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BDNF attenuates IL-1beta-induced F-actin remodeling by inhibiting NF-kappaB signaling in hippocampal neurons

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Abstract

OBJECTIVES: To examine the effect of BDNF on F-actin during the stimulation of IL-1β in hippocampal neurons.
MATERIALS & METHODS: We cultured hippocampal neurons from rat embryos. Cell stimulation was induced by IL-1β. Cell culture success was evaluated by an activity analysis of CCK-8, staining of gliocyte by immunohistochemistry. Changes in F-actin, BDNF and NF-κB were examined using molecular analyses.
RESULTS: Our results demonstrate that a high concentration of IL-1β exaggerates the stimulation-induced degradation of F-actin by BDNF, whereas a low concentration of IL-1β protects F-actin against this degradation. These beneficial effects might be associated with the inhibition or exaggeration of the NF-κB signaling cascade.
CONCLUSIONS: Taken together, our findings indicate that BDNF acts as an F-actin-protective regulator during stimulation by IL-1β and that this function largely occurs via the regulation of NF- κB signaling. These results suggest that interventions targeting the BDNF signaling system may be of therapeutic value against major depressive disorder (MDD).

Abbreviations:

MDD	- major depressive disorder
IL-1β	- interleukin-1 beta
BDNF	- brain-derived neurotrophic factor
HBSS	- Hank's Balanced Salt Solution
DMEM/HG	- Dulbecco's modified Eagle's medium/high glucose
DSMO	- dimethylsulfoxide
NSE	- neuron-specific enolase
CCK-8	- Cell Counting Kit-8
ELISA	- enzyme-linked immunoassay
TBST	- Tris-buffered saline containing Tween 20
ANOVA	- analysis of variance

ORIGINAL ARTICLE

INTRODUCTION

Major depressive disorder (MDD) is a debilitating, life-threatening disease that is increasing in prevalence (Kupfer 2012). At present, it is widely recognized that a loss of hippocampal volume is a major risk factor for the development of MDD (Ye *et al.* 2011; Yang *et al.* 2009; Dagytė *et al.* 2011; Eisch & Petrik 2012; Cobb *et al.* 2013; Workmann *et al.* 2011). At the cellular level, MDD is characterized by decreased F-actin (Zhang 2013). Although the multiple signaling mechanisms that control the program of MDD have been extensively studied, the molecular mechanisms that mediate the development of MDD are not completely understood.

The avalilable evidence suggests that immune system plays an important role in regulating psychiatric disorders (Kaneko *et al.* 2006). The expression level of Interleukin-1 beta (IL-1β), which is known as one of the proinflammatory cytokine, is widely changed in various situations in brain (Koo & Duman 2008; Zunszain *et al.* 2012). In our group studys, we identify IL-1β as a critical regulator of hippocampus neuron by stimulating F-actin (Zhang *et al.* 2013). However, our understanding of this pathogenesis at the molecular level remains extremely limited.

Brain-derived neurotrophic factor (BDNF) was discovered in 1982 by the German chemist Y.A. Barde, who was the first to isolate and purify BDNF from the porcine brain (Barde *et al.* 1982). The monomer BDNF molecule is composed of 119 amino acid residues and is a mature polypeptide-secreted protein. BDNF, which has a molecular weight of 13.15 Kd, is mainly composed of a β sheet and a random N-stage structure with three disulfide bonds (Barde *et al.* 1982). The BDNF protein is synthesized in the brain and widely distributed throughout the central nervous system. BDNF plays important roles in the central nervous system during development by contributing to neuronal survival, differentiation and growth. It also can prevent the death of injured neurons, improve neuropathological conditions and promote the regeneration of injured neurons (Lu *et al.* 2013). Thus, BDNF is necessary for neuronal survival in the mature central and peripheral nervous systems.

Therefore, BDNF may be an attractive target for therapeutic intervention to prevent MDD. The current study included the following objectives: 1) to determine whether BDNF is altered in a cell culture model of MDD and 2) to identify the mechanisms underlying any observed effects.

MATERIALS AND METHODS

Animals

All of the experimental animals were used in accordance with the Chinese Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Institutional Animal Care and Use Committee of Wuhan University. Pregnant Wistar rats at the 16th–18th day of gestation (body weight: 350–380 g) were purchased from the Experimental Animal Research Center of Disease Control Center of Hubei Provincial. The rats were housed under standard conditions (22±2 °C, 60% relative humidity) in a facility with a 12-h/12-h light/dark cycle (lights on from 07:00 to 19:00 h) and had free access to food and water. Every effort was made to minimize animal suffering and the number of rats used.

Primary hippocampal neuron culture

Rats at the 16th–18th day of gestation were sacrificed by cervical dislocation. The primary hippocampal neurons were isolated from these embryos (E16-E18) as described previously (Carlisle *et al.* 2011). Briefly, the brains were removed and dissociated from the meninges, and the hippocampi were isolated. The hippocampi were washed three times in Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS), cut into small pieces of approximately 1 mm^3 with scissors and digested for 10 min at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium/high glucose (DMEM/HG, GNM12800, Gibco, California, USA) with 2 mg/ml papain and 10 units/ml DNase I (EN0521, Fermentas, Vilnius, Lithuania). Digestion was terminated by adding 10% fetal bovine serum. The hippocampal tissue was washed three times in DMEM/HG and dissociated with a flame-polished Pasteur pipette. The cells were counted, resuspended in plating medium (DMEM/HG) and plated on poly-L-lysine-coated coverslips (0.1 mg/ml) at a density of 1×10^5 cells per coverslip for most of the experiments or at a density of $2\times10^5/cm^2$ in poly-L-lysine-coated 6-well plates for the biochemical experiments. Four hours after plating, the medium was replaced with neurobasal medium (21103-49, Gibco, California, USA; 2% B-27, 1% glutamine, 100 units/ml penicillin and streptomycin), and Ara-C (5 μM) was added to the culture medium after 2 days *in vitro*. The hippocampal neurons were used in experiments after a culturing period of 7 to 9 days.

Drug administration

IL-1β (400-01B, Peprotech, New Jersey, USA) was dissolved in dimethylsulfoxide (DSMO), and the aliquots were stored at −80 °C. Subsequently, the aliquots were diluted in fresh PBS before use. After the neurons were cultured for 8 days, IL-1β was administered at a final concentration of 0 (PBS was added to control neurons at a concentration of 0.1%), 0.01, 0.1, 1, 10, 20, 50 or 100 ng/ml. After 12 h of treatment, the neurons were harvested for use in other experiments.

Immunocytochemistry

After treatment, the neuronal identity and morphology of the cultures were confirmed by immunocytochemical labeling for neuron-specific enolase (NSE) (BA0535, Boster, Hubei, China) (Cabras *et al.* 2010). Hippocam-

pal neurons were placed on six-well plates at a density of 0.7–1 × 10^6 cells/ml, rinsed three times with 0.01 M PBST for 5 min and fixed with 4% paraformaldehyde for 20 min at 4 °C. The neurons were then washed three times with 0.01 M PBST for 5 min at room temperature. The neurons were subsequently permeabilized with 0.1% Triton X-100 for 20 min in 0.01 M PBST and 3% H_2O_2 for 15 min at room temperature. The neurons were blocked in a wet box with 5% goat serum for 30 min at 37 °C. The cells were incubated overnight at 4 °C with a rabbit anti-NSE (1:100) primary antibody. After washing three times with 0.01 M PBST for 5 min, a goat secondary antibody (sc-2091, Santa Cruz Biotechnology) was applied for 30 min at room temperature. Subsequently, the cells were stained with DAB for 20 min and hematoxylin for 1 min. After dehydration with a gradient of ethanol, the cells were mounted and observed under an inverted microscope.

Neuron viability assays

The viability assay assesses the ability of neurons to maintain or recover their viability after treatment (Hou *et al.* 2007). Neuron viability assays were performed in three independent experiments. Hippocampal neurons were plated in quintuplicate onto 96-well plates at a density of 1×10^5 cells/well. Hippocampal neuron growth was detected with a Cell Counting Kit-8 (CCK-8) following the manufacturer's protocol (C0038, Beyotime, Shanghai, China). A total of 10 µl of CCK-8 reagent was added to each well and allowed to incubate for 4 h. The amount of CCK-8 reagent reduced to formazan by cellular dehydrogenase indicates cell viability, and this value was determined by measuring the absorbance at 450 nm in a 96-well plate reader. The absorbance metric was subtracted from the background control and averaged over three measurements.

ELISA Measurement

At the end of the treatment, the culture medium was collected from each well. BDNF levels in the neurobasal medium were measured with the solid-phase sandwich, two-site, enzyme-linked immunoassay (ELISA) technique using the Rat BDNF ELISA kit (E-EL-R1235, Eli Reiter, Hubei, China) according to the manufacturer's instructions (Wu *et al.* 2008). The collected medium samples were treated either with IL-1β for 12 h or with IL-1ra for 12 h following a 12 h treatment with IL-1β. BDNF levels were calculated from the standard curves prepared from each plate using SOFTmax PRO® software version 4.3 (Molecular Devices, Sunnyvale, CA, USA). The standard curves were linear within the range used, and the quantities of BDNF in the experimental samples were always within the linear range of the standard curve.

Western blotting

To analyze the effect of different treatment concentrations of IL-1β on F-actin, BDNF and NF-KB protein levels in hippocampal neurons, Western blotting was performed (Zhang *et al.* 2013). Cultured hippocampal neurons were homogenized in ice-cold lysis buffer containing PMSF, NaF, Na3VO4, complete PhoStop and EDTA. The neurons were homogenized with an ultrasonic wave cell disruption instrument (KS-600, Kesheng, Zhejiang, China) and centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatant was collected and stored at −80 °C. The protein concentration was measured using a Bradford Protein Assay Kit (301003, Best-Bio, Shanghai, China) according to the manufacturer's protocol. The cell extracts were analyzed by 4–10% SDS/PAGE and blotted onto PVDF membranes. The membranes were first blocked in 5% non-fat dry milk in Tris-buffered saline containing Tween 20 (TBST) for 2 h at room temperature. After three washes of 5 min each in TBST buffer, the membranes were incubated with primary antibodies (F-actin 1:500, ab205, Abcam, Massachusetts, USA), (BDNF 1:500, SC-546, Santa Cruz, USA) or (NF-KB 1:400, BS1257, Bioworld, USA) overnight at 4 °C with gentle rocking. The membranes were washed three times for 5 min as described above; then, the secondary antibodies were added, and the membranes were gently rocked for 60 min at room temperature. To analyze the relative protein quantity, GAPDH was used as a loading control.

Statistical methods

All of the data comparisons were performed using one-way analysis of variance (ANOVA) followed by post hoc LSD or Tamhane's T2 tests. These tests were used to evaluate significant differences between the different groups. All of the analyses were performed using the statistics software SPSS 13.0. All of the data were acquired from separate experiments. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Successful primary hippocampal neuron culture

To confirm that the hippocampal neurons were growing normally, we observed them under an inverted microscope. The cells cultured on cover slips were grown for one to nine days and observed under an inverted microscope (Figure 1A). Protrusions were visibly growing from cell bodies. After a culture period of seven days, the cell edges were large and smooth and exhibited strong refraction. Additionally, neural synapses were connected with each other to form a network. We utilized two approaches to further confirm that the primary hippocampal neuron culture was successful. In the first approach, we used immunocytochemistry to assess the purity of the hippocampal neurons (Figure 1B). Primary hippocampal neurons were stained with positive markers. We observed that the positive cells were connected to the surrounding cells. Positive cells were present throughout the entire field of view, and the culture was enriched in neurons

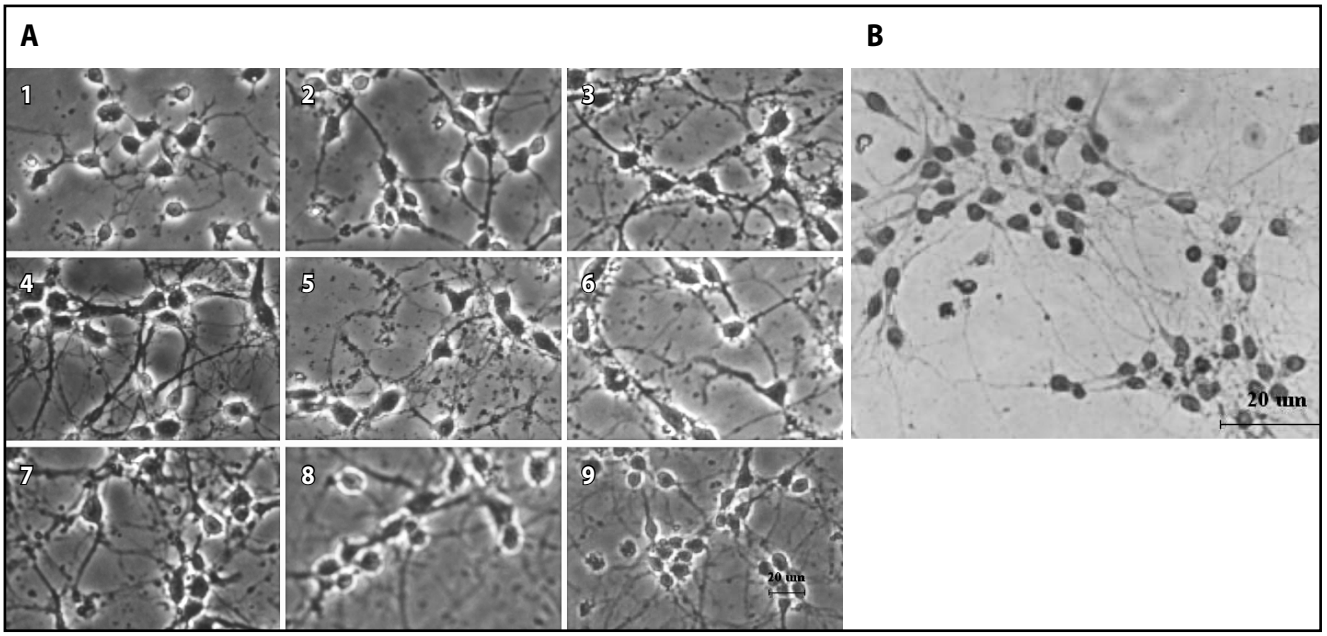

Figure 1. Successful cell culture. **A**. Hippocampal neuron culture at 1, 2, 3, 4, 5, 6, 7, 8 and 9 days. **B**. Immunocytochemistry was performed to detect the purity of the hippocampal neurons at 8 days.

Tab. 1. Neuron viability detected by the CCK-8 assay in different time at the cell culture.

	Day 1	Day 3	Day 5	Day 7	Day 9
3h50m	0.39	0.4	0.49	0.74	0.98
4h	0.41	0.44	0.53	0.73	0.91

(>95%). In the second approach, neuron viability was assessed using the CCK-8 assay at 1, 3, 5, 7 and 9 days. Viability was markedly increased in cell cultures from five to nine days, and the highest viability was observed at 9 days (Table 1).

Effects of IL-1β on hippocampal neuron viability and F-actin

To determine the effect of IL-1β on rat hippocampal neurons, we exposed neurons that were cultured for 8 days to different concentrations of IL-1β and determined their viability and the change of F-actin. Initially, the viability and F-actin was increased at 0.01 ng/ml, and significant differences in viability and F-actin were observed between the neurons cultured under this concentration and those in the control group. When the concentration was increased to 0.1 or 1 ng/ml, hippocampal neurons exhibited a more significant increase in viability and F-actin after 12 h of IL-1β treatment, with the highest viability observed at 0.1 ng/ml. Above this concentration, viability and F-actin was significantly reduced with increasing concentrations of IL-1β (Figure 2, Table 2).

Taken together, these data show that IL-1β increases neuronal survival and the expression fo F-actin in a dose-dependent manner at low doses and is less effective in increasing neuronal survival in a dose-dependent manner at high doses. The increase in neural survival and F-actin was significant at 0.1 ng/ml ($p<0.01$).

Effects of IL-1β on BDNF secretion in hippocampal neurons

To investigate the role of different concentrations of IL-1β on hippocampal neuron secretion, we measured the BDNF content in the neurobasal medium after treatment with different concentrations of IL-1β for 12 h.

Tab. 2. Different parameters in different concentration of IL-1β.

IL1-β(ng/ml)	0	0.01	0.1	1	10	20	50	100
Viability	0.80±0.12	0.87±0.18	1.16±0.14	1.32±0.17	1.1±0.23	0.87±0.08	0.75±0.15	0.65±0.21
F-actin(WB)	0.80±0.01	0.87±0.03	1.17±0.02	1.31±0.03	1.03±0.03	0.92±0.01	0.78±0.00	0.70±0.00
BDNF(WB)	0.30±0.01	0.60±0.04	0.47±0.02	0.41±0.02	0.39±0.03	0.18±0.01	0.16±0.01	--
BDNF(EL)	5.08±0.26	9.01±0.48	8.11±0.42	7.14±0.36	6.46±0.33	1.49±0.12	1.23±0.11	0.83±0.02
NF-κB(WB)	0.56±0.05	0.23±0.03	0.26±0.01	0.36±0.02	0.48±0.03	0.64±0.02	0.65±0.03	--

WB: Western blot; EL: ELISA.

The ELISA test results showed that the BDNF content was higher in the 0.01 ng/ml group compared to the control group. Similarly, the BDNF content was higher in the 0.1 ng/ml and 1 ng/ml groups than in the control group but lower than in the 0.01 ng/ml group. However, these increases were severely blunted at higher concentrations. In particular, the BDNF levels were diminished at a concentration of 50 ng/ml (Table 2).

Effects of IL-1β on BDNF in hippocampal neurons

One of the specific objectives of this study was to investigate the effects of different IL-1β concentrations on hippocampal neurons. We explored BDNF protein expression and morphology by performing Western blotting. The analysis of the Western blots demonstrated that protein expression was higher in the 0.01 ng/ml group compared to the control group. Similarly, protein expression was higher in the 0.1 ng/ml group than in the control group but lower than in the 0.01 ng/ml group. However, these increases were severely blunted at higher concentrations. In particular, the level of BDNF was diminished at 50 ng/ml (Table 2).

IL-1β-mediated NF-κB change in BDNF expression

The Western blot analysis demonstrated that NF-κB protein expression was higher in the 0.01 ng/ml group compared to the control group. Similarly, protein expression was lower in the 0.1 ng/ml group compared to the 0.01 ng/ml group. With elevated concentrations of IL-1β, NF-κB protein expression exhibited a dose-dependent increase. In particular, the level of NF-κB was the highest at 50 ng/ml (Figure 3, Table 2).

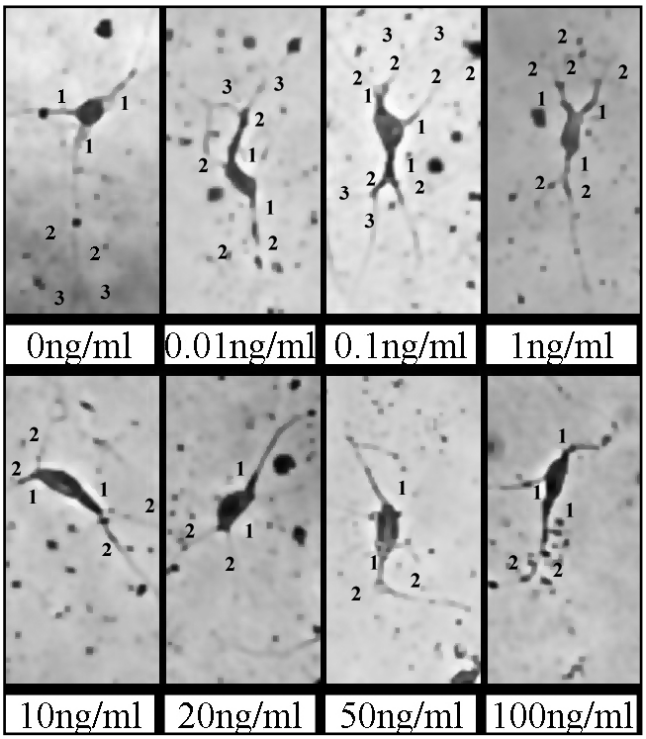

Figure 2. The effects of IL-1β on hippocampal neurons. The changes in F-actin by immunocytochemistry analysis at different concentrations of IL-1β.

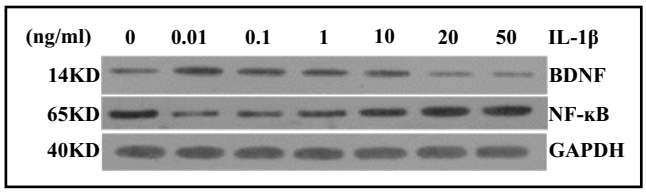

Figure 3. The effects of IL-1β on F-actin in hippocampal cells might be connected with BDNF and NF-κb. Top: representative Western blotting for BDNF in experiments after treatment with different concentrations of IL-1β. Middle: representative Western blotting for NF-κb in experiments after treatment with different concentrations of IL-1β. Bottom: GAPDH was used as a loading control.

DISCUSSION

Our group has demonstrated that an MDD rat model shows cytoskeletal impairment in the hippocampus, further suggesting that IL-1β has a critical role in regulating hippocampal neuron viability by stimulating F-actin (Yang *et al.* 2009; Zhang *et al.* 2013). The aim of the present study was to examine the mechanism of F-actin alteration in hippocampal neurons stimulated by IL-1β. The main result of the present study is that BDNF functions as a protective factor in the pathological effects of F-actin on hippocampal neurons stimulated by a low concentration of IL-1β. Conversely, the disruption of BDNF resulted in an exaggerated, pathological IL-1β-induced response in hippocampal neurons. These data demonstrate an important role for BDNF in the regulation of F-actin by NF-κB on hippocampal neurons under inflammatory stimulation.

Our results indicate that BDNF plays a critical role in protecting F-actin against the responses to inflammation stress, a finding that is consistent with previous studies on many types of cells (Bakos *et al.* 2013; Tong *et al.* 2012; Catarino *et al.* 2013; Hale *et al.* 2011; Pal *et al.* 2008; Lykissas *et al.* 2007; Rex *et al.* 2007; Moreno-López *et al.* 2006; Avwenagha *et al.* 2003; Schmidt 2004; Smart *et al.* 2003; Paves & Saarma 1997). For example, oxytocin receptor ligands induce changes in BDNF as well as a dense recruitment of F-actin filaments in neuroblastoma cells (Bakos *et al.* 2013). Additionally, an IL-1β concentration of 50 ng/μl suppressed the BDNF-dependent regulation of signaling to prevent the formation of F-actin in spines, a treatment that also impaired consolidation in hippocampal slices (Tong *et al.* 2012). Furthermore, the influence of BDNF on F-actin is retained into adulthood, when it serves to positively modulate the time-dependent LTP consolidation process in the adult hippocampus (Catarino *et al.* 2013). BDNF also induces a rapid, transient three- to four-fold increase in the F-actin concentration at the central part of the growth cone in NGF-dependent neurons (Hale *et al.* 2011). However, BDNF expression variation during

light stimulation is not known. Our study suggests that in response to inflammation, the brain may initially activate an adaptive physiological response to the increased stimulation by expressing more BDNF and F-actin in a larger hippocampal volume; however, over time, the increased concentration of inflammation and the burden of continuous stimulation may ultimately promote a loss of BDNF and F-actin, decreased hippocampal volume and MDD.

Mechanistically, the F-actin-protective effect of BDNF on pathological cell cultures might be largely mediated by inhibiting NF-κB signaling. Our study suggests that stress induced by the inflammation induced by IL-1β triggers the change in NF-κB, which in turn regulates the expression of genes that participate in immune, apoptotic and oncogenic processes (Rayet & Gelinas 1999). Other studies have demonstrated that NF-κB signaling pathways are often activated in response to inflammation through IL-1β stresses and have been shown to contribute to inflammation (Jung *et al.* 2003). The production of IL-1β and other proinflammatory cytokines might play a role in establishing pregnancy by modulating the NF-κB system in a number of species.

In conclusion, the present study defines the role of BDNF upon F-actin in response to inflammation through IL-1β. The molecular mechanism for the protective effect of BDNF on F-actin may be dependent on the inhibition of the NF-κB signaling pathway. Our observations may help to develop novel therapeutic strategies for the treatment of MDD.

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