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ORIGINAL PAPER

The Dual Role of HIV-1 gp120 V3 Loop-Induced Autophagy in the Survival and Apoptosis of the Primary Rat Hippocampal Neurons

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Abstract

HIV-1 gp120, an important subunit of the envelope spikes that decorate the surface of virions, is known to play a vital role in neuronal injury during HIV-1-associated neurocognitive disorder (HAND), although the pathological mechanism is not fully understood. Our previous studies have suggested that the V3 loop of HIV-1 gp120 (HIV-1 gp120 V3 loop) can induce neuronal apoptosis in the hippocampus, resulting in impairment in spatial learning and memory in Sprague–Dawley (SD) rats. In this study, we demonstrated that autophagy was significantly increased in rat primary hippocampal neurons in response to treatment of HIV-1 gp120 V3 loop. Importantly, HIV-1 gp120 V3 loop-induced autophagy played a dual role in the cell survival and death. An increase in autophagy for a short period inhibited apoptosis of neurons, while persistent autophagy over an extended period of time played a detrimental role by augmenting the apoptotic cascade in rat primary hippocampal neurons. In addition, we found that the HIV-1 gp120 V3 loop induced autophagy via AMPK/mTOR-dependent and calpain/mTOR-independent pathways, and the ERK/mTOR pathway plays a partial role. These findings provide evidence that HIV-1-induced autophagy plays a dual role in the survival and apoptosis of the primary rat hippocampal neurons and persistent autophagy may contribute to the pathogenesis of HAND, and autophagy modulation may represent a potential therapeutic strategy for reducing neuronal damage in HAND.

Keywords HIV-1- associated neurocognitive disorders · HIV-1 gp120 V3 loop · Autophagy · Apoptosis · AMPK · ERK · Calpain

Abbreviations

HIV-1	Human immunodeficiency virus type 1
HAND	HIV-1- associated neurocognitive disorders
GAPDH	Glyceraldehyde-3-phosphatedehydrogenase
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex 1
MAP1LC3 (LC3)	Microtubule-associated protein 1 light chain 3
ATG	Autophagy-related gene
SQSTM1 (p62)	Sequestosome 1 (a ubiquitin-binding scaffold protein)
MAPK1 (ERK2)	Mitogen-activated protein kinase 1
MAPK3 (ERK1)	Mitogen-activated protein kinase 3
LC3B-I	Unlipidated form of LC3B
LC3B-II	Phosphatidylethanolamine-conjugated form of LC3B

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Cas-3	Caspase 3
3-MA	3-Methyladenine
AMPK	AMP activated protein kinase
MDC	Monodansylcadaverine
NMDA	*N*-Methyl-D-Aspartate
TNF-α	Tumor necrosis factor-α

Introduction

Nearly 35 million people have succumbed to human immunodeficiency virus type 1 (HIV-1) since the first AIDS cases were reported in 1981, and another 36.9 million people are living with HIV-1. HIV-1 not only infects and impairs the immune system, but also invades the central nervous system (CNS), leading to a broad spectrum of motor impairments and cognitive deficits, termed HIV-1-associated neurocognitive disorders (HAND) [1]. HIV-1 gp120, a subunit of the viral envelope protein (Env), is known to play a crucial role in the pathological process of HAND [2], although the mechanisms remain largely unclear.

Many efforts have been made to decipher the molecular mechanism by which HIV-1 gp120 induces apoptosis in neurons. Our group and others have demonstrated that HIV-1 gp120 can induce apoptosis in rat hippocampal neurons [3], leading to an impairment in spatial learning and memory in Sprague–Dawley (SD) rats [4]. gp120 can induce neuronal apoptotic death through the CXCR4/CCR5 receptor [5], interact with *N*-Methyl-D-Aspartate (NMDA) receptors, and influence the influx of Ca^{2+}, thus triggering further neuronal injury [6]. Furthermore, gp120 can activate glial cells to produce various types of cytokines, chemokines and other soluble factors, which can directly lead to neuronal injury [7]. The V3 loop is the most vital region among the whole structure of HIV-1 gp120, which is essential for virus infectivity [8].

Macroautophagy, hereafter referred to as autophagy, is a highly conserved lysosomal degradation pathway in which unnecessary byproducts and damaged organelles are engulfed into double-membrane vesicles termed autophagosomes and transported to lysosomes. Autophagosomes fuse with lysosomes, and inner cargo is degraded and recycled. Thus, autophagy is necessary for maintaining homeostasis and plays a pro-survival role. In other circumstances, autophagy can stimulate a pro-death signaling pathway [9] and thus accelerate cell death. Data have shown that autophagy may also participate in the progression of HIV. Simian immunodeficiency virus (SIV)-infected microglia can inhibit autophagy and reduce the survival rate of neurons, while rapamycin, an inducer of autophagy, acts as a protective agent in neuron [10]. Zhou et al. [11] have found autophagy was increased in the postmortem brains of HIV-1 encephalitis patients, and their further studies showed gp120 can enhance the level of autophagy in SK-N-SH neuroblastoma cells. These findings suggest that homeostasis of autophagy is a reliable security factor for neurons, and the dysregulation of autophagy may be important for the pathogenesis of HAND.

However, it remains unclear how HIV-1 gp120 induces autophagy, and the role it plays in cell death in rat primary hippocampal neurons has yet to be determined. This study set out to assess whether HIV-1 gp120 induces autophagy and its role in apoptosis of rat primary hippocampal neurons. We also set out to study the exact mechanism if autophagy is increased by the presence of gp120 by looking at the relationship of apoptosis and autophagy. Previous studies have reported that autophagy is regulated by a multitude of signaling pathways, such as class I PtdIns 3-kinase-AKT1 signaling, the mechanistic target of rapamycin (mTOR) kinase, the response to endoplasmic reticulum (ER) stress and the energy sensor AMP-activated protein kinase (AMPK) [12–14]. Based on these studies, we used specific inhibitors of AMPK, ERK and calpain to see whether these molecules have effects on autophagy, and the impact of autophagy on apoptosis in rat primary neurons induced by the HIV-1 gp120 V3 loop.

Results

The HIV-1 gp120 V3 Loop Induces Apoptosis in Rat Primary Hippocampal Neurons

At first, we confirmed the purification of rat primary neurons via immunofluorescent staining against MAP2, which is a specific marker for neurons. As shown in red in Fig. 1a, most of cells are MAP2 positive. Then we treated primary rat neurons with gp120 V3 loop at concentrations ranging from 0.1 to 10 μg/mL. MTT assay revealed 0.1 μg/mL gp120 V3 loop had a slight negative effect on neurons, while 1 and 10 μg/mL gp120 V3 loop treatment can result in an inhibition of cell growth in a time dependent manner (Fig. 1b). Although it has been reported that the levels of gp120 in the plasma of HIV patients is approximately 796 ng/mL [15], other groups in this field have used a range from 1 pg/mL to 1 μg/mL for in vitro experiments. Based on the results from the MTT assay and our previous results [16], 1 μg/mL was the optimal concentration and used for subsequent experiments. Hoechst 33342 nucleic acid stain is a cell-permeable nuclear counterstain for dsDNA and is often used to distinguish condensed pyknotic nuclei in apoptotic cells. To test whether gp120 V3 loop induces neuronal apoptosis, we treated rat primary hippocampal neurons with 1 μg/mL gp120 V3 loop for various times and employed Hoechst 33342 staining to assess apoptosis in neurons. After treatment with

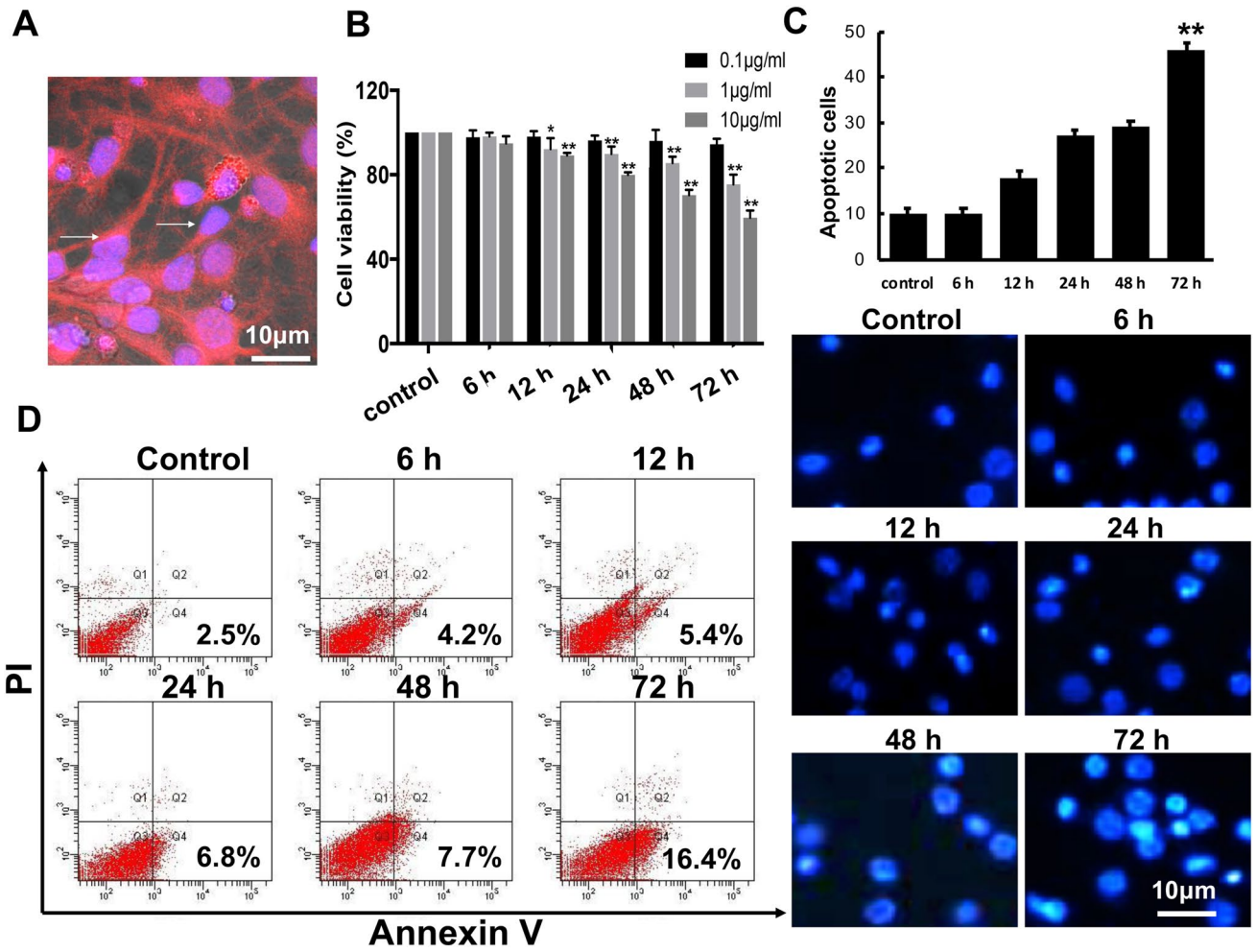

Fig. 1 The HIV-1 gp120 V3 loop induces apoptosis in primary rat hippocampal neurons. **a** MAP2/Hochest33342 Immunofluorescence method is used to identify the primary rat hippocampal neurons, MAP2 (red) is specific for neurons, and cell nuclei (in blue) were labeled with Hoechst 33342. **b** Neurons were seeded in 96-well plates (5×10^3 cells/well). On the 7th day, cells were stimulated with different concentrations of gp120 V3 loop (0, 0.1, 1 and 10 μg/mL) for 6 h, 12 h, 24 h, 48 h and 72 h and subjected to cell viability using the MTT assay. **c** Neurons were treated with 1 μg/mL HIV-1 gp120 V3 loop for various times. The apoptotic rate was analyzed using Hoechst 33342 staining. **d** Neurons were treated with 1 μg/mL HIV-1 gp120 V3 loop for various times were stained with Annexin V/PI and detected by flow cytometry. Scale bars: 10 μm. n = 6. $*p<0.05$ vs. control, $**p<0.01$ vs. control

HIV-1 gp120 V3 loop, the number of bright fragmented nuclei with condensed chromatin increased significantly with time, which is typical of cells undergoing apoptosis (Fig. 1c). To further quantify the apoptosis level induced by gp120 V3 loop, we used Annexin V-FITC and propidium iodide (PI) staining followed by flow cytometry to assess the neuronal apoptotic rate. The data show the percentage of early phase apoptosis cells (Q4, Annexin V^+ PI^-) increased after exposure to gp120 V3 loop for 72 h (Fig. 1d), revealed that treatment with 1 μg/mL gp120 V3 loop resulted in a significant increase in the percentage of apoptotic cells. These results suggest that the HIV-1 gp120 V3 loop can induce apoptosis in primary hippocampal neurons in a time-dependent manner.

The HIV-1 gp120 V3 Loop Induces Autophagy in Primary Rat Hippocampal Neurons

To determine whether the HIV-1 gp120 V3 loop induces autophagy, we treated primary rat hippocampal neurons with 1 μg/mL gp120 V3 loop for 6, 12, 24, 48 and 72 h and tested the autophagic level using monodansylcadaverine (MDC) staining. MDC labels late-stage autophagosomes or autophagic vesicles allowing for the monitoring of increases in autophagic activity in many studies [17]. As shown in Fig. 2a, the number of autophagic vacuoles, which appeared as distinct dot-like structures distributed within the cytoplasm or localized in the perinuclear zone, was increased at 6 h, followed by a slight decrease at 12 h and

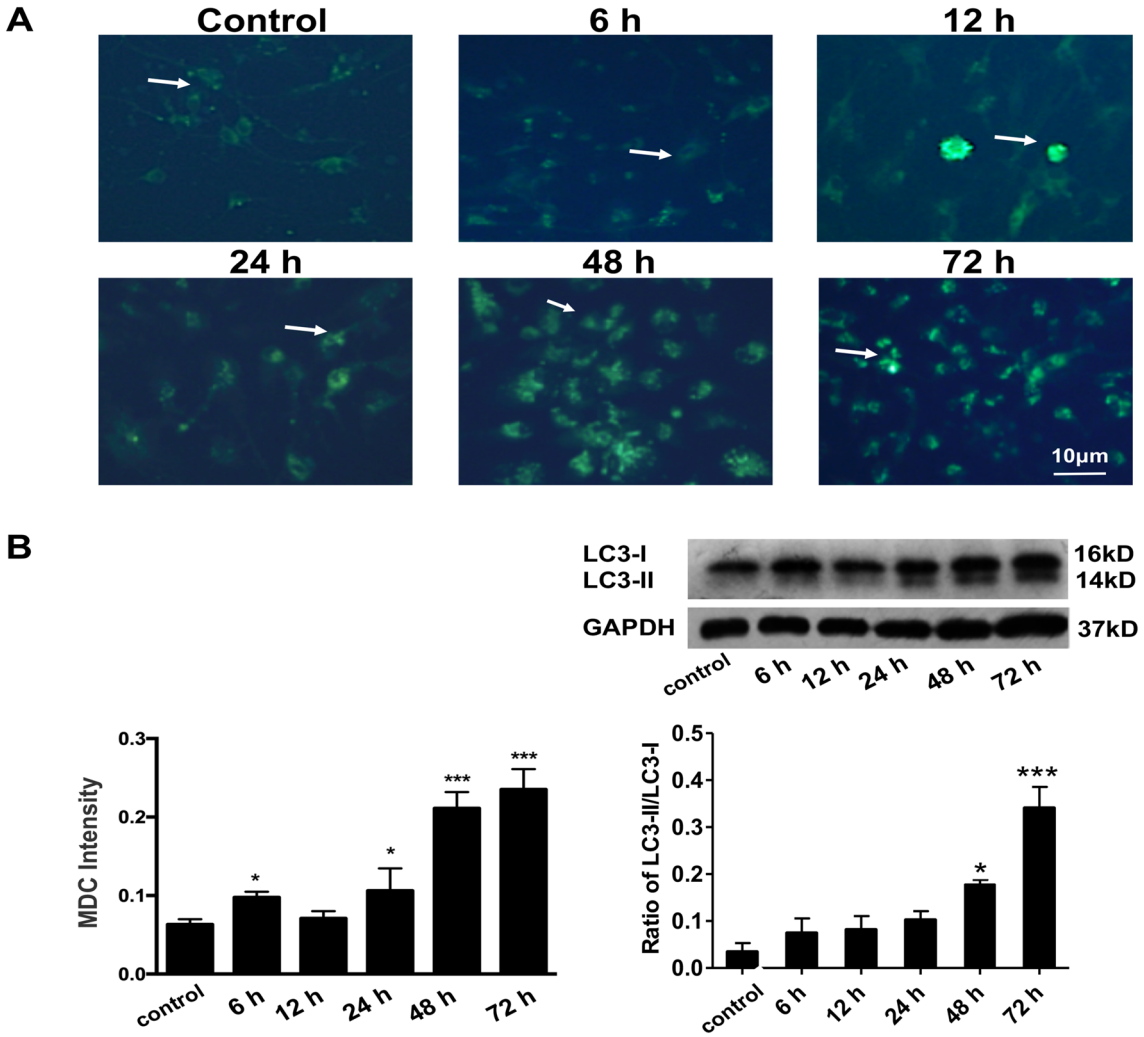

Fig. 2 The HIV-1 gp120 V3 loop induces autophagy in primary rat hippocampal neurons. **a** Neurons treated with HIV-1 gp120 V3 loop for various times, the levels of autophagic vacuoles were evaluated using MDC dye to measure. **b** After treatment with HIV-1 gp120 V3 loop for the indicated times, the neurons were harvested and protein was extracted using an assay kit. The concentration was determined using a Bio-Rad Protein Assay. Protein expression was analyzed by western blotting using antibodies against LC3B and GAPDH. Scale bars: 10 μm. n = 5. *$p < 0.05$ and ***$p < 0.001$ vs. control group

a subsequent increase at 24 h until 72 h. To further clarify the effect of gp120 on rat neuronal autophagy, we examined the microtubule-associated protein 1 light chain 3 (LC3) via western blot analysis. Under basal conditions, LC3 is cytosolic, but during autophagy it becomes conjugated to phosphatidylethanolamine (PE) and changes to the LC3-II form, which localizes to the membrane of phagophores and autophagosomes [18]. LC3-II is the only known protein in higher eukaryotes that is specifically associated with autophagosomes and acts as a reliable marker of autophagy. After treatment with gp120 V3 loop, the expression level of LC3-II protein increased in rat hippocampal neurons in a time dependent manner (Fig. 2b). These data indicate that HIV-1 gp120 V3 loop can induce cell autophagy in primary rat hippocampal neurons.

Autophagy Enhancement at Early Time Points Prevents Neuronal Apoptosis, but Promotes Apoptosis at Late Time Points

Recent studies suggest that autophagy is also a process of cell death, which is similar to apoptosis, and depends on the kind of stress the cell is undergoing. Furthermore, apoptosis and autophagy do not act as counterparts to each other, complicated interactions are between them when cells go through some stress. Autophagy can cooperate with or suppress apoptosis depending on the circumstance [19]. However, the role of autophagy in HAND remains unclear. To investigate the role of autophagy induced by the gp120 V3 loop in rat primary hippocampal neurons, we inhibited autophagy by using 3-MA. 3-MA is a

specific inhibitor of class III PI3 K, which is involved in autophagosome formation, and can inhibit autophagy by blocking autophagosome formation [20, 21].

As previous data has shown, the HIV-1 gp120 V3 loop induced an increase in neuronal autophagy after 6 h of treatment, decreased slightly after 12 h of exposure, and increased again in the following time. Considering that different levels of autophagy may affect cell survival distinctly, 6 h and 72 h were used as the major time points to assess whether autophagy played varying roles depending on time in neuronal apoptosis induced by HIV-1 gp120 V3 loop. After treating with gp120 V3 loop and 3-MA for 6 h, the level of autophagy and apoptosis in rat primary rat hippocampal neurons were examined using fluorescence microscope to measure autophagosomes with the LC3 antibody. As shown in Fig. 3a, b, treatment with HIV-1 gp120 V3 loop alone increased autophagic activity in rat neurons, while co-incubation with 3-MA displayed a reduction in autophagic activity. Western blot analysis also indicated that the gp120 V3 loop can increase the expression of LC3 and Beclin-1 and decrease the level of p62, while co-incubation with 3-MA can reverse these effects in neurons (Fig. 3c). After treating with 3-MA for 72 h, similar results are seen, indicating that HIV-1 gp120 V3 loop can induced neuronal autophagy, while 3-MA can reverse this enhancement (Fig. 4a–c).

To investigate the impact of autophagy blockade on HIV-1 gp120 V3 loop-induced apoptosis, the Hoechst 33342 staining, flow cytometry and western blotting were used. Interestingly, at 6 h time point, pretreatment with 3-MA can somehow exacerbate the neuronal death induced by HIV-1 gp120 V3 loop. Hoechst 33342 staining demonstrated that 3-MA plus gp120 V3 loop can significantly increase neuronal apoptosis compared to gp120 V3 loop alone (Fig. 5a). Moreover, the rate of apoptosis (Q4) in rat primary hippocampal neurons after treatment with HIV-1 gp120 V3 loop was 4.4%, whereas the rate of apoptosis was 13.2% in cells treated with gp120 V3 loop and 3-MA when evaluated by Annexin V/PI staining and flow cytometry (Fig. 5b). Caspase-3, a member of the cysteine-aspartic acid protease (caspase) family, plays a central role in apoptosis via the caspase cascade. Activation of caspase-3 by caspase-8 and caspase-9 is a hallmark of apoptosis. Caspase 3 cleavage is also indicated to be vital for the execution of apoptosis during advanced stages of neurodegenerative disorders. Caspase-3 consists of three chains with molecular weights of 35, 19 and 17 kDa, the latter two of which are the active chains. We used the 17-kDa caspase-3 chain as a proxy for caspase-3 activity [22]. Western blot analysis revealed that the expression level of caspase-3 in hippocampal neurons did not significantly change during treatment with the HIV-1 gp120 V3 loop when compared with the controls, but increased significantly when the neurons were co-treated with 3-MA (Fig. 5c).

Unexpectedly, 3-MA was able to protect neurons from HIV-1 gp120 V3 loop-induced apoptosis when assessed neuronal apoptosis treatment with 3-MA for 72 h. Hoechst 33342 staining demonstrated less apoptotic cells after 3-MA treatment (Fig. 6a). Moreover, the rate of apoptosis in rat primary hippocampal neurons after treatment with HIV-1 gp120 V3 loop was 20.2%, whereas the rate of apoptosis was 12.3% when combinates 3-MA with gp120 V3 loop (Fig. 6b). Western blot analysis revealed the expression level of caspase-3 in hippocampal neurons significantly increased during treatment with the HIV-1 gp120 V3 loop compared to the controls, while caspase-3 expression decreased significantly when co-treatment with 3-MA compared to the gp120 V3 loop alone group (Fig. 6c). Therefore, we concluded that early enhancement of autophagy by the HIV-1 gp120 V3 loop could inhibit the caspase-dependent neuronal apoptosis. In contrast, at 72 h time point, co-incubation with HIV-1 gp120 V3 loop and 3-MA can somehow inhibit neuronal apoptosis and prevent the damage to rat hippocampal neurons, which means that late autophagic enhancement induced by HIV-1 gp120 V3 loop can increase neuronal apoptosis and aggravate the damage to rat hippocampal neurons.

AMPK, ERK and Calpain are Involved in the Early Autophagy Induced by the Presence of HIV-1 gp120 V3 Loop in Neurons

Under normal conditions, autophagy is kept at a low level to maintain cellular homeostasis, but during stress conditions, such as heart disease, cancer and infectious diseases, autophagy reacts rapidly to protect tissues from damage. The formation of autophagic vacuoles is stimulated by a variety of intracellular and extracellular stressors, including amino acid starvation, the aggregation of misfolded proteins, and the accumulation of damaged organelles [23]. Several signaling pathways control the formation of autophagic vacuoles in mammalian cells. The Ser/Thr protein kinase Tor (target of rapamycin, mTOR) is a critical regulator of autophagy induction; activated mTOR (MAPK/ERK signaling) suppresses autophagy, and negative regulation of mTOR (AMPK signaling) promotes it [14].

In addition to mTOR-dependent signaling pathways, other mTOR-independent pathways can also influence autophagy. Calpains are a family of calcium-activated cysteine proteases that inhibit autophagy. Reducing the level of calpain activity in cultured cells can increase autophagy and decrease the levels of autophagy substrates. The core autophagy protein ATG5 has also been demonstrated to be directly cleaved and inactivated by calpains, suggesting that calpains may act on a number of substrates to negatively regulate autophagy [24].

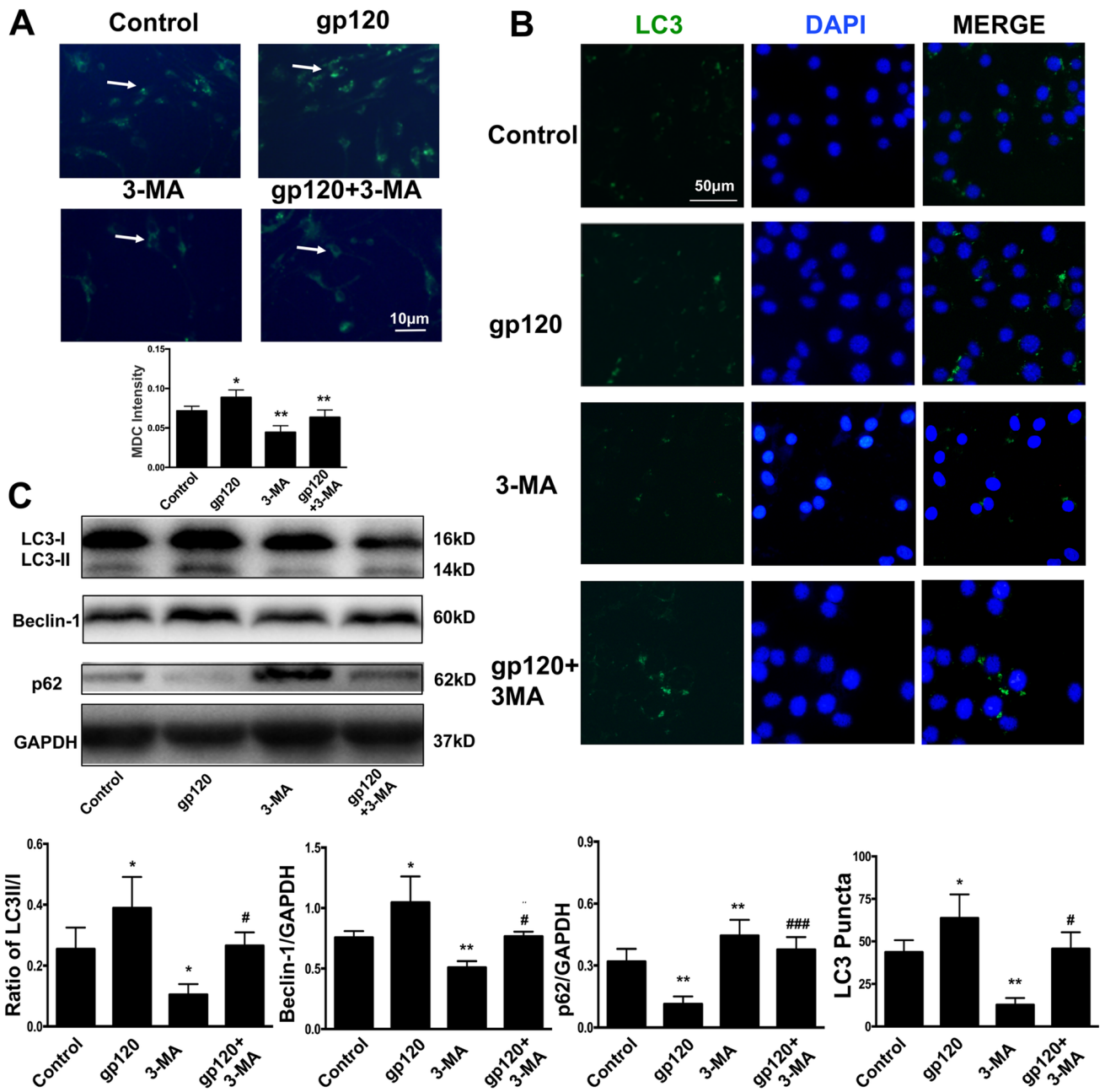

Fig. 3 3-MA inhibits autophagy in primary rat hippocampal neurons induced by HIV-1 gp120 V3 loop. Neurons were treated with 1 μg/mL HIV-1 gp120 V3 loop and/or 40 mM 3-MA for 6 h, and **a** the amount of autophagic vacuoles was evaluated using MDC dye, MDC intensity was quantified by Image J. **b** punctate structures stand for autophagosomes in cell immunostained with anti-LC3 (green) using fluorescence microscope, average number of punctate structures per cell was quantified by Image J. **c** Protein expression levels were analyzed by western blotting using antibodies against LC3B, Beclin-1, p62 and GAPDH. Scale bars: 10 μm. n = 6. * $p < 0.05$, ** $p < 0.01$ vs. control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. gp120 V3 group

Both AMPK and ERK can regulate the activity of mTORC1, while calpain is the main regulator of mTOR-independent autophagy [25, 26]. To investigate the role of ERK, AMPK and calpain in the autophagy induced by HIV-1 gp120 V3 loop in neurons, we treat neurons with specific ERK, AMPK and calpain antagonists PD98059, Compound C and calpeptin and examined their effects on autophagy and apoptosis.

Assessment of LC3 by immunofluorescence showed an increase in the number of puncta in the cytoplasm of hippocampal neurons in the gp120 V3 loop, PD98059- and calpeptin-treated groups when compared with controls; however, the number of puncta decreased in the Compound C-treated group. Compared with the group treated with the gp120 V3 loop alone, the number of puncta in cells

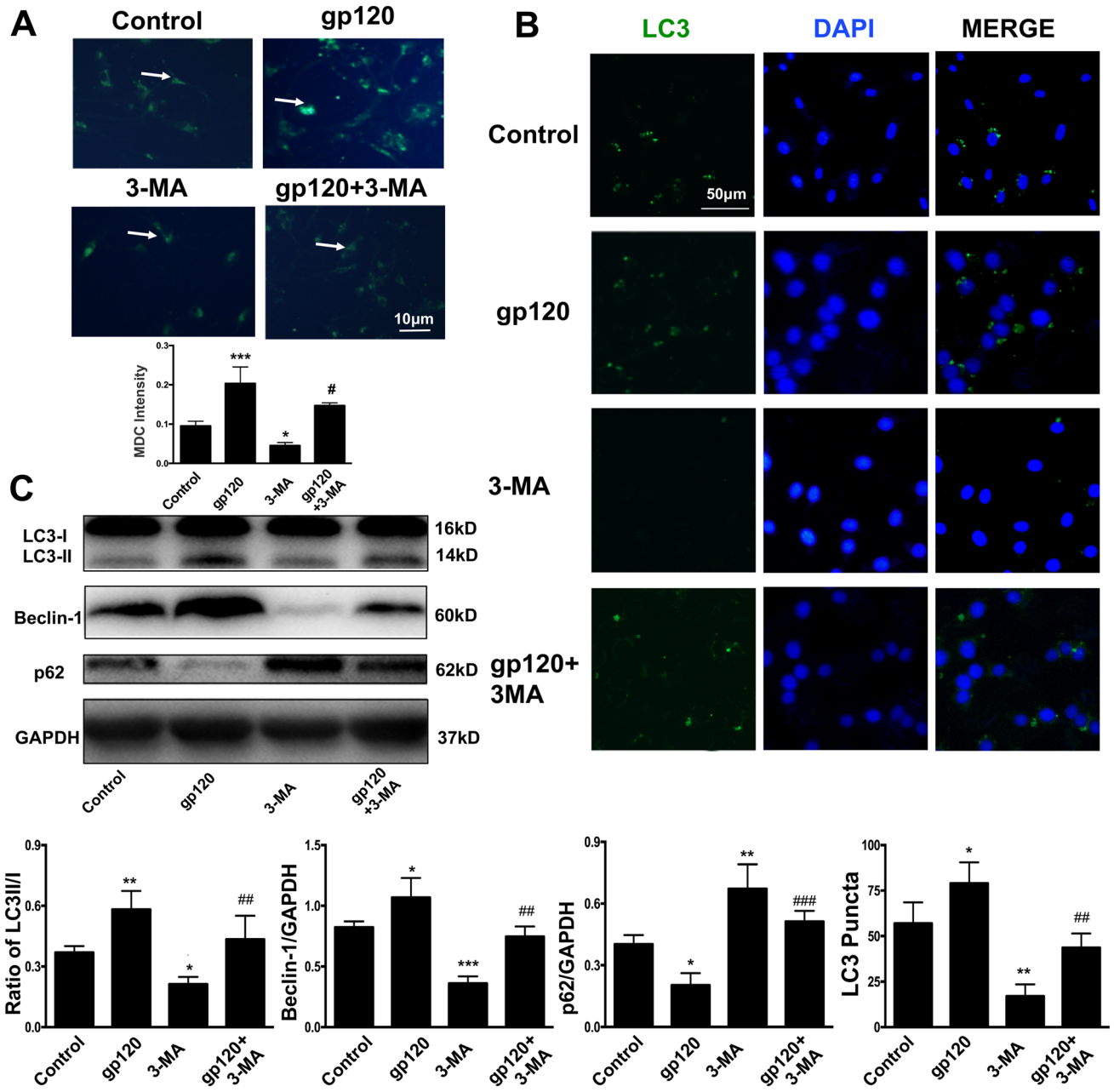

Fig. 4 3-MA inhibits autophagy in primary rat hippocampal neurons induced by HIV-1 gp120 V3 loop. Neurons were treated with HIV-1 gp120 V3 loop and/or 3-MA for 72 h and, **a** the amount of autophagic vacuoles was evaluated using MDC dye, MDC intensity was quantified by Image J. **b** Punctate structures stand for autophagosomes in cell immunostained with anti-LC3 (green) using fluorescence microscope, average number of punctate structures per cell was quantified by Image J. **c** Protein expression levels were analyzed by western blotting using antibodies against LC3B, Beclin-1, p62 and GAPDH. Scale bars: 10 μm. n = 6. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. gp120 V3 group

treated with gp120 V3 loop plus PD98059 or calpeptin was increased, whereas it was decreased in cells treated with the gp120 V3 loop plus Compound C (Fig. 7a). Western blot analysis indicated that the expression of LC3 and Beclin-1 increased and p62 expression decreased in the gp120 V3 loop-, PD98059- and calpeptin-treated groups as compared to the control group. The expression of LC3 and Beclin-1 decreased, while p62 expression increased in the Compound C-treated group compared with the control. The expression of LC3 and Beclin-1 increased while the expression of p62 decreased in the cells treated with the gp120 V3 loop plus PD98059 or calpeptin compared with the cells treated with the gp120 V3 loop alone. The expression of LC3 and Beclin-1 decreased and the expression of p62 increased in the

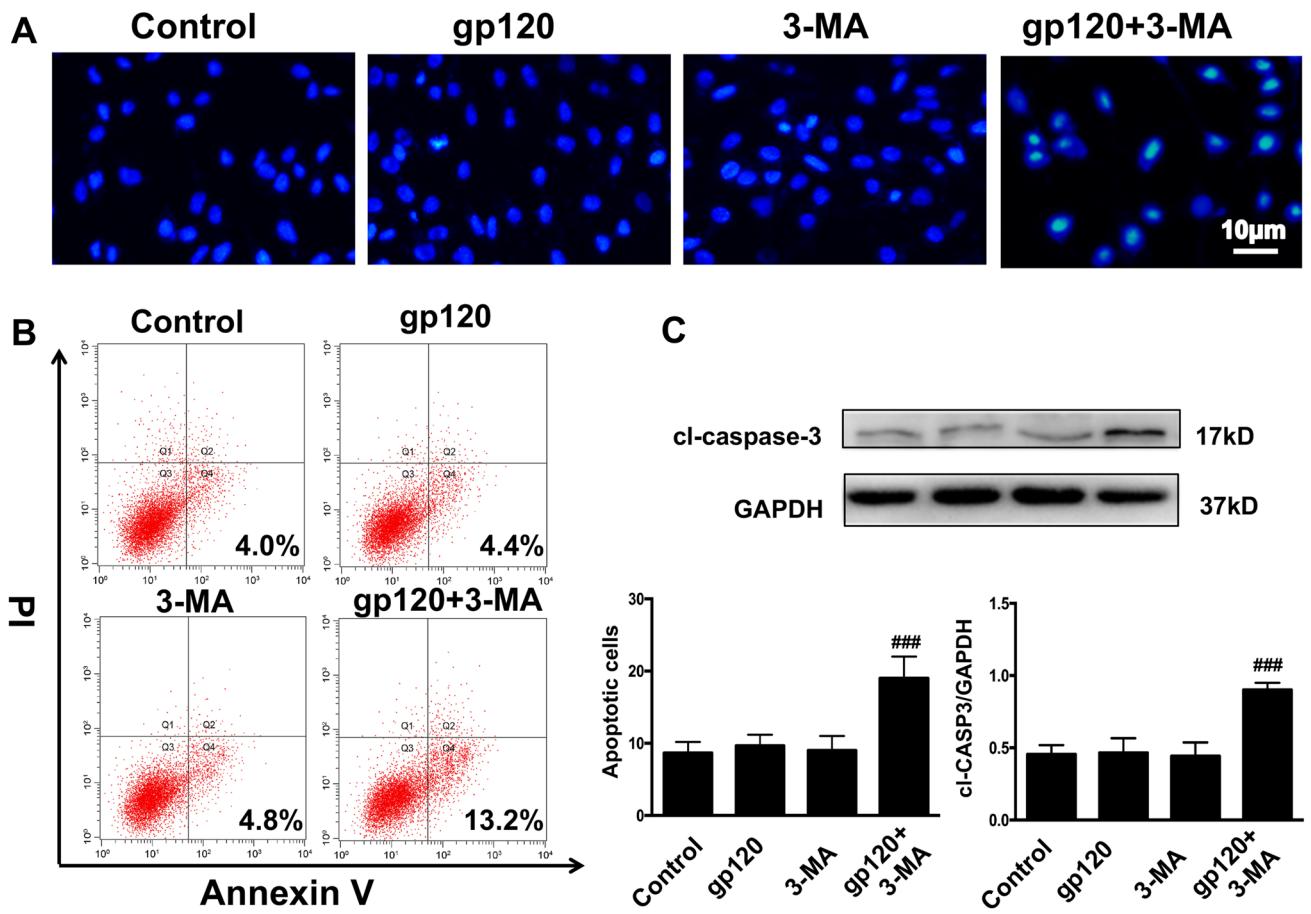

Fig. 5 3-MA increases apoptosis in primary rat hippocampal neurons induced by HIV-1 gp120 V3 loop at 6 h. Neurons were treated with HIV-1 gp120 V3 loop and/or 3-MA for 6 h and apoptotic level were detected. **a** Cell nuclei (in blue) were labeled with Hoechst 33342. **b** The cells were stained with Annexin V/PI and detected by flow cytometry analysis. **c** After incubation with HIV-1 gp120 V3 loop/3-MA for 6 h, neurons were harvested. Protein expression levels were analyzed by western blotting using antibodies against caspase-3 and GAPDH. Scale bars: 10 μm. n = 6. $^{###}p < 0.001$ vs. gp120 V3 group

group treated with gp120 V3 loop plus Compound C compared with the gp120 V3 loop-treated group (Fig. 7b).

The autophagy inhibited by Compound C, and induced by PD98059 and calpeptin, those inhibitors have influence on the apoptosis of HIV-1 gp1b20V3 loop treated neurons. Flow cytometry indicated that the rate of apoptosis in hippocampal neurons remained unchanged in the group treated with the gp120 V3 loop, compared to non-treated cells. While after co-incubation with PD98059 or Compound C, the apoptosis rate significantly increased compared with gp120 V3 group, and decreased in the group treated with the gp120 V3 loop plus calpeptin compared with the gp120 V3 loop-treated group (Fig. 8a). Western blot analysis showed the caspase-3 expression was elevated in the gp120 V3 loop, PD98059 and Compound C-treated groups, but decreased in the calpeptin-treated group compared with the control group. Caspase-3 expression was increased in the cells treated with the gp120 V3 loop plus PD98059 or Compound C, and it was decreased in the cells treated with the gp120 V3 loop plus calpeptin compared with the gp120 V3 loop-treated group (Fig. 8b).

AMPK and Calpain, but not ERK are Involved in the Late Autophagy Enhancement Induced by the HIV-1 gp120 V3 Loop in Neurons

Interestingly, ERK seems not work when looked into pathways get involved in neuron autophagy in the late time point. Assessment of LC3 by immunofluorescence revealed that the number of puncta increased in the cytoplasm of hippocampal neurons in the gp120 V3 loop-, PD98059- and calpeptin-treated groups when compared with controls, however it decreased in the Compound C-treated group. Compared with the group treated with the gp120 V3 loop alone, the number of puncta in cells treated with gp120 V3 loop plus calpeptin was increased, whereas it was decreased in cells treated with the gp120 V3 loop plus Compound C, and no apparent difference was detected in cells treated with

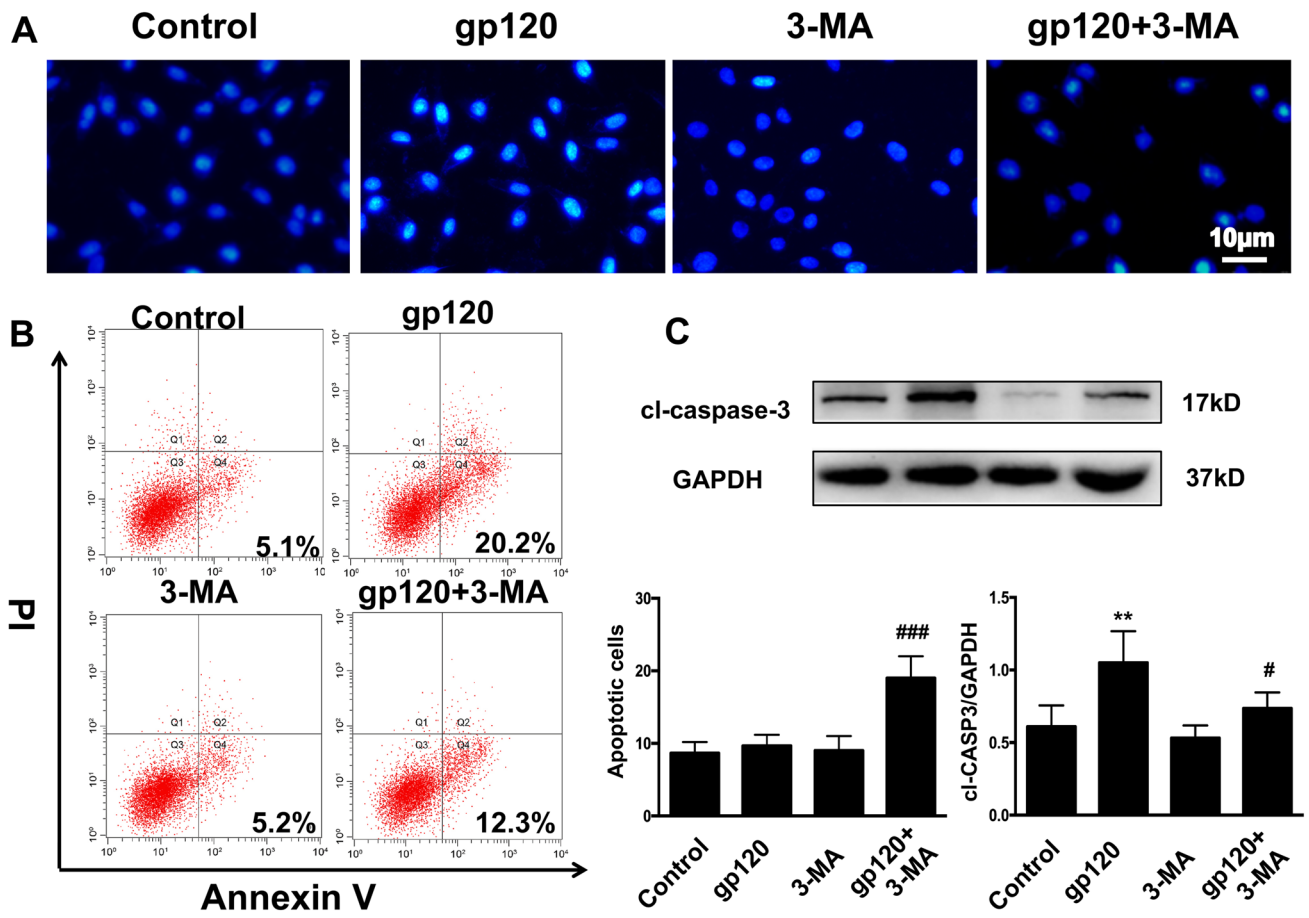

Fig. 6 3-MA decreases apoptosis in primary rat hippocampal neurons induced by HIV-1 gp120 V3 loop at 72 h. Neurons were treated with HIV-1 gp120 V3 loop and/or 3-MA for 72 h and apoptotic level were detected. **a** Cell nuclei (in blue) were labeled with Hoechst 33342. **b** The cells were stained with Annexin V/PI and detected by flow cytometry analysis. **c** After incubation with HIV-1 gp120 V3 loop/3-MA for 6 h, neurons were harvested. Protein expression levels were analyzed by western blotting using antibodies against caspase-3 and GAPDH. Scale bars: 10 μm. n = 6. $^{**}p < 0.01$ vs. control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. gp120 V3 group

the gp120 V3 loop plus PD98059 (Fig. 9a). Western blot analysis indicated that the expression of LC3 and Beclin-1 increased and p62 expression decreased in the gp120 V3 loop-, PD98059- and calpeptin-treated groups compared to the control group. The expression of LC3 and Beclin-1 decreased and p62 expression increased in the Compound C-treated group compared with the control. The expression of LC3, Beclin-1 and p62 was not significantly changed in cells treated with the gp120 V3 loop plus PD98059 compared with cells treated with the gp120 V3 loop alone. The expression of LC3 and Beclin-1 increased while the expression of p62 decreased in the cells treated with the gp120 V3 loop plus calpeptin compared with the cells treated with the gp120 V3 loop alone. The expression of LC3 and Beclin-1 decreased and the expression of p62 increased in the group treated with gp120 V3 loop plus Compound C compared with the gp120 V3 loop-treated group (Fig. 9b).

Interestingly, those autophagy inhibitors can influence neuron apoptosis induced by HIV-1 gp120 V3 loop. Flow cytometry indicated that the rate of apoptosis in hippocampal neurons was increased in the gp120 V3 loop-, PD98059- and Compound C-treated groups, and it was decreased in the calpeptin-treated group compared with the control group. The rate of apoptosis in hippocampal neurons remained unchanged in the group treated with the gp120 V3 loop plus PD98059 compared with the gp120 V3 loop-treated group, but the rate was increased in the group treated with the gp120 V3 loop plus Compound C and decreased in the group treated with the gp120 V3 loop plus calpeptin compared with the gp120 V3 loop-treated group (Fig. 10a). Western blots showed that the expression of caspase-3 was elevated in the gp120 V3 loop-, PD98059- and Compound C-treated groups, and caspase-3 was decreased in the calpeptin-treated group compared with the control group. There was little change in the expression of caspase-3 in the cells treated with gp120 V3 loop plus PD98059 compared with the gp120 V3 loop-treated cells, but the expression of caspase-3 was increased in the cells treated with the gp120 V3

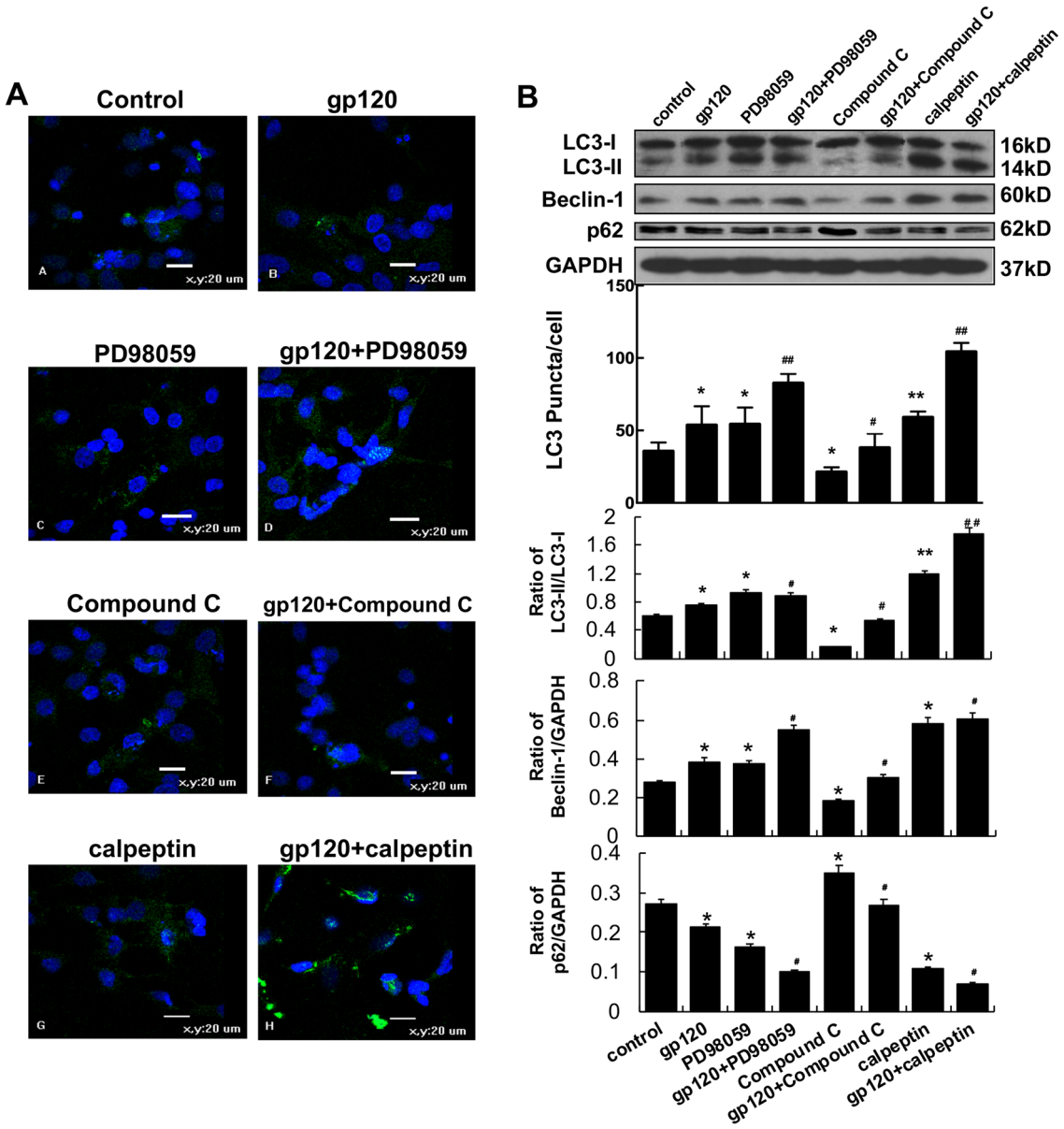

Fig. 7 AMPK, ERK and Calpain are involved in the early autophagy induced by the HIV-1 gp120 V3 loop in neurons. Neurons were treated with HIV-1 gp120 V3 loop and/or PD98059, Compound C or Calpeptin for 6 h. **a** Cell nuclei (in blue) were labeled with DAPI, and the cytoplasmic LC3 protein (in green) was labeled with FITC, average number of punctate structures per cell was quantified by Image J. **b** Protein expression levels were analyzed by western blotting using antibodies against LC3B, Beclin-1, p62 and GAPDH. Scale bars: 20 μm. n = 5. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group, $^{\#}p < 0.05$ vs. gp120 V3 group; $^{\#\#}p < 0.01$ vs. gp120 V3 group

loop plus Compound C, and it was decreased in the cells treated with the gp120 V3 loop plus calpeptin compared with the gp120 V3 loop-treated group (Fig. 10b). These data indicate that the AMPK and calpain signaling pathways are not only involved in the whole autophagy, but they may also take part in the interaction between autophagy and apoptosis induced by the HIV-1 gp120 V3 loop in primary hippocampal neurons, whereas the ERK pathway is not involved in the autophagy enhancement induced by the HIV-1 gp120 V3 loop.

Discussion

HIV-1 gp120 is a glycoprotein on the surface of HIV and is an important toxin that affects HAND patients. In our research, we focused on the third variable region of the gp120 molecule, the gp120 V3 loop. This region contains the binding site on HIV-1 for its co-receptors CXCR4 and CCR5, and it is the main factor responsible for the tropism of the virus, which plays a vital role for gp120-induced neuronal injury [27]. Numerous researches have reported

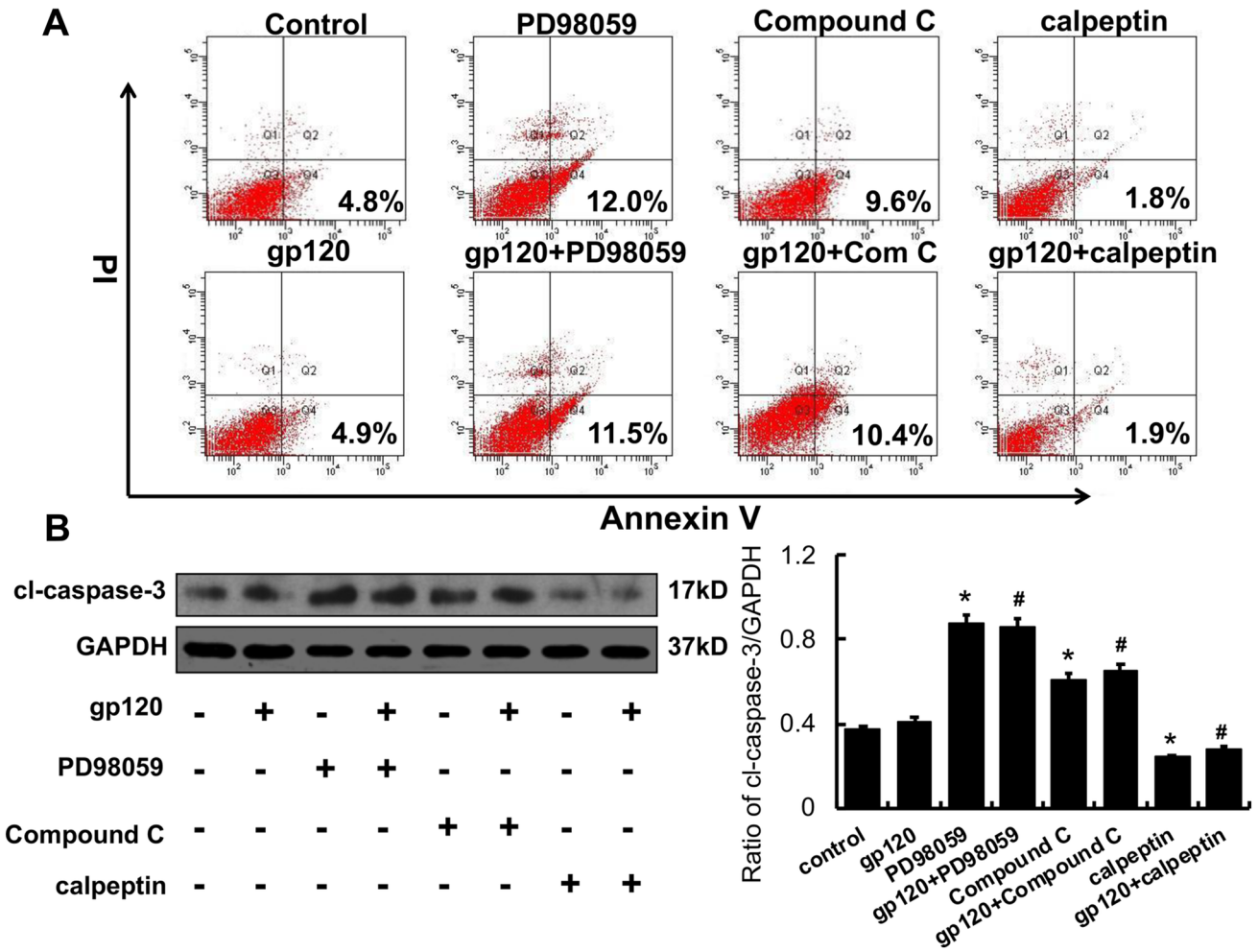

Fig. 8 AMPK, ERK and Calpain are involved in the early apoptosis induced by the HIV-1 gp120 V3 loop in neurons. Neurons were treated with HIV-1 gp120 V3 loop and/or PD98059, Compound C or Calpeptin for 6 h. **a** The cells were stained with Annexin V/PI and detected by flow cytometry. **b** Protein expression levels were analyzed by western blotting using antibodies against caspase-3 and GAPDH. n = 5; $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. gp120 V3 group

the pathogenic mechanisms of HIV-1 gp120, and our previous studies also confirmed that the HIV-1 gp120 V3 loop can induce apoptosis in rat hippocampal neurons [4] and cause impairments in spatial learning and memory in Sprague–Dawley (SD) rats [3].

With the importance of autophagy in various diseases being discovered, researchers have also paid attention to its role in HIV pathogenesis. It has been found that autophagy is increased in HIV encephalitis patients and gp120 treated neuroblastoma cells. However, the effect of autophagy on neuronal cell death induced by HIV-1 gp120 remains unclear, and the exact mechanism is still unknown. Hence, we focused on investigating the role of autophagy induced by HIV-1 gp120 in rat primary neurons and the interaction between autophagy and apoptosis. We, for the first time, demonstrate that long period exposure of the HIV-1 gp120 V3 loop can induce high levels of autophagy in rat primary hippocampal neurons, and this excessive autophagy aggravates the neuronal apoptosis induced by the HIV-1 gp120 V3 loop.

Autophagy is one of the key mechanisms in which cells degrade and recycle damaged or harmful cellular components, a process that mediates metabolic adaptations and maintains energy homeostasis during an integrated stress response. Our data demonstrate that the HIV-1 gp120 V3 loop can stimulate the conversion of LC3B-I to LC3B-II and increase the number of autophagic vacuoles in rat primary hippocampal neurons. In addition, we examined whether further autophagy induction have effects on apoptosis in neurons. Using the Class III PI3 K inhibitor 3-MA as a specific autophagy inhibitor, we co-treated the neurons with the HIV-1 gp120 V3 loop and examined the changes in autophagy and apoptosis within these neurons. Results indicate that incubation with 3-MA can reverse the increase

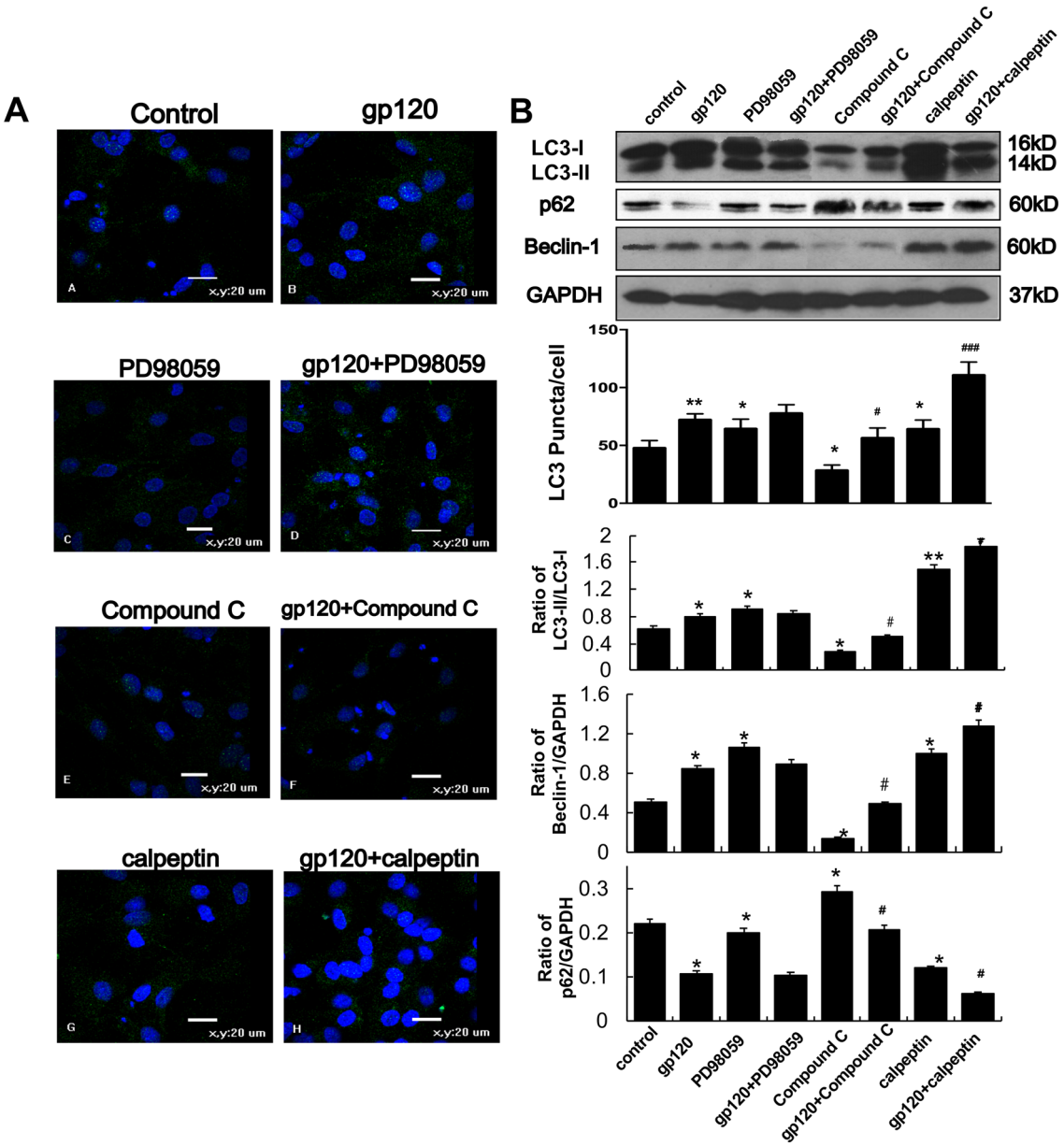

Fig. 9 AMPK and Calpain, but not ERK are involved in the late autophagy induced by the HIV-1 gp120 V3 loop in neurons. Neurons were treated with 1 μg/mL HIV-1 gp120 V3 loop and/or PD98059, Compound C or Calpeptin for 72 h. **a** Cell nuclei (in blue) were labeled with DAPI, and the cytoplasmic LC3 protein (in green) was labeled with FITC, average number of punctate structures per cell was quantified by Image J. **b** Protein expression levels were analyzed by western blotting using antibodies against LC3B, Beclin-1, p62 and GAPDH. Scale bars: 20 μm. n = 5. $^{*}p < 0.05$, $^{**}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. gp120 V3 group; $^{\#\#\#}p < 0.001$ vs. gp120 V3 group

in the number of autophagic vacuoles, LC3 puncta and the levels of LC3B-II and Beclin-1 induced by the HIV-1 gp120 V3 loop. Meanwhile, Hoechst 33342 and Annexin V-FITC/PI staining demonstrated that apoptosis increases in neurons after 3-MA treatment for 6 h, while western blot revealed a similar trend for the expression level of caspase-3. However, 72 h of 3-MA incubation can dramatically cause opposite effect, which means that it can decrease the apoptotic rate in neurons treated with HIV-1 gp120 V3 loop. This is the first time that we found autophagy can affect neuronal death distinctly and time-dependently.

CXCR4, CCR5 and many other chemokine receptors expressed on the cell surface of neurons, and all of them are seven-pass transmembrane heterotrimeric GTP binding proteins. HIV-1 gp120 can bind to these receptors directly and activate the downstream signaling pathways without the presence of the CD4 receptor [28]. By binding to the co-receptor CXCR4 in neurons, gp120 can stimulate the

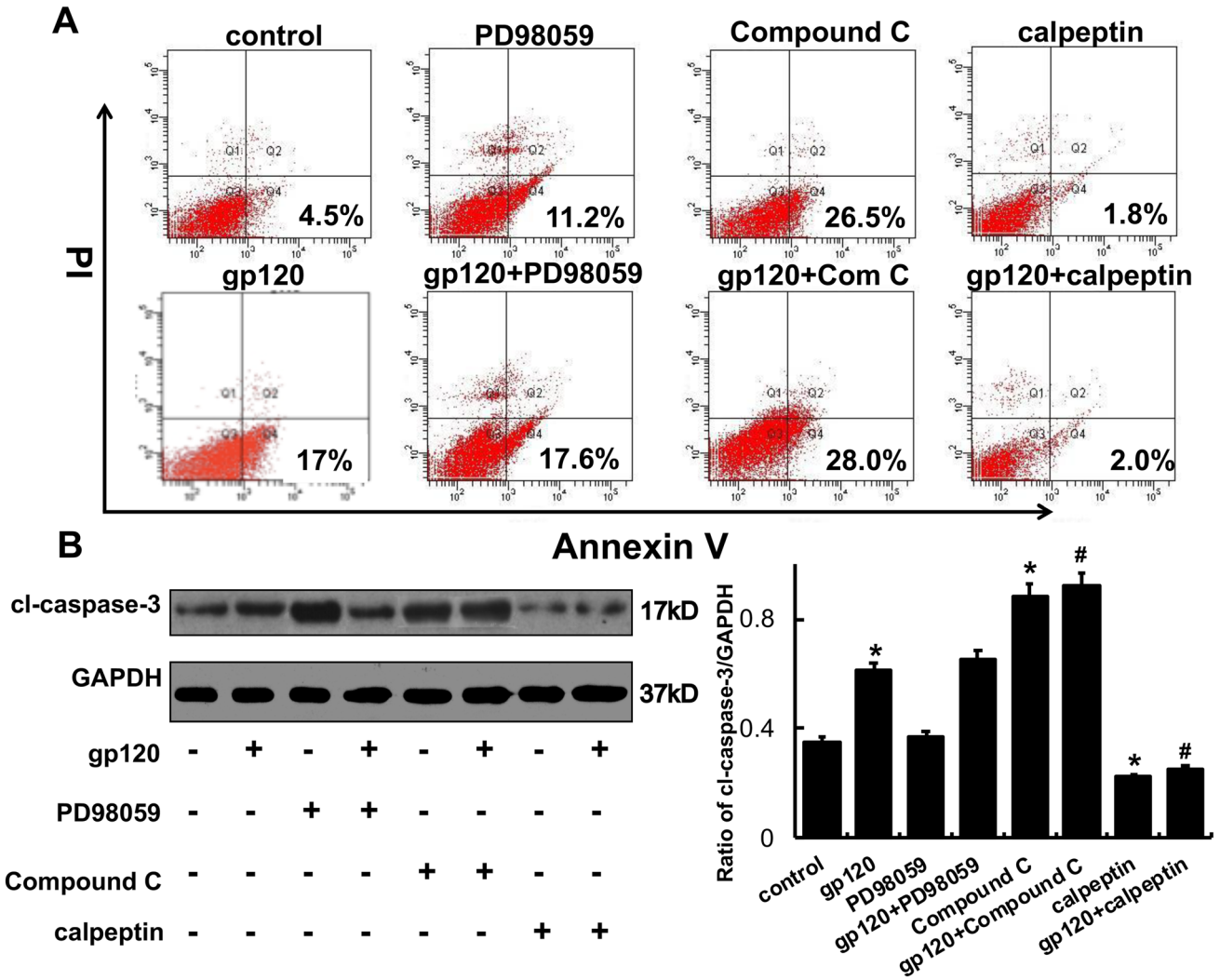

Fig. 10 AMPK and Calpain, but not ERK are involved in the late apoptosis induced by the HIV-1 gp120 V3 loop in neurons. Neurons were treated with HIV-1 gp120 V3 loop and/or PD98059, Compound C or Calpeptin for 72 h. **a** The cells were stained with Annexin V/PI and detected by flow cytometry. **a** Protein expression levels were analyzed by western blotting using antibodies against cl-caspase-3 and GAPDH. n = 5. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. gp120 V3 group

mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway [29], which can also regulate autophagy. Recent studies have shown that sustained MEK/ERK activation results in the complete disassembly of both mTORC1 and mTORC2, strongly enhancing Beclin-1 activity, resulting in cyto-destruction [14].

Research has shown that p53 is required both in neurons and microglia during HIV-associated neurodegeneration, and AMP-activated kinase (AMPK) acts upstream of p53 [30], thus AMPK also took participate in the pathogenesis of HAND. Moreover, the increasing concentration of intracellular calcium can activate autophagy via AMPK [31]. Data have also shown that AMPK can sense changes in the intracellular ATP/AMP ratio and directly phosphorylate TSC2, thereby providing the priming phosphorylation for the subsequent phosphorylation of TSC2 by glycogen synthase kinase 3 (GSK-3) to inhibit mTOR signaling [32]. As such, we focused our research on AMPK as an additional protein target.

gp120 can also activate the NMDA receptor of hippocampal neurons, which can further stimulate calpain [33]. Calpains are a family of calcium-activated cysteine proteases that inhibit autophagy. Strategies that reduce calpain activity in cell culture increase autophagy and decrease the levels of autophagy substrates, such as mutant Htt. These effects are likely to be mediated by Gsα, a heterotrimeric G-protein subunit that is activated by calpain cleavage [34]. In addition to this mechanism of autophagy up-regulation by calpains, the core autophagy protein ATG5 has also been demonstrated to be cleaved and inactivated by calpains, suggesting

that calpains may act on a number of substrates to negatively regulate autophagy.

As a result, we employed the ERK inhibitor PD98059, the AMPK inhibitor Compound C, and the calpain inhibitor calpeptin, to regulate the respective autophagic signaling pathways and to examine whether these pathways are involved in the changes in autophagy induced by HIV-1 gp120 in rat primary hippocampal neurons. In accordance with previous research, our data show that Compound C inhibited the enhancement of autophagy induced by HIV-1 gp120 V3 loop, and calpeptin increased the autophagy induction. While Compound C treatment aggravates the neuronal apoptosis and calpeptin decreases the apoptosis. These results refer that AMPK up-regulates autophagy and inhibits apoptosis, however, calpain down-regulates the autophagy and therefore increase apoptosis. Conversely, ERK1/2 inhibition did not affect HIV-1 gp120 V3 loop-induced neuronal autophagy or apoptosis, especially after long period exposure (72 h). Additionally, the activity of these signaling molecules may vary at different stages, which requires further investigation.

To conclusion, in the present study, we have demonstrated that exposing neurons to the HIV-1 gp120 V3 loop for 72 h causes excessive autophagy, which in turn, can aggravate neuronal apoptosis. Therefore, the HIV-1 gp120 V3 loop has a negative effect on cell survival in rat primary hippocampal neurons when exposure for a long time. The underlying mechanism may involve both AMPK/mTOR-dependent and mTOR-independent calpain pathways. Prior studies have shown that autophagy and apoptosis interact both positively and negatively, and extensive crosstalk exists between the two. Our study demonstrates that excessive autophagy can promote apoptosis, which supports the use of a combinatorial treatment with an autophagy inhibitor as a possible therapeutic strategy for HAND.

Experimental Procedures

Antibodies and Reagents

All commercial antibodies and chemicals were purchased from the following resources: anti-MAP-2 antibody (M5670) was from Sigma-Aldrich (St Louis, MO, USA); anti-caspase-3 (9662S), anti-LC3 (3868S), anti-p62 (5114S), anti-Beclin-1 (3738S) and anti-GAPDH (2118S) antibodies were from Cell Signaling Technology (Danvers, MA, UAS); The HIV-1 gp120 V3 loop was synthesized by the Shanghai Apeptide Co. (Shanghai, China; sequence: Asn-Asn-Thr-Arg-Lys-Ser-Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys-Ile-Gly; molecular formula: $C_{114}H_{199}N_{41}O_{31}$; molecular weight: 2640.06, 1 μg/mL); Compound C (P5499, 40 μM, pretreatment for 30 min), PD98059 (215, 25 μM, pretreatment for 1 h) and 3-MA (189,490, 10 mM, pretreatment for 4 h) were from Sigma-Aldrich; Calpeptin (0448, 20 μM, pretreatment for 30 min) was from Tocris (Bristol, UK).

Primary Culture of Rat Neurons

Rat primary hippocampal neurons were isolated and cultured according to methods previously described [3] with some modifications. A total of 10^5 or 10^6 cells/well in 96- or 6-well plates were cultured with 90% Dulbecco's modified Eagle medium/F12 and 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China) in a sterile environment. After 4 h, the culture media was replaced with serum free media containing 98% Neurobasal medium (Gibco, Carlsbad, California, USA) and 2% B27 (Gibco). Finally, 50% of the media was replaced every 3 days.

Immunocytochemical Identification in Hippocampal Neurons

Neurons were cultured for 4 h or 3 days and imaged under an inverted phase contrast microscope (Olympus, Tokyo, Japan). On 7th day the primary rat hippocampal neurons seeded on the plate, Hoechst 33342 (Beyotime, Haimen, Jiangsu, China) and MAP-2 (Sigma, St. Louis, MO, USA) staining were performed to identify the neurons. Briefly, the cells were fixed with ice-cold 4% paraformaldehyde (PFA) and incubated with the neuron-specific MAP-2 antibody in blocking serum overnight at 4 °C. After incubation with a species-specific IgG conjugated with Cy3 for 1 h, the cells were washed with PBS, followed by Hoechst 33342 staining at 37 °C for 5 min and imaged with a confocal laser scanning microscope (Zeiss, LSM 510, Oberkochen, Baden Wurttemberg, Germany).

Cell Cytotoxicity Assay

The effect of HIV-1 gp120 on cell growth was assayed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method, as previously described [22]. Briefly, cells were seeded in 96-well plates (5×10^3 cells/well) until the cells adhered. After 7 days, the hippocampal neurons were stimulated with different concentrations of gp120 (0, 0.1, 1 and 10 μg/mL) in the culture media. After 6 h, 12 h, 24 h, 48 h and 72 h of gp120 stimulation, 20 μL MTT was added to each well (5 mg/mL final concentration) and incubated for an additional 4 h. The number of viable cells was directly proportional to the production of formazan following the solubilization with isopropanol. The color intensity was measured at 570 nm. Each condition was performed in triplicate, and the data were obtained from at least three separate experiments.

Detection of Apoptotic Cells by Hoechst 33342 Staining

Neurons were plated at a density of 5000 cells per well in a 24-well plate with sterile glass cover slips. Apoptotic cells were detected using Hoechst 33342 (Beyotime, Haimen, China), as previously described [23]. The cells were fixed with 4% PFA for 10 min, washed with ice-cold PBS three times and then stained with Hoechst 33342 for 5 min. Cells with bright blue fragmented nuclei, indicating condensed chromatin, were identified as apoptotic cells. The morphological changes in apoptotic nuclei were evaluated under a fluorescence microscope (excitation wavelength 350 nm, emission filter 460 nm) (Olympus Fluoview, Japan).

Detection of Cell Death Using Flow Cytometry

Cell death was evaluated using an Annexin V-FITC apoptosis detection kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions [22]. Briefly, the neurons from various treatment conditions were collected and resuspended in 1 × binding buffer. The cells were transferred to a 5 mL culture tube containing Annexin V-FITC and incubated for 10 min at room temperature in the dark. After centrifugation, the supernatants were discarded. The neurons were stained with Annexin V-FITC binding buffer and propidium iodide (PI) and then incubated for 15 min at room temperature in the dark. The stained cells were analyzed using flow cytometry (FACScan, Becton–Dickinson, San Jose, CA). and the apoptotic rate were assessed by FlowJo 7.

Immunofluorescence Analysis for Autophagy Related Proteins

After incubation according to the study design, the neurons were fixed with 4% PFA in PBS for 30 min, washed with PBS three times and then permeabilized with Triton-X-100 for 15 min. After washing with PBS three times, the cells were blocked with 5% BSA for 1 h at room temperature and then incubated with anti-LC3 antibody (Cell Signaling Technologies Inc., Danvers, MA, USA) in 5% BSA overnight at 4 °C. Subsequently, the cells were washed in PBS three times and incubated with fluorescein isothiocyanate-labeled secondary antibody for 1 h at 37 °C. The cells were then washed and incubated with DAPI for 15 min and then washed again and mounted. The cells were then imaged using fluorescence microscopy (LSM 510 META, Carl Zeiss, Oberkochen, Germany).

Lysosome Detection by Monodansycadaverine (MDC) Staining

Following treatment with gp120, the cells were washed three times with PBS and then stained with monodansylcadaverine (MDC, Sigma, USA), an autofluorescent dye that accumulates in autophagic vacuoles, by incubating the cells with 0.01 mM MDC at 37 °C for 1 h. Subsequently, the cells were washed three times with PBS and analyzed using fluorescence microscopy (Olympus Fluoview, Japan).

Western Blot Analysis

The cells were treated according to the study design and harvested at the indicated time. Protein was extracted using a protein extraction kit (Beyotime, China), and the concentration was determined using a Bio-Rad Protein Assay. Immunoblot analysis was performed as previously described [3]. The cell lysates were separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were incubated with TBS containing 0.1% Tween-20 (TBST) and 5% bovine serum albumin for 1 h at room temperature. After washing three times with TBST for 10 min each, the blots were incubated with the indicated primary antibodies overnight at 4 °C, followed by a horseradish peroxidase-labeled secondary antibody for 1 h. The detection of specific proteins was carried out with an enhanced chemiluminescent detection system, and the bands were quantified by densitometry using Image-ProPlus 6.0.

Statistical Analysis

The data are presented as mean ± SD (standard deviation) for at least three independent experiments. The statistical analyses were performed using the SPSS 21 statistical software (SPSS Inc., IL, USA). Multiple comparisons of data among the groups were performed using a one-way ANOVA followed by the least significant difference test (Fisher test). The significance was evaluated using the unpaired Student's t test for comparisons between two means. A p value less than 0.05 was considered statistically significant.

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Compliance with Ethical Standards

Conflict of Interest There are no conflicts of interest associated with the present study.

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