Curcumin ameliorates hippocampal neuron damage induced by human immunodeficiency virus-1⋆

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Abstract

Our previous studies have shown that infection with the gp120 V3 loop can cause human immunodeficiency virus-1 associated neurocognitive disorders. Curcumin has been shown to improve these effects to some degree, but the precise mechanisms remain unknown. The present study analyzed the neuroprotective effect and mechanism of curcumin in relation to hippocampal neurons. Results showed that 1 nmol/L gp120 V3 loop suppressed the growth of synapses. After administration of 1 μmol/L curcumin, synaptic growth improved. Curcumin is neuroprotective against gp120 V3 loop-induced neuronal damage by inhibiting the activation of L-type calcium currents, relieving intracellular Ca^{2+} overload, promoting Bcl-2 expression, and inhibiting Bax activation. The effect of curcumin was identical to nimodipine, suggesting that curcumin has the same neuroprotective effects against gp120 V3 loop-induced neuronal damage.

Key Words

neural regeneration; traditional Chinese medicine; curcumin; gp120 V3 loop; human immunodeficiency virus-1 associated neurocognitive disorders; Ca^{2+}; synapse; apoptosis; grants-supported paper; neuroregeneration

Research Highlights

(1) Our previous studies showed that the application of curcumin could reverse the neurocognitive impairment caused by human immunodeficiency virus-1. In this study, we analyzed the neuroprotective effect and mechanism of curcumin on gp120 V3-induced neuronal injury in cultured neurons.
(2) Curcumin improved the growth of synapses, relieved intracellular Ca^{2+} overload, promoted Bcl-2 expression, and inhibited Bax activation.

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INTRODUCTION

Infection with human immunodeficiency virus (HIV)-1 can induce cognitive disorders, and motor and behavioral abnormalities.

These changes are termed as HIV-associated neurocognitive disorder[1-3]. Currently, as many as 50–60% of HIV-1 infected patients are suffering from neurocognitive disorders, despite highly active antiretroviral therapy being available

against HIV infection[4]. However, HIV seldom infects neurons[5]. Research suggests that direct and indirect activation of the immune system may account for neuronal degeneration and development of neurological symptoms in HIV-associated neurocognitive disorder[6-7]. Notable neuronal apoptosis in the brain is a feature during HIV infection[8], illustrating one of the many reasons why continuing HIV-associated neurocognitive disorder research is necessary to understand the complications that may develop.

gp120 is a viral protein that is released from HIV infected mononuclear phagocytes. The neurotoxic activity of this protein occurs by interacting with uninfected mononuclear phagocytes to release cellular neurotoxins (such as glutamate, pro-inflammatory cytokines and chemokines)[9]. Animal studies have revealed that gp120 causes amnesia and memory dysfunction[10-11]. Further findings have shown that the released neurotoxins can activate N-methyl-D-aspartate receptors and induce calcium influx, disrupting calcium homeostasis and triggering the pathway for intrinsic neuronal apoptosis[12-14]. Our previous work revealed that intracerebroventricular injection of gp120 V3 loop peptide inhibited long-term potentials, induced behavioral alteration and cognition impairment *in vivo* [15]. These results also confirmed that gp120 contributed to the pathology of neuronal damage and synaptic plasticity.

Curcumin, a substance derived from the roots of curcuma plants, can suppress HIV replication through inhibition of HIV integrase and protease[16-17]. In addition, the pharmacological properties of curcumin include anti-inflammation[18], anti-oxidant[19], and neuroprotective effects[20-21]. Our previous *in vivo* research confirmed the neuroprotective effect of curcumin against neuronal damage induced by gp120[15]. However, it remains unclear whether this neuroprotection occurs *via* regulating calcium homeostasis. This study seeks to explore the protective mechanism of curcumin on gp120 V3 loop-induced neural damage by using the L-type calcium channel antagonist, nimodipine, as a positive control.

RESULTS

Quantitative analysis of experimental animals
One hundred and two day-old male neonatal rats were used for cell culture. Six groups were created randomly, and included the control, curcumin, nimodipine, gp120 V3 loop, curcumin + gp120 V3 loop and nimodipine + gp120 V3 loop groups. All rats were included in the final analysis.

Curcumin prevented gp120 V3 loop-induced morphological changes in hippocampal neurons
Modulation of Ca^{2+} channel activity is known to have dramatic effects on the survival of neurons, and in particular plays a crucial role in apoptosis[22]. We, therefore, studied neuronal morphology in all experimental groups to determine the effect of curcumin. Hippocampal neurons were cultured for 3 days and stained with neuron specific microtubule-associated protein 2. Immunofluorescence revealed that microtubule- associated protein 2-positive neurons in the control, curcumin and nimodipine groups possessed more synapses, with intensive networks between each synapse. The gp120 V3 loop caused a decreased number and a shortened length of synapses ($P < 0.05$). Treatment with curcumin and nimodipine significantly reduced the injury caused by the gp120 V3 loop ($P < 0.05$; Figure 1).
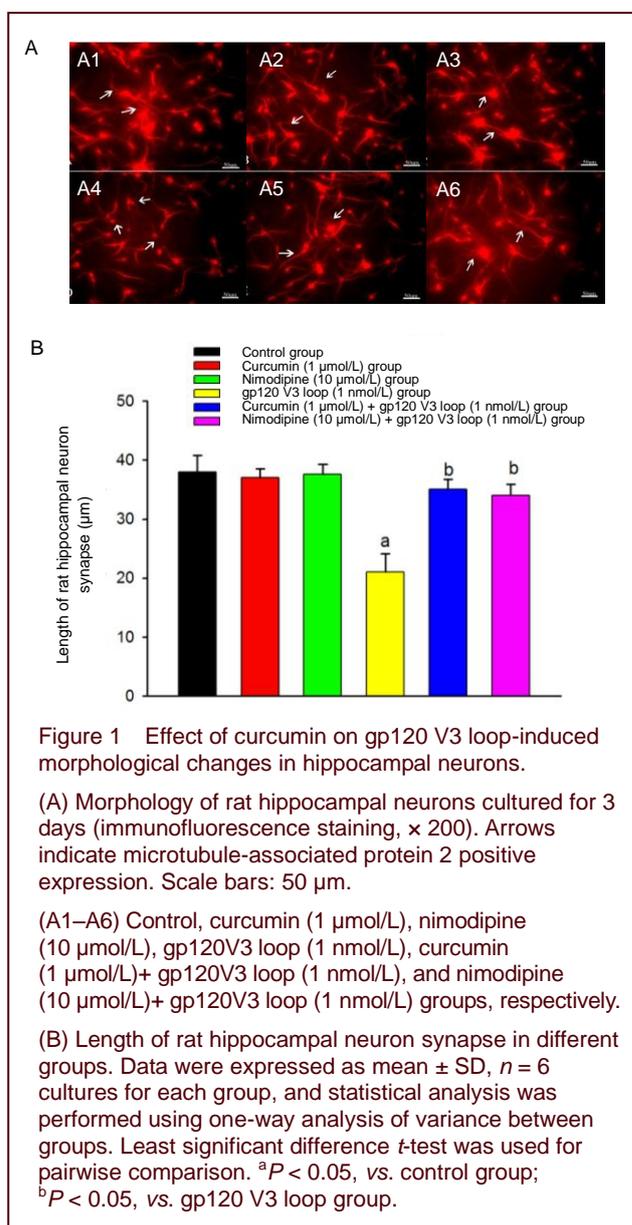
Figure 1   Effect of curcumin on gp120 V3 loop-induced morphological changes in hippocampal neurons.

(A) Morphology of rat hippocampal neurons cultured for 3 days (immunofluorescence staining, × 200). Arrows indicate microtubule-associated protein 2 positive expression. Scale bars: 50 µm.

(A1–A6) Control, curcumin (1 µmol/L), nimodipine (10 µmol/L), gp120V3 loop (1 nmol/L), curcumin (1 µmol/L)+ gp120V3 loop (1 nmol/L), and nimodipine (10 µmol/L)+ gp120V3 loop (1 nmol/L) groups, respectively.

(B) Length of rat hippocampal neuron synapse in different groups. Data were expressed as mean ± SD, $n = 6$ cultures for each group, and statistical analysis was performed using one-way analysis of variance between groups. Least significant difference *t*-test was used for pairwise comparison. [a]$P < 0.05$, *vs.* control group; [b]$P < 0.05$, *vs.* gp120 V3 loop group.

Curcumin prevented gp120 V3 loop-induced upregulation of L-type Ca^{2+} currents in rat hippocampal neurons

Morphological results demonstrated that gp120 depressed synaptic plasticity. We further investigated the influence of calcium ion channels, which are strongly associated with synaptic plasticity[23]. Whole cell Ca^{2+} currents were elicited by a series of depolarizing voltage clamp steps from a holding potent potential of –40 mV, in which potential Na^{+} channels were almost inactivated. Tetraethylammonium and Cs^{2+} were added to make sure K^{+} channels were blocked. According to the voltage protocol described in the methods section, we successfully recorded a slowly inactivated inward L-type Ca^{2+} current, the negative value of Ca^{2+} current represents the in-ward direction

other than its actual value. The average L-type Ca^{2+} currents in the gp120 V3 loop group represented a persistent increase in hippocampal neurons ($P < 0.05$; Figure 2), while the application of curcumin and nimodipine reversed the increased currents induced by gp120 ($P < 0.05$).

To analyze the difference in voltage dependence of channel activity, current-voltage curves were fitted between each group. L-type Ca^{2+} currents in the gp120 V3 loop group were more precipitous than those in the control group under different holding potentials. L-type Ca^{2+} channel currents in the curcumin + gp120 V3 loop group and nimodipine + gp120 V3 loop group were smaller than those in the gp120 V3 loop group from the same patch in whole cell patch clamp recordings.
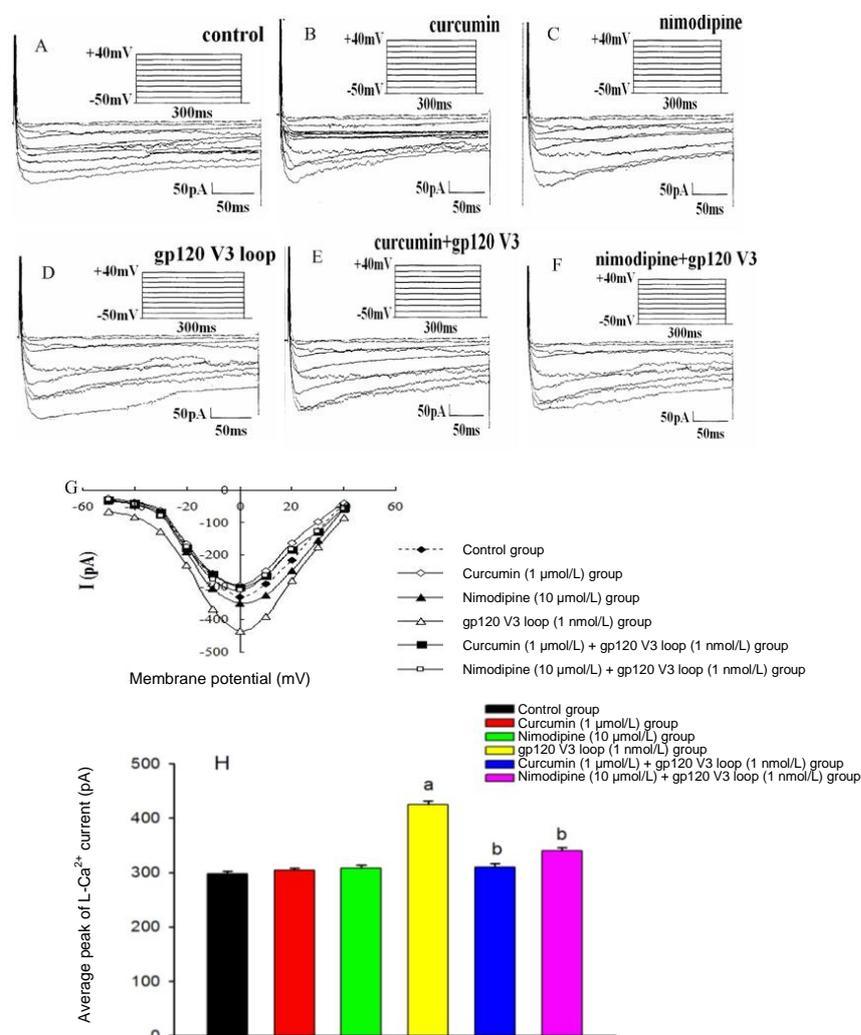
Figure 2   Effect of curcumin on gp120 V3 loop-induced L-type Ca^{2+} currents of rat hippocampal neurons.

(A–F) L-type Ca^{2+} curves in the control, curcumin, nimodipine, gp120 V3 loop, curcumin + gp120 V3 loop, nimodipine + gp120 V3 loop groups, respectively; (G) current-voltage curves; (H) average peak of L-Ca^{2+} currents. [a]$P < 0.05$, *vs*. control group; [b]$P < 0.05$, *vs*. gp120 V3 loop group. Data were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance between groups, and least significant difference *t*-test was used for pairwise comparison. Six cells were used in each group for recording L-Ca^{2+} currents.

Curcumin decreased the high concentration of intracellular calcium induced by the gp120 V3 loop in rat hippocampal neurons

Previous data have shown that gp120 increased L-type Ca^{2+} currents. Whether these changes in L-type Ca^{2+} currents influence intracellular calcium in each neuron remains to be elucidated. To this end, we used a calcium ion probe, Fura-2/AM, which binds with dissociated intracellular Ca^{2+}. The compound of Fura-2/AM and Ca^{2+} emits red fluorescence, and fluorescence intensity can be calculated by a microplate reader. The $[Ca^{2+}]_i$ fluorescence intensity in the gp120 V3 loop group was higher than that in the control group ($P < 0.05$). The presence of curcumin or nimodipine reduced $[Ca^{2+}]_i$ concentration ($P < 0.05$; Figure 3). These data together suggested that gp120 V3 increased $[Ca^{2+}]_i$, and curcumin and nimodipine eliminated the over-expression of $[Ca^{2+}]_i$.
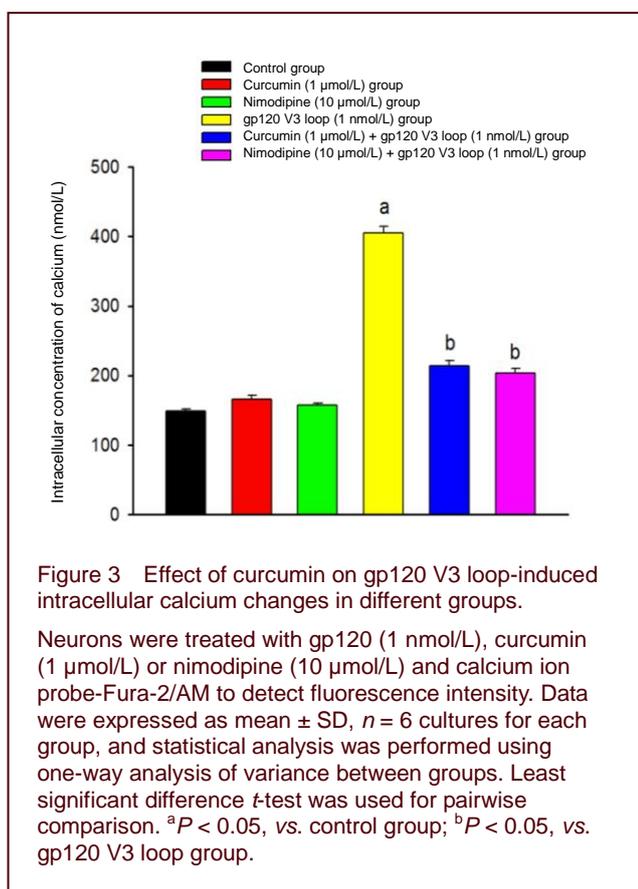
Figure 3 Effect of curcumin on gp120 V3 loop-induced intracellular calcium changes in different groups.

Neurons were treated with gp120 (1 nmol/L), curcumin (1 µmol/L) or nimodipine (10 µmol/L) and calcium ion probe-Fura-2/AM to detect fluorescence intensity. Data were expressed as mean ± SD, $n = 6$ cultures for each group, and statistical analysis was performed using one-way analysis of variance between groups. Least significant difference t-test was used for pairwise comparison. [a]$P < 0.05$, vs. control group; [b]$P < 0.05$, vs. gp120 V3 loop group.

Curcumin inhibited the expression of hippocampal Bax mRNA and increased Bcl-2 mRNA

To investigate the mechanism of gp120 V3-induced neuronal injury, we tested the expression of Bcl-2 and Bax mRNA. Quantitative real time reverse transcription-PCR results showed that mRNA level of the anti-apoptotic indicator Bcl-2 decreased remarkably in the gp120 V3 loop group compared with the control group ($P < 0.05$; Figure 4), and the apoptotic indicator Bax

increased significantly ($P < 0.05$). Incubation with curcumin and nimodipine together contributed to high expression of Bcl-2 mRNA and less Bax compared with the gp120 V3 loop group ($P < 0.05$).
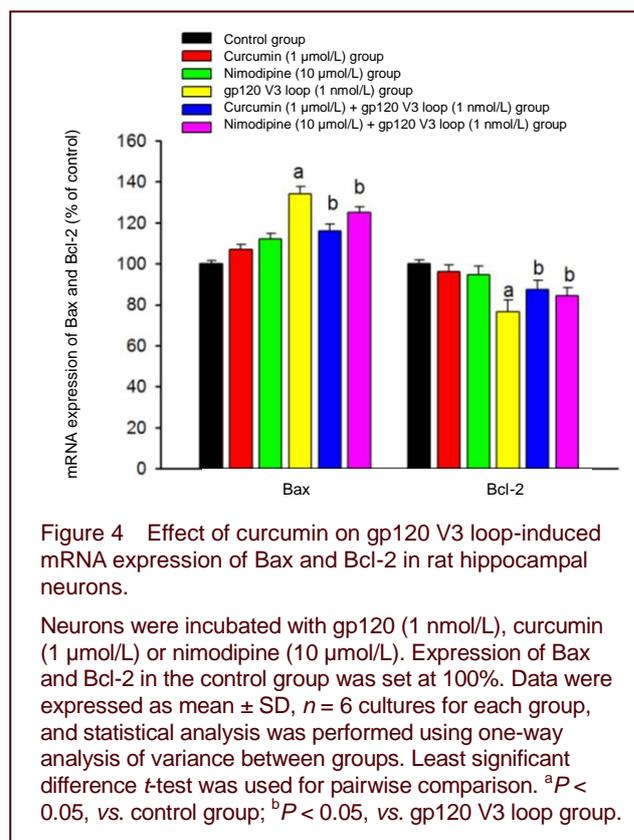
Figure 4 Effect of curcumin on gp120 V3 loop-induced mRNA expression of Bax and Bcl-2 in rat hippocampal neurons.

Neurons were incubated with gp120 (1 nmol/L), curcumin (1 µmol/L) or nimodipine (10 µmol/L). Expression of Bax and Bcl-2 in the control group was set at 100%. Data were expressed as mean ± SD, $n = 6$ cultures for each group, and statistical analysis was performed using one-way analysis of variance between groups. Least significant difference t-test was used for pairwise comparison. [a]$P < 0.05$, vs. control group; [b]$P < 0.05$, vs. gp120 V3 loop group.

DISCUSSION

The main aim of therapeutic trials for the treatment of human HIV-associated neurocognitive disorder is to produce complete viral suppression in both plasma and the central nervous system. This aim is in addition to the application of highly active antiretroviral therapy[24], and adjunctive therapies including N-methyl-D-aspartate antagonists[25], calcium channel blockers[26], antioxidants[9] and anti-inflammatory drugs[27]. Central nervous system penetration, modest price, minor side effects and effective delivery of metabolites are some other factors that need to be considered for the use of these drugs. Currently, the strategy for new drug development is to find novel therapeutic targets or find new drugs that display all required properties to create a selective and efficacious compound. In this experiment, we chose to investigate curcumin given that prior findings have shown its potent action as an inhibitor of HIV protease and integrase[28]. Additionally, curcumin has been shown to effectively traverse the blood-brain barrier[29], and also to possess anti-oxidant

and, anti-inflammatory activity[30]. Our previous experiments showed that curcumin improved the learning ability of gp120 impaired rats through anti-oxidation[15], and ameliorated tumor necrosis factor-α-induced neuronal damage. We also demonstrated that the expression of caspase-3 and mitochondrial membrane potential (△ Ψm) was decreased in the presence of curcumin[31]. Our current study focused on the possible neuroprotective mechanisms of curcumin.

Plasticity is a crucial feature in the brain, which accommodates neuronal structure and function to patterns of electrical activity. Activity of L-type calcium channels is linked to neuronal survival and death *via* regulating calcium and its signaling events to the nucleus. Morphological results demonstrated that the gp120 V3 loop caused a decreased number and a shortened length of synapses, while neurons in the curcumin and nimodipine groups displayed more synapses that formed more complex networks.

Calcium overload has been implicated in loss of synaptic plasticity and showed adverse effects on neuronal function and survival[32]. Exaggerated or deregulated calcium flux can promote cellular dysfunction and death by activating death-inducing signaling pathways. Bax is a proapoptotic protein that enhances endoplasmic reticulum and mitochondrial Ca^{2+} cross-talk[33]. Bax increases the permeabilization of mitochondrial outer membranes and the release of mitochondrial factors, such as calcium and cytochrome c, into the cytosol[34]. Survival signaling in the nucleus related to the L-type calcium channel refers to the expression of c-Fos, cAMP-response element binding protein, brain-derived neurotrophic factor and Bcl-2[35]. These genes allow neurons to have a wide dynamic range of responses to surrounding stimulation. Our results showed that gp120 increased L-type calcium currents, but curcumin and nimodipine reduced calcium currents, suggesting the neuroprotective role of curcumin occurs *via* blocking calcium channels. Results of reverse transcription-PCR also confirmed that gp120 may disturb calcium homeostasis by upregulating the expression of Bax mRNA and downregulating Bcl-2 mRNA. This change in expression may result in a further increase in the release of cytochrome c from mitochondria *via* a direct enhancement of calcium, which would finally cause neuronal death or apoptosis.

In summary, gp120 caused hippocampal neuron damage *via* activating calcium and related pro-apoptotic genes. In turn, curcumin reversed the neuronal and synaptic damage. These findings demonstrate that the application of curcumin may be a suitable therapy for HIV-associated neurocognitive disorders.

MATERIALS AND METHODS

Design
A controlled, randomized cell culture experiment.

Time and setting
This experiment was performed from February 2009 to October 2011 at the Key Laboratory of State Administration of Traditional Chinese Medicine, Medical College of Jinan University, China.

Materials
Animals
A total of 102 male neonatal Sprague-Dawley rats born within 24 hours were required for cell culture. These animals were obtained from the Experimental Animal Center of Southern Medical University in China (license No. SCXK (Yue) 2006B023, 2009-0002). All protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[36].

Virus
HIV-1MN V3 loop peptide (V3, 23-mer, cyclic, AATALS IAIGAGPGAAPVTIG LIG-NH$_2$, with the chemical formula of $C_{114}H_{199}N_{41}O_{31}$, and a molecular weight of 2 640.06) was purchased from Sigma (St. Louis, MO, USA).

Drugs
Curcumin was obtained from Fluka ($C_{21}H_{20}O_6$, a molecular weight of 368.37, density with 93%; Buchs, Grisons, Switzerland). The molecular structure of curcumin is shown as follows:
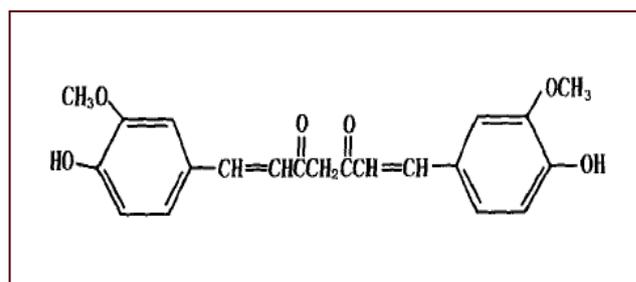
Methods
Hippocampal neuronal culture and drug intervention
After anesthesia with diethyl ether, hippocampal neurons

of neonatal rats were dissected and placed in Ca^{2+} and Mg^{2+}-free hydroxyethyl piperazine ethanesulfonic acid-buffered Hanks salt solution, pH 7.45. The Hank's balanced salt solution was composed of the following: hydroxyethyl piperazine ethanesulfonic acid 20 mmol/L, NaCl 137 mmol/L, $CaCl_2$ 1.3 mmol/L, $MgSO_4$ 0.4 mmol/L, $MgCl_2$ 0.5 mmol/L, KCl 5.0 mmol/L, KH_2PO_4 0.4 mmol/L, Na_2HPO_4 0.6 mmol/L, $NaHCO_3$ 3.0 mmol/L, and glucose 5.6 mmol/L. A total of 10^5 or 10^6 cells/well were plated into 96- or 6-well plates containing 90% Dulbecco's modified Eagle's medium/F12 (DMEM/F12) and 10 % fetal bovine serum (Sijiqing, Hangzhou, Zhejiang Province, China). After 4 hours, culture medium was replaced by serum-free medium containing 98% neurobasal medium (Gibco, Carlsbad, CA, USA) and 2% B27 (Gibco). Subsequently, 50% medium changes occurred twice weekly. The dose and time of gp120 and curcumin have been assessed using a methyl thiazolyl tetrazolium assay[31]. Therefore, neurons were incubated with 1 nmol/L gp120, 1 μmol/L curcumin or 10 μmol/L nimodipine for 24 hours before being subjected to the following experiments.

Assessment of nuclear morphology using microtubule-associated protein 2 and immunofluorescence staining

gp120 (1 nmol/L), curcumin (1 μmol/L) and/or nimodipine (10 μmol/L) were used in the gp120 V3 loop, curcumin + gp120 V3, nimodipine and nimodipine + gp120 V3 groups. Microtubule-associated protein 2 staining was used to identify neurons[37]. Briefly, cells were fixed in ice-cold 4% paraformaldehyde and incubated with the neuron specific mouse anti-rat microtubule-associated protein 2 antibody (1:200; Sigma, St. Louis, MO, USA) in blocking serum at 4°C overnight. The following day, cells were washed and incubated with rabbit anti-mouse IgG conjugated with Cy3 (Sigma) at room temperature in a humid dark box for 1 hour. Cells were then washed with PBS, and images were obtained using a confocal laser scanning microscope (Zeiss, LSM 510, Oberkochen, Baden-Württemberg, Germany). Image-Pro Plus 6.0 software (Bethesda, Maryland, USA) was used to measure and count synapses. Three visual fields of each sample were randomly selected under the fluorescence microscope.

Whole cell recordings

The resistance of the recording pipettes for whole-cell recording was 2–4 MΩ. Recordings were obtained according to standard patch-clamp methods using an Axon 200B amplifier (Molecular Devices, Union, CA, USA). Voltage commands were generated, and current

responses were recorded and analyzed using a pCLAMP computerized acquisition and storage system (Molecular Devices). Whole-cell recordings were filtered at 2 kHz and digitized at 2–3 kHz. Voltage pulses were delivered at 5-second intervals. L-type Ca^{2+} currents were evoked by depolarizing pulse commands: voltage clamps were –50 mV and traces were obtained by stepping the voltages to potentials from –50 to +40 mV for 300 ms in duration, and each depolarization step was –10 mV. The internal solution included 130 mmol/L CsCl, 10 mmol/L TEA-Cl, 2 mmol/L $MgCl_2$, 10 mmol/L hydroxyethyl piperazine ethanesulfonic acid (HEPES), 10 mmol/L glucose, 10 mmol/L K_2-ATP, and 10 mmol/L ethylene glycol tetraacetic acid (EGTA), pH 7.2. The external solution included 120 mmol/L NaCl, 5 mmol/L CsCl, 10 mmol/L tetraethylammonium-Cl, 10 mmol/L $BaCl_2$, 10 mmol/L HEPES, 1 mmol/L $MgCl_2$, and 0.5 μmol/L TTX was added to block sodium currents, pH 7.3–7.4. The osmolarity of all recording solutions was adjusted to 320–325 mOsm as necessary.

Concentration of calcium in hippocampal neurons as measured by calcium imaging

Cultured hippocampal neurons were washed with PBS, then incubated with 2 mmol/L Fura-2/AM (Molecular Probes) for 30 minutes at 37°C and subsequently transferred to a microscope stage for imaging. External solution contained 150 mmol/L NaCl, 10 mmol/L hydroxyethyl piperazine ethanesulfonic acid, 3 mmol/L KCl, 2 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, 5.5 mmol/L glucose, pH 7.4, and osmolality was adjusted to 325 mOsm. Measurement of Ca^{2+}-induced fluorescence at both 340 and 380 nm allows for calculation of calcium concentrations based on 340 nm/380 nm ratios. The use of the ratio automatically cancels out certain variables for imaging calcium concentrations in cells. After excited at 340 and 380 nm, Fura-2 emission intensity at 488 nm was acquired using an Olympus IX71 fluorescence microscope (Olympus, Hicksville, NY, USA) and a PD70 intensified camera.

Quantitative real-time reverse transcription-PCR of Bax and Bcl-2 mRNA in hippocampal neurons

Total cellular RNA was extracted using Trizol (Takara, Kyoto, Japan) and reverse transcribed to cDNA according to standard methods[38]. Real-time PCR was carried out using a Roche Light Cycler 480 in a total volume of 20 μL comprising 2 μL of 1:10 diluted strand cDNA, 0.4 μL each of the forward and reverse primers, 10 μL SYBR Green I Master (Takara) and 7.2 μL of nuclease-free water. The sequences of the primers are listed below:

Gene	Primer sequence	Product size (bp)
Bcl-2	Forward: 5′-CAT CTT CTC CTT CCA GCC-3′ Reverse: 5′-GGA GAA ATC AAA CAG ACG AC-3′	118
Bax	Forward: 5′-TGC TGA TGG C AAC TTC AAC T-3′ Reverse: 5′-GTG AGG ACT CCA GCC ACA AA-3′	204
β-actin	Forward: 5′-ATT GTA ACC AAC TGG GAC G-3′ Reverse: 5′-TTG CCG ATA GTG ATG ACC T-3′	236

The PCR mixture was heat denatured at 95°C for 30 minutes, followed by 40 cycles of 5 seconds at 95°C, 20 seconds at 60°C. Fluorescent signals were acquired at the last step of each cycle. A melting curve was calculated at the end of the cycles using a Light Cycler 480 PCR Instrument System (Roche, Basel, Switzerland). β-actin was used as the internal control.

Statistical analysis

All data were expressed as mean ± SD. Statistical analyses were carried out using one-way analysis of variance between groups, and differences between groups were compared using least significant difference *t*-test with SPSS 18.0 (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Author contributions: Hongmei Tang, Rui Pan, and Wenli Fang designed and performed the study. Yanyan Xing, Junbing Wang, Zheng Gong and Guoyin Xiong conducted partial experiments and provided material support. Dexi Chen provided technical support. Xiaobao Chen and Yuanyuan Yu were responsible for figure trimming. Jun Dong was responsible for funding, study design, and provided study guidance. All authors have read and agreed to the manuscript as written.

Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Ethics Committee of Jinan University, Guangzhou, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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