Annballaw Bridget Leigh for proof-reading our manuscript. This work was

supported by grants from National Natural Science Foundation of China (31741035,81373387,31570856), Shandong Province Natural Science Foundation (ZR2017MH117), Key Research and Development Projects of Sichuan Science and Technology Department (2017SZ0201), Scientific Research Project of Beijing Educational Committee (KM201610025003),and Beijing Institute of Brain Disorders & the Capital Medical University Research Center for Brain Disorders Open Project Funding (2014NZDJ06).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.01.002.

References

Botella, J.A., Kretzschmar, D., Kiermayer, C., Feldmann, P., Hughes, D.A., Schneuwly, S., 2003. Deregulation of the Egfr/Ras signaling pathway induces age-related brain degeneration in the Drosophila mutant vap. Mol. Biol. Cell 14, 241–250.

Chen, S., Luo, M., Zhao, Y., Zhang, Y., He, M., Cai, W., Liu, A., 2015. Fasudil stimulates neurite outgrowth and promotes differentiation in C17.2 neural stem cells by modulating notch signalling but not autophagy. Cell. Physiol. Biochem. 36, 531–541.

Cheung, Z.H., Chin, W.H., Chen, Y., Ng, Y.P., Ip, N.Y., 2007. Cdk5 is involved in BDNF-stimulated dendritic growth in hippocampal neurons. PLoS Biol. 5, e63.

Cohen-Cory, S., Kidane, A.H., Shirkey, N.J., Marshak, S., 2010. Brain-derived neurotrophic factor and the development of structural neuronal connectivity. Dev Neurobiol 70, 271–288.

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., Han, Y., 2011a. 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.

Cui, W., Li, W., Zhao, Y., Mak, S., Gao, Y., Luo, J., Zhang, H., Liu, Y., Carlier, P.R., Rong, J., Han, Y., 2011b. Preventing H(2)O(2)-induced apoptosis in cerebellar granule neurons by regulating the VEGFR-2/Akt signaling pathway using a novel dimeric antiacetylcholinesterase bis(12)-hupyridone. Brain Res. 1394, 14–23.

Day, J.S., O'Neill, E., Cawley, C., Aretz, N.K., Kilroy, D., Gibney, S.M., Harkin, A., Connor, T.J., 2014. Noradrenaline acting on astrocytic beta(2)-adrenoceptors induces neurite outgrowth in primary cortical neurons. Neuropharmacology 77, 234–248.

Frimat, J.P., Sisnaiske, J., Subbiah, S., Menne, H., Godoy, P., Lampen, P., Leist, M., Franzke, J., Hengstler, J.G., van Thriel, C., West, J., 2010. The network formation assay: a spatially standardized neurite outgrowth analytical display for neurotoxicity screening. Lab Chip 10, 701–709.

Hasan, M.R., Kim, J.H., Kim, Y.J., Kwon, K.J., Shin, C.Y., Kim, H.Y., Han, S.H., Choi, D.H., Lee, J., 2013. Effect of HDAC inhibitors on neuroprotection and neurite outgrowth in primary rat cortical neurons following ischemic insult. Neurochem. Res. 38, 1921–1934.

Hensel, N., Schon, A., Konen, T., Lubben, V., Forthmann, B., Baron, O., Grothe, C., Leifheit-Nestler, M., Claus, P., Haffner, D., 2016. Fibroblast growth factor 23 signaling in hippocampal cells: impact on neuronal morphology and synaptic density. J. Neurochem. 137, 756–769.

Hu, S., Wang, R., Cui, W., Zhang, Z., Mak, S., Xu, D., Choi, C., Tsim, K.W., Carlier, P.R., Lee, M., Han, Y., 2015. Inhibiting beta-amyloid-associated Alzheimer's pathogenesis in vitro and in vivo by a multifunctional dimeric bis(12)-hupyridone derived from its natural analogue. J. Mol. Neurosci. 55, 1014–1021.

Huang, E.J., Reichardt, L.F., 2001. Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. 24, 677–736.

Huang, J.Z., Chen, Y.Z., Su, M., Zheng, H.F., Yang, Y.P., Chen, J., Liu, C.F., 2010. dl-3-n-Butylphthalide prevents oxidative damage and reduces mitochondrial dysfunction in an MPP(+)-induced cellular model of Parkinson's disease. Neurosci. Lett. 475, 89–94.

Ip, F.C., Zhao, Y.M., Chan, K.W., Cheng, E.Y., Tong, E.P., Chandrashekar, O., Fu, G.M., Zhao, Z.Z., Ip, N.Y., 2016. Neuroprotective effect of a novel Chinese herbal decoction on cultured neurons and cerebral ischemic rats. BMC Complement Altern. Med. 16, 437.

Lei, H., Zhao, C.Y., Liu, D.M., Zhang, Y., Li, L., Wang, X.L., Peng, Y., 2014. l-3-n-Butylphthalide attenuates beta-amyloid-induced toxicity in neuroblastoma SH-SY5Y cells through regulating mitochondrion-mediated apoptosis and MAPK signaling. J. Asian Nat. Prod. Res. 16, 854–864.

Liao, K.K., Wu, M.J., Chen, P.Y., Huang, S.W., Chiu, S.J., Ho, C.T., Yen, J.H., 2012. Curcuminoids promote neurite outgrowth in PC12 cells through MAPK/ERK- and PKC-dependent pathways. J. Agric. Food Chem. 60, 433–443.

Ling-Sing Seow, S., Naidu, M., David, P., Wong, K.H., Sabaratnam, V., 2013. Potentiation of neuritogenic activity of medicinal mushrooms in rat pheochromocytoma cells. BMC Complement Altern. Med. 13, 157.

Loers, G., Astafiev, S., Hapiak, Y., Saini, V., Mishra, B., Gul, S., Kaur, G., Schachner, M., Theis, T., 2017 Aug. The polysialic acid mimetics idarubicin and irinotecan stimulate neuronal survival and neurite outgrowth and signal via protein kinase C. J. Neurochem. 142 (3), 392–406. https://doi.org/10.1111/jnc.14076. [Epub 2017 Jun 20].

Lu, X.L., Luo, D., Yao, X.L., Wang, G.L., Liu, Z.Y., Li, Z.X., Li, W., Chang, F.J., Wen, L., Lee, S.M., Zhang, Z.J., Li, L., Zeng, J.S., Huang, R.X., Pei, Z., Ou, J.S., 2012. dl-3n-Butylphthalide promotes angiogenesis via the extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase/Akt-endothelial nitric oxide synthase signaling pathways. J. Cardiovasc. Pharmacol. 59, 352–362.

Lu, Z., Cui, M., Zhao, H., Wang, T., Shen, Y., Dong, Q., 2014. Tissue kallikrein mediates neurite outgrowth through epidermal growth factor receptor and flotillin-2 pathway in vitro. Cell. Signal. 26, 220–232.

Manecka, D.L., Mahmood, S.F., Grumolato, L., Lihrmann, I., Anouar, Y., 2013. Pituitary adenylate cyclase-activating polypeptide (PACAP) promotes both survival and neuritogenesis in PC12 cells through activation of nuclear factor kappaB (NF-kappaB) pathway: involvement of extracellular signal-regulated kinase (ERK), calcium, and c-REL. J. Biol. Chem. 288, 14936–14948.

More, V.S., Sushruta, K., Kim, I.S., Kumar, H., Kim, B.W., Choi, D.K., 2012. The role of bioactive compounds on the promotion of neurite outgrowth. Molecules 17, 6728–6753.

Myers, J.P., Gomez, T.M., 2011. Focal adhesion kinase promotes integrin adhesion dynamics necessary for chemotropic turning of nerve growth cones. J. Neurosci. 31, 13585–13595.

Nagahara, Y., Suzuki, E., Sekine, Y., Uchiro, H., Yoshimi, Y., Shinomiya, T., Ikekita, M., 2012. SUTAF, a novel beta-methoxyacrylate derivative, promotes neurite outgrowth with extracellular signal-regulated kinase and c-jun N-terminal kinase activation. Eur. J. Pharmacol. 694, 53–59.

Nardicchi, V., Ferrini, M., Pilolli, F., Angeli, E.B., Persichetti, E., Beccari, T., Mannucci, R., Arcuri, C., Donato, R., Dorman, R.V., Goracci, G., 2014. NGF induces the expression of group IIA secretory phospholipase A2 in PC12 cells: the newly synthesized enzyme is addressed to growing neurites. Mol. Neurobiol. 50, 15–25.

Peng, Y., Hu, Y., Xu, S., Li, P., Li, J., Lu, L., Yang, H., Feng, N., Wang, L., Wang, X., 2012. L-3-n-butylphthalide reduces tau phosphorylation and improves cognitive deficits in AbetaPP/PS1-Alzheimer's transgenic mice. J. Alzheimers Dis. 29, 379–391.

Peng, Y., Xu, S., Chen, G., Wang, L., Feng, Y., Wang, X., 2007. l-3-n-Butylphthalide improves cognitive impairment induced by chronic cerebral hypoperfusion in rats. J. Pharmacol. Exp. Therapeut. 321, 902–910.

Phan, C.W., Lee, G.S., Hong, S.L., Wong, Y.T., Brkljaca, R., Urban, S., Abd Malek, S.N., Sabaratnam, V., 2014. Hericium erinaceus (Bull.: Fr) Pers. cultivated under tropical conditions: isolation of hericenones and demonstration of NGF-mediated neurite outgrowth in PC12 cells via MEK/ERK and PI3K-Akt signaling pathways. Food Funct 5, 3160–3169.

Polleux, F., Snider, W., 2010. Initiating and growing an axon. Cold Spring Harb. Perspect. Biol. 2, a001925.

Qin, C., Zhou, P., Wang, L., Mamtilahun, M., Li, W., Zhang, Z., Yang, G.Y., Wang, Y., 2018 May 15. Dl-3-N-butylphthalide attenuates ischemic reperfusion injury by improving the function of cerebral artery and circulation. J. Cerebr. Blood Flow Metabol. https://doi.org/10.1177/0271678X18776833. 271678X18776833, [Epub ahead of print].

Rodrigues, T., Reker, D., Schneider, P., Schneider, G., 2016. Counting on natural products for drug design. Nat. Chem. 8, 531–541.

Sarma, T., Koutsouris, A., Yu, J.Z., Krbanjevic, A., Hope, T.J., Rasenick, M.M., 2015. Activation of microtubule dynamics increases neuronal growth via the nerve growth factor (NGF)- and Galphas-mediated signaling pathways. J. Biol. Chem. 290, 10045–10056.

Song, Q., Gou, W.L., Zou, Y.L., 2017. FAM3A protects against glutamate-induced toxicity by preserving calcium homeostasis in differentiated PC12 cells. Cell. Physiol. Biochem. 44, 2029–2041.

Stockert, J.C., Horobin, R.W., Colombo, L.L., Blazquez-Castro, A., 2018. Tetrazolium salts and formazan products in Cell Biology: viability assessment, fluorescence imaging, and labeling perspectives. Acta Histochem. 120, 159–167.

Tang, G., Liu, X., Ma, N., Huang, X., Wu, Z.L., Zhang, W., Wang, Y., Zhao, B.X., Wang, Z.Y., Ip, F.C., Ip, N.Y., Ye, W.C., Shi, L., Chen, W.M., 2016. Design and synthesis of dimeric securinine analogues with neuritogenic activities. ACS Chem. Neurosci. 7, 1442–1451.

Tyson, D.R., Larkin, S., Hamai, Y., Bradshaw, R.A., 2003. PC12 cell activation by epidermal growth factor receptor: role of autophosphorylation sites. Int. J. Dev. Neurosci. 21, 63–74.

Wang, S., Ma, F., Huang, L., Zhang, Y., Peng, Y., Xing, C., Feng, Y., Wang, X., Peng, Y., 2018. Dl-3-n-Butylphthalide (NBP): a promising therapeutic agent for ischemic stroke. CNS Neurol. Disord. - Drug Targets 17, 338–347.

Xiang, Y.Y., Dong, H., Wan, Y., Li, J., Yee, A., Yang, B.B., Lu, W.Y., 2006. Versican G3 domain regulates neurite growth and synaptic transmission of hippocampal neurons by activation of epidermal growth factor receptor. J. Biol. Chem. 281, 19358–19368.

Xiao, J., Liu, Y., 2003. Differential roles of ERK and JNK in early and late stages of neuritogenesis: a study in a novel PC12 model system. J. Neurochem. 86, 1516–1523.

Xie, X., Zhao, Y., Ma, C.Y., Xu, X.M., Zhang, Y.Q., Wang, C.G., Jin, J., Shen, X., Gao, J.L., Li, N., Sun, Z.J., Dong, D.L., 2015. Dimethyl fumarate induces necroptosis in colon cancer cells through GSH depletion/ROS increase/MAPKs activation pathway. Br. J. Pharmacol. 172, 3929–3943.

Xiong, N., Huang, J., Chen, C., Zhao, Y., Zhang, Z., Jia, M., Zhang, Z., Hou, L., Yang, H., Cao, X., Liang, Z., Zhang, Y., Sun, S., Lin, Z., Wang, T., 2012. Dl-3-n-butylphthalide, a natural antioxidant, protects dopamine neurons in rotenone models for Parkinson's disease. Neurobiol. Aging 33, 1777–1791.

Xu, H.L., Feng, Y.P., 2000. Inhibitory effects of chiral 3-n-butylphthalide on inflammation following focal ischemic brain injury in rats. Acta Pharmacol. Sin. 21, 433–438.

Xu, J., Wang, Y., Li, N., Xu, L., Yang, H., Yang, Z., 2012. L-3-n-butylphthalide improves cognitive deficits in rats with chronic cerebral ischemia. Neuropharmacology 62, 2424–2429.

Xu, M.F., Zhou, H., Hu, C.Y., Liang, Y.Q., Hu, L., Chen, D., 2014. The mechanisms of EGFR in the regulation of axon regeneration. Cell Biochem. Funct. 32, 101–105.

Yang, L.C., Li, J., Xu, S.F., Cai, J., Lei, H., Liu, D.M., Zhang, M., Rong, X.F., Cui, D.D.,

Wang, L., Peng, Y., Wang, X.L., 2015. L-3-n-butylphthalide promotes neurogenesis and neuroplasticity in cerebral ischemic rats. CNS Neurosci. Ther. 21, 733–741.
Yang, M., Dang, R., Xu, P., Guo, Y., Han, W., Liao, D., Jiang, P., 2018. Dl-3-n-Butylphthalide improves lipopolysaccharide-induced depressive-like behavior in rats: involvement of Nrf2 and NF-kappaB pathways. Psychopharmacology (Berlin) 235, 2573–2585.
Zhao, C.Y., Lei, H., Zhang, Y., Li, L., Xu, S.F., Cai, J., Li, P.P., Wang, L., Wang, X.L., Peng, Y., 2016. L-3-n-Butylphthalide attenuates neuroinflammatory responses by down-regulating JNK activation and upregulating Heme oxygenase-1 in lipopolysaccharide-treated mice. J. Asian Nat. Prod. Res. 18, 289–302.
Zhao, J., Cheng, Y.Y., Fan, W., Yang, C.B., Ye, S.F., Cui, W., Wei, W., Lao, L.X., Cai, J., Han, Y.F., Rong, J.H., 2015a. Botanical drug puerarin coordinates with nerve growth factor in the regulation of neuronal survival and neuritogenesis via activating ERK1/2 and PI3K/Akt signaling pathways in the neurite extension process. CNS Neurosci. Ther. 21, 61–70.
Zhao, Y., Xu, P., Hu, S., Du, L., Xu, Z., Zhang, H., Cui, W., Mak, S., Xu, D., Shen, J., Han, Y., Liu, Y., Xue, M., 2015b. Tanshinone II A, a multiple target neuroprotectant, promotes caveolae-dependent neuronal differentiation. Eur. J. Pharmacol. 765, 437–446.
Zhao, Y.M., Sun, L.N., Zhou, H.Y., Wang, X.L., 2006. Voltage-dependent potassium channels are involved in glutamate-induced apoptosis of rat hippocampal neurons. Neurosci. Lett. 398, 22–27.
Zhong, M., Song, W.L., Xu, Y.C., Ye, Y., Feng, L.Y., 2015. Paeoniflorin ameliorates ischemic neuronal damage in vitro via adenosine A1 receptor-mediated transactivation of epidermal growth factor receptor. Acta Pharmacol. Sin. 36, 298–310.
Zhu, B.L., Xie, C.L., Hu, N.N., Zhu, X.B., Liu, C.F., 2018. Inhibiting of GRASP65 phosphorylation by DL-3-N-butylphthalide protects against cerebral ischemia-reperfusion injury via ERK signaling. Behav. Neurol. 2018, 5701719.