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Neuroprotective effects of vitexin by inhibition of NMDA receptors in primary cultures of mouse cerebral cortical neurons

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Abstract The accumulation of glutamate can excessively activate the *N*-methyl-D-aspartate (NMDA) receptors and cause excitotoxicity. Vitexin (5, 7, 4-trihydroxyflavone-8-glucoside, Vit) is a c-glycosylated flavone which was found in the several herbs, exhibiting potent hypotensive, anti-inflammatory, and neuroprotective properties. However, little is known about the neuroprotective effects of Vit on glutamate-induced excitotoxicity. In present study, primary cultured cortical neurons were treated with NMDA to induce the excitotoxicity. Pretreatment with Vit significantly prevented NMDA-induced neuronal cell loss and reduced the number of apoptotic neurons. Vit significantly inhibited the neuronal apoptosis induced by NMDA exposure by regulating balance of Bcl-2 and Bax expression and the cleavages of poly (ADP-ribose) polymerase and pro-caspase 3. Furthermore, pretreatment of Vit reversed the up-regulation of NR2B-containing NMDA receptors and the intracellular Ca^{2+} overload induced by NMDA exposure. The neuroprotective effects of Vit are related to inhibiting the activities of NR2B-containing NMDA receptors and reducing the calcium influx in cultured cortical neurons.

Keywords Vitexin · Excitotoxicity · Neuron · Apoptosis · Calcium

Abbreviation

Caspase	Cysteinyl aspartate-specific protease
MTT	(3-(4,5-Dimethylthiazol-2-yl-)-2,5-diphenyl-tetrazolium bromide
NMDA	*N*-methyl-D-asparate
NMDAR	NMDA receptor
Vit	Vitexin

Introduction

Vitexin (5, 7, 4-trihydroxyflavone-8-glucoside, Vit) is a c-glycosylated flavone (Fig. 1a), which was found in the *Passion flower* [1], *Phyllostachys nigra* bamboo leaves [2], *Vitex agnus-castus* (chaste tree or chasteberry) [3], *Pearl millet* (Pennisetum millet) [4], and *Hawthorn* [5]. Vit has received much attention because of its wide spectrum of pharmacological effects, such as anticancer effect [6, 7], antioxidant activity [8], antidepressant-like effect [9], antinociceptive activity [10], and anti-inflammatory activity [11]. In addition, Vit has neuroprotective effects on pentylenetetrazole-induced seizure in rats. Vit reduces minimal clonic seizures and generalized tonic–clonic seizures by increasing the seizure onset time, which possibly through interaction at the benzodiazepine site of the γ-aminobutyric acid type A receptor complex [12]. However, the effect of Vit on neural excitotoxicity induced by glutamate is not well known.

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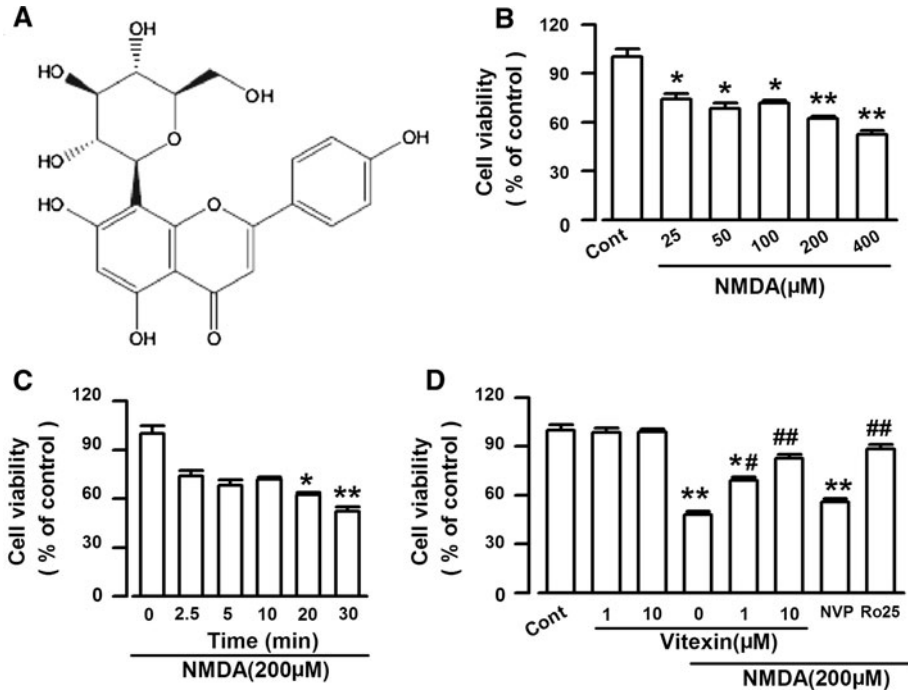

Fig. 1 Effects of Vit on cell viability in cultured cortical neurons**. a** Chemical structure of vitexin. **b** Concentration-dependent cytotoxic effects of NMDA on the cell viability of cortical neurons. $*P < 0.05$, $**P < 0.01$ compared with control. **c** Time-dependent cytotoxic effects of NMDA on the cell viability of cortical neurons. $*P < 0.05$, $**P < 0.01$ compared with control. **d** Effects of Vit (1 and 10 μM), NVP-AAM077 (0.4 μM), and Ro 25-6981 (0.3 μM) on the cell viability of cortical neurons after exposure to NMDA. $*P < 0.05$, $**P < 0.01$ compared with NMDA alone

Glutamate is a primary excitatory neurotransmitter and plays a key role in synaptic plasticity, learning and memory, and excitotoxicity [13]. Glutamate receptors are divided into three categories: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid (AMPA), and kainate (KA) receptors. Glutamate accumulation can cause NMDA receptor-mediated excitotoxicity, which has been implicated in neurodegeneration [14]. NMDA receptors, a kind of glutamate-gated ion channels, are widely expressed in the central nervous system and highly permeable to calcium ions [15]. The overactivation of glutamate receptors can trigger high calcium ion (Ca^{2+}) influx, which activates a number of enzymes that damage cell structures such as the cell membrane, cytoskeleton components, and DNA. This Ca^{2+} influx is thought to contribute to Ca^{2+}-mediated excitotoxic neuronal cell death in the above-mentioned disease processes [15].

The aim of this study is to investigate the possible protective efficacy of Vit in neuronal apoptosis induced by NMDAR activation and to elucidate the underlying mechanisms. We found that pretreatment of Vit significantly attenuated exitotoxicity by depressing the apoptotic signaling pathways.

Materials and methods

Materials

Vitexin was purchased from the ShangHai PureOne Biotechnology (Shanghai, China). Purity: 98 % by High-performance liquid chromatography (HPLC). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium-bromide], NVP-AAM077, Ro25-6981 and anti-β-actin antibody were purchased from Sigma (St. Louis, MO, USA). Anti-NR2A, and anti-procaspase-3 were purchased from Millipore (Billerica, MA, USA). Anti-NR2B, anti-Bax, and anti-Bcl-2 antibodies were purchased from Chemicon (Temecula, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Hyclone (Logan, UT, USA). Anti-PARP (poly ADP-ribose polymerase) antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Fetal bovine serum, Neurobasal medium, and B27 were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). All of the other chemicals and reagents were standard commercially available biochemical quality.

Primary mouse cortical neuronal culture

The Animal Care and Use Committee of the Fourth Military Medical University approved all animal protocols. Cultured prefrontal cortex neurons were derived from E18 C57Bl/6 mice as Wang et al. [16] described. Briefly, the prefrontal cortex was dissected, minced, and trypsinized for 15 min using 0.125 % trypsin (Invitrogen, Carlsbad, USA). Cells were then seeded onto 96-well plates, 24-well plates, 6-well plates, or 100 mm dishes. All plates were precoated with 50 μg/ml poly-D-lysine (Sigma) and grown in Neurobasal medium (Invitrogen) supplemented with B27 and 2 mM glutamine (Invitrogen). In B27/Neurobasal medium, glial growth was reduced to less than 0.5 % as assessed by immunocytochemistry for glial fibrillary acidic protein

(GFAP). The vast majority of cultured cells were immunoreactive for neuron-specific enolase [17]. The cultures were incubated at 37 °C in 95 % air/5 % carbon dioxide with 95 % humidity. The media was changed every 2 days during the maturation of cortical neurons. Cultures were used for experiments on the 10th day in vitro (DIV 10). The neurons were briefly rinsed with phosphate-buffered saline (PBS) and added new Neurobasal medium without B27, and then treated with Vit for 24 h. Then, NMDA and glycine (10 μM) were added to the medium with Vit for another 30 min. The cells were washed twice and returned to the original culture medium for another 24 h. Then, cell viability, staining, western-blot, and imaging were performed.

Cell viability analysis

The MTT assay was used to detect cell viability as Liu et al. [18] described. Neurons were cultured in 96-well plates at a density of 1×10^4 per well. The substrate MTT was dissolved in DMEM medium and added to each well at a final concentration of 0.5 mg/ml and then incubated at 37 °C for 4 h. Then the medium was then replaced by 150 μl dimethyl sulfoxide (DMSO) to dissolve the formazan product. The optical density (OD) was read on a Universal Microplate Reader (Elx 800, Bio-TEK instruments Inc., USA) at 570 nm (using 630 nm as a reference). Cell viability was presented as a percentage of the absorbance of untreated cultures. All data are expressed as mean ± SEM of three independent experiments and each mean included data from six wells.

Hoechst 33258 and PI double staining

Cell death was determined by propidium iodide (PI, Sigma) and Hoechst 33258 (Sigma) double fluorescent staining as Zhang et al. [19] described. Neurons were cultured in 24-well plates at a density of 600 cells/mm^2. The cells were exposed to NMDA for 30 min. 24 h after NMDA treatment, the cells were stained with PI (10 μg/ml) and Hoechst 33258 (10 μg/ml) for 15 min, and then fixed in 4 % paraformaldehyde for 10 min. Hoechst 33258 is excited by UV light at around 350 nm and emits blue fluorescence light at 461 nm. Hoechst 33258 is often used to distinguish the compact chromatin of apoptotic nuclei from that of normal cells [20]. Propidium iodide, a red-fluorescence dye (excited at 620 nm), is only permeant to dead cells. Staining was imaged and analyzed using Olympus Fluoview FV100 (Olympus, Japan). To assess apoptotic nuclei and dead/dying neurons, three visual fields were randomly selected from each well.

Western blot analysis

In order to further explore the mechanisms involved in VIT-mediated neuroprotection, we examined the effects of VIT on signaling pathways related to survival by Western blot analysis as described previously [21]. Neurons were cultured in 6-well plates at a density of 2×10^6 cells/well. After each treatment, cells were rinsed twice with PBS and lysed by M-PER Protein Extraction Buffer containing 1× protease inhibitor cocktail. Cell protein was quantified by a BCA Kit and equal amounts of protein (50 μg) separated on 10 % polyacrylamide gel followed by transferred onto an Immun-Blot PVDF membrane. The membrane was blocked for 1 h with 5 % non-fat milk in Tris–phosphate buffer containing 0.05 % Tween 20 (TBS·T). It was further incubated overnight at 4 °C with primary antibodies including anti-NR2A (dilution ratio1:400), anti-NR2B (dilution ratio1:1,000), anti-Bax (dilution ratio 1:400), anti-Bcl-2(dilution ratio 1:400), Anti-PARP (dilution ratio 1:1,000), anti-pro-caspase-3 (dilution ratio 1:1,000), and β-actin (dilution ratio1:10,000) as a loading control. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG for the primary antibodies), and bands were visualized using an ECL system (Perkin Elmer).

Calcium imaging

Calcium imaging was performed as previously described [22]. Neurons were cultured in 3.5-mm plates made especially for laser scanning microscope at a density 3×10^5 per plate. Cultured cells were washed twice using Mg^{2+}-free extracellular solution (ECS) containing (in mM): NaCl, 140; KCl, 3; $CaCl_2$, 2; HEPES, 10; and glucose, 10. The pH was adjusted to 7.2–7.3 with NaOH and osmotic pressure adjusted to 310 ± 5 with sucrose. Then, the neurons were incubated with 2 μM fluo-3/AM at 37 °C. After 30 min, the cultures were washed twice and returned to the original culture medium for an additional 30 min. The dye-loaded cells were measured for fluorescence using a confocal laser scanning microscope (Olympus, Japan). Prior to NMDA application, the dye-loaded cells were scanned for approximately 1 min to obtain a basal level of intracellular Ca^{2+}. Vit (10 μM) was added 24 h before the detection of calcium imaging and present in the whole experimental process. Then, 200 μM NMDA was applied to the cultures, and an equal amount of ECS was added as a placebo. The change of Ca^{2+} concentration was estimated by the fluorescence ratio of the fluo-3/AM-loaded neurons for another 4 min. The results are expressed as changes relative to basal levels, and five cells were selected randomly for analysis.

Data analysis

Data were expressed as the mean ± SEM. Statistical comparisons were evaluated by a *t* test and one-way

ANOVA was used for comparison among multiple groups. $P<0.05$ was considered to be statistically significant.

Results

Effects of Vit on cell viability in cultured cortical neurons

NMDA has been proven to be involved in the pathogenesis of neurodegenerative disorders associated with glutamate excitotoxicity. The neurons were exposed to increasing concentrations of NMDA (0, 50, 100, 200, and 400 μM) for 30 min. As expected, NMDA decreased cell viability in a concentration-dependent (Fig. 1b) and time-dependent (Fig. 1c) manner, as measured by the MTT assay. The neurons were exposed to 200 μM NMDA for 30 min in subsequent experiments because cell injury was significant in this paradigm (cell viability in NMDA treatment: 52.3 % ± 2.5 %). Cultured cortical neurons were pre-treated with Vit for 24 h, and then treated with NMDA (200 μM) and glycine (10 μM) for another 30 min. The cells were returned to the original culture medium for 24 h. However, treatment with Vit significantly increased the cell viability of neurons in a concentration-dependent manner. Neuroprotection of 10 μM Vit was most effective. Vit (1 and 10 μM) alone showed no effect on cell viability. In addition, NR2B-selective antagonist Ro 25-6981 (0.3 μM) blocked cell death by NMDA exposure; however, NR2A-selective antagonist NVP-AAM077 (0.4 μM) did not affect the cell viability by NMDA exposure (Fig. 1d), suggesting the different the role of NMDA receptor subtypes in mediating the excitotoxicity.

Effects of VIT on NMDA-induced apoptosis in cultured cortical neurons

Hoechst 33258 and PI double-staining were performed to further determine the neuroprotective effects of Vit on NMDA-induced apoptosis in cultured cortical neurons. Hoechst 33258 and PI are popular fluorescent nucleic acid dye. Hoechst 33258 and PI emit blue and red fluorescence, respectively, when bound to double-stranded DNA. Hoechst 33258 can penetrate the cell membrane of necrotic and living cells, but PI cannot pass through the living cells. Therefore, PI is usually used to detect cell apoptosis and necrosis. Data demonstrated that 5.9 ± 4.5 % of the cells in the control group underwent cell death or apoptosis compared with 35.1 ± 2.6 % of the cells in the NMDA injury group (Fig. 2a, b). Treatment with Vit (1 μM) significantly decreased the number of apoptotic and necrotic cells (Fig. 2a, b). These data suggest that Vit protects neurons from excitotoxicity mediated by NMDA.

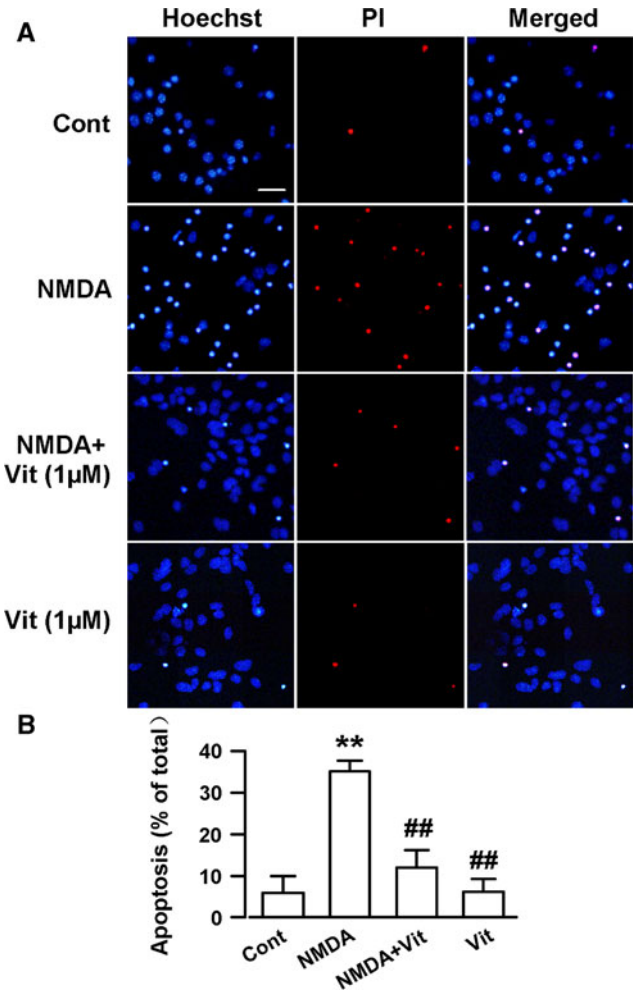

Fig. 2 Hoechst 33258 and PI double-staining in cultured cortical neurons. **a** The representative fluorescence images obtained after Hoechst 33258, PI, and double-staining in cortical neurons. *Scale bar* 20 μm. **b** The percentage of apoptotic neurons in total neurons for control, NMDA, and NMDA + Vit (1 μM) treated groups. The apoptotic numbers were counted from three independent experiments. $^{**}P < 0.01$ compared with control, $^{\#\#}P < 0.01$ compared with NMDA alone

Effects of Vit on Bcl-2/Bax expression

Bcl-2 family members include both anti-apoptotic (e.g., Bcl-2 and Bcl-xl) and pro-apoptotic (e.g., Bax, Bad, Bak, and Bid) proteins. The ratio between the two subsets determines the susceptibility of the cells to a death signal [23]. Next, we detected the effects of Vit on the levels of Bcl-2 and Bax, and the ratio of Bax/Bcl-2. Western blot analysis showed that Bcl-2 and Bax were both expressed in non-injured cortical neurons, and Vit (10 μM) treatment alone did not alter the expression of these proteins. Compared with control, 200 μM NMDA exposure in cultured cortical neurons increased the levels of Bax (Fig. 3a, b), decreased the levels of Bcl-2 (Fig. 3a, b) and increased the ratio of Bax/Bcl-2 (Fig. 3a, b). However, pretreatment of Vit (10 μM) before NMDA

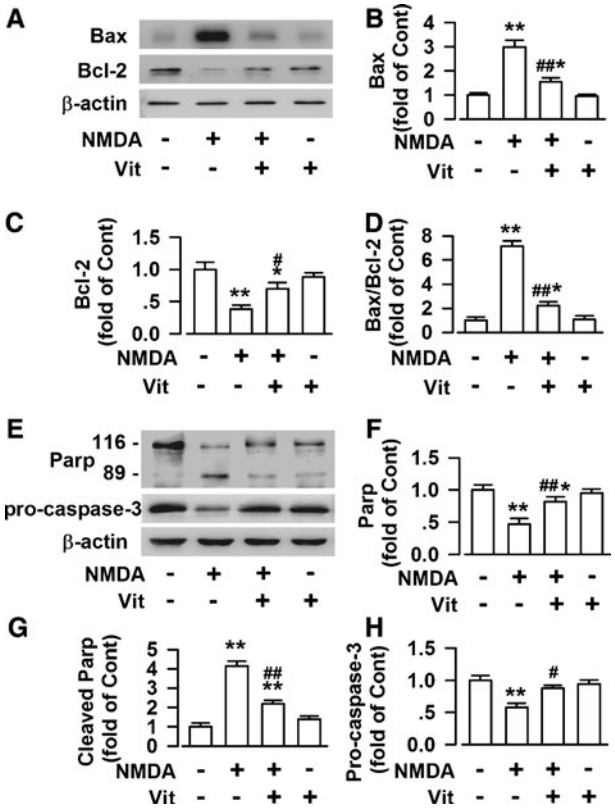

Fig. 3 Effects of Vit on the levels of Bax/Bcl-2, Parp, and pro-caspase-3 **a** Expression levels of Bax and Bcl-2 proteins were detected by Western blot. **b** The result showed that the levels of Bax markedly increased after exposure to NMDA in cultured cortical neurons. Pretreatment of 10 μM Vit significantly decreased the levels of Bax. **c** The levels of Bcl-2 significantly decreased after exposure to NMDA in cultured cortical neurons. Pretreatment of 10 μM Vit evidently increased the levels of Bcl-2. **d** The ratio of Bax/Bcl-2 was notably increased after NMDA exposure; however, the 10 μM Vit reversed the ratio. **e** Expression levels of Parp and pro-caspase-3 proteins were detected by Western blot. **f** The levels of Parp 116 significantly decreased after exposure to NMDA in cultured cortical neurons. Pretreatment of 10 μM Vit evidently prevented the decrease of Parp 116 by NMDA exposure. **g** The result showed that the levels of Parp 89 markedly increased after exposure to NMDA in cultured cortical neurons. Pretreatment of 10 μM Vit significantly decreased the levels of Parp 89. **h** The levels of pro-caspase-3 significantly decreased after exposure to NMDA in cultured cortical neurons. Pretreatment of 10 μM Vit evidently prevented the decrease of pro-caspase-3 by NMDA exposure. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with NMDA alone

exposure significantly reversed the levels of Bax (Fig. 3a, b) and Bcl-2 (Fig. 3a, c), and the ratio of Bax/Bcl-2 (Fig. 3a, d).

Effects of Vit on Parp and caspase-3 activation

Next, we detected the effects of Vit on the levels of cleavages of poly (ADP-ribose) polymerase (Parp) and caspase 3. Vit (10 μM) treatment alone did not alter the levels of Parp and pro-caspase 3 (Fig. 3e). Compared with control, 200 μM NMDA exposure increased the cleavages of Parp (116) shown as the decrease of Parp (116) levels (Fig. 3e, f) and the increase of Parp (89) levels (Fig. 3e, g), and decreased the levels of pro-caspase-3 (Fig. 3e, h). Pretreatment of Vit (10 μM) significantly reversed the levels of Parp (116) (Fig. 3e, f) and Parp (89) (Fig. 3e, g), and the pro-caspase-3 (Fig. 3e, h). These results indicate that Vit effectively prevents NMDA-induced Parp cleavage and caspase 3 activation.

Effects of VIT on expression of NR2A- and NR2B-containing NMDARs

NR2A- and NR2B-containing NMDARs are reported to link to different intracellular cascades and participate in different functions in neuronal cell survival or death after exposure to NMDA [24]. Blockades of NR2B-containing NMDA receptors promote neuronal survival, exerting a protective action against NMDA receptor-mediated neuronal damage [24–26]. This is consistent to our results that NR2B specific antagonist Ro25-6981 abolished the NMDA-induced cell loss (Fig. 1d). Next, Western blot was performed to further examine the effects of Vit on the expression of NMDAR subtypes. Levels of NR2B subtype expression was markedly increased after exposure to NMDA (Fig. 4a, b). Pretreatment of Vit (10 μM) significantly reduced the over-expression of NR2B subtype induced by NMDA exposure (Fig. 4a, b). However, neither NMDA exposure nor Vit (10 μM) treatment altered the levels of NR2A subtype receptors (Fig. 4a, c). Thus, downregulation of NR2B-containing NMDARs by Vit is suggested to be, at least partly, responsible for the neuroprotective effects against NMDA-induced excitotoxicity.

Inhibition of NMDA-induced Ca^{2+} overload by Vit

Sustained intracellular Ca^{2+} overload contributes to neuronal injury following excessive activation of NMDARs [27]. Next, we measured the calcium influx in cultured cortical neurons with treatment of NMDA or Vit. The fluorescence intensity can be regarded as an indicator of cytoplasmic Ca^{2+} concentration [28]. The Ca^{2+} concentration in cultured neuron was stable during our experiments (Fig. 4d, e). NMDA (200 μM) evoked a fast elevation of Ca^{2+} concentration for the next four minutes (Fig. 4d, f). Pretreatment with Vit (10 μM) could attenuate the amplitude of elevation in Ca^{2+} influx after NMDA exposure (Fig. 4d, f).

Discussion

In this study, the anti-excitotoxicity effect of Vit was investigated in cultured cortical neurons. Expressions of

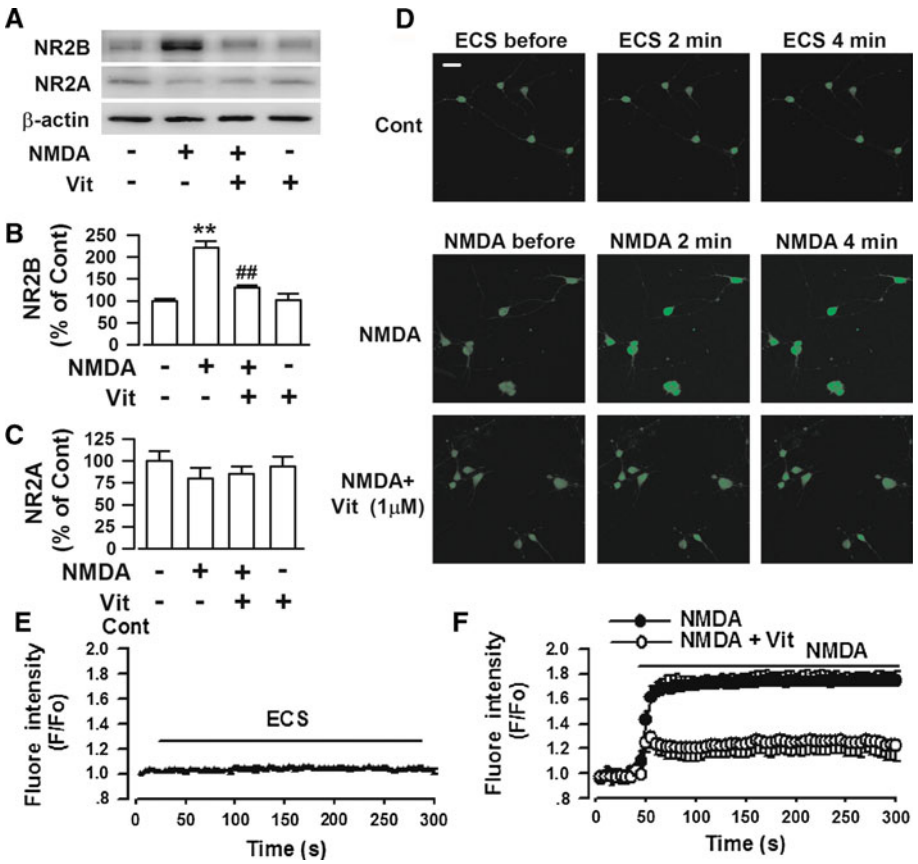

Fig. 4 Effects of Vit on the expression of NR2A and NR2B and calcium imaging **a** Expression levels of NR2A and NR2B proteins were detected by Western blot. **b** The result showed that the levels of NR2B markedly increased after exposure to NMDA in cultured cortical neurons. Pretreatment of 10 μM Vit significantly prevented the increase of NR2B by NMDA exposure. **c** Both NMDA and Vit treatment did not influence the levels of NR2A. $^{**}P < 0.01$ compared with control, $^{\#\#}P < 0.01$ compared with NMDA alone. **d** The green fluorescence under the laser scanning microscope at different time showed the concentration of calcium in neurons, which was stable during the experiment. **e** The fluorescence intensity showing the Ca^{2+} concentration was stable during detection by a laser scanning microscope for 300 s ($n = 42$ neurons). **f** 200 μM NMDA could evoke strong fluorescence intensity ($n = 54$ neurons) and pretreatment of Vit (10 μM) could significantly reduce fluorescence intensity in neurons ($n = 46$ neurons). *Scale bar* 20 μm

NMDA-induced apoptotic markers, such as Bcl-2 degradation, Bax expression, cleavages of Parp and pro-caspase 3, were all attenuated by Vit treatment. Our results suggest that Vit protects neurons from excitotoxicity through inhibiting cell apoptosis and calcium overload induced by NMDA exposure.

Glutamate plays a key role in neural transmission, development, and synaptic plasticity. Glutamate can binds to ionotropic receptors (iGluRs) and metabotropic receptors (mGluRs). The ionotropic GluRs include NMDA, KA, and AMPA receptors. Excessive accumulation of glutamate results in over activation of NMDA receptors, which induces excitotoxicity and causes neuronal damage [29]. Furthermore, activation of glutamate receptors triggers large increases in Ca^{2+} levels into neurons. Ca^{2+} acts as an important second messenger, activating several intracellular signaling cascades. Intracellular Ca^{2+} accumulation results in glutamate-induced neurotoxicity [30]. Excitotoxicity has been linked to several pathological states of the nervous system such as seizures, ischemia, anoxia, inflammation, and neurodegenerative disorders [30]. Vit has been found to exhibit antioxidant activity [8], antidepressant-like effect [9], and neuroprotective effects [12]. In this study, NMDA exposure greatly decreased the cell viability in cultured cortical neurons. However, Vit treatment concentration-dependently attenuated the cell loss. Specific NR2A- and NR2B-containing NMDA receptor antagonists NVP-AAM077 and Ro25-6981 were used to identify the NMDA receptor subunit roles in the excitotoxicity. The IC50 of NVP-AAM077 is 14 nM and 1.8 μM for NR2A and NR2B, respectively [31], and the IC50 of Ro25-6981 is 9 nM and 52 μM for NR2B and NR2A, respectively [32]. The concentration (0.4 μM) of NVP-AAM077 is high enough to antagonize NR2A and 0.3 μM of Ro25-6981 is specific for antagonize NR2B. In the previous and present study, we found NR2B specific antagonist Ro25-6981(0.3 μM) could abolished the excitotoxicity [33].

Hoechst 33258 and PI double-staining further testified the neuroprotective effects of Vit, which was related to regulation of Bcl-2 and Bax expression and the cleavages of Parp and pro-caspase 3. The ratio between Bax and Bcl-2 determines the susceptibility of cells to a death signal [34], and is widely used to show the effects of treatment on cell injury and survival. Vit decreased Bax expressin level while increased Bcl-2 level. Therefore, it significantly decreased the ratio of Bax/Bcl-2. Western blot revealed the different roles of NR2A- and NR2B-containing NMDARs in the neuroprotection of Vit. Treatments of NMDA and Vit did not change the expression of NR2A receptors, but NMDA increased the expression of NR2B, which is implied in mediating the excitotoxicity [24]. NMDAR overactivation is a critical step in the glutamate-induced excitotoxicity of CNS neurons. Both NR2A- and NR2B-containing NMDARs are linked to different intracellular cascades and have distinct roles in cell survival and excitotoxicity. The membrane distribution of NMDARs also influences excitotoxicity. Synaptic NMDARs have anti-apoptotic activities, whereas extrasynaptic NMDARs reportedly activate signs that promote neuronal death following ischemia [35, 36]. Furthermore, NR1/NR2B receptors are known to be expressed predominantly at extrasynaptic sites [37] and excitotoxicity is triggered by the selective activation of NMDARs containing the NR2B subunit [24, 25, 38, 39]. Pretreatment of Vit selectively reversed the protein levels of NR2B induced by NMDA exposure, implying that the neuroprotection of Vit is likely to antagonize a particular NMDAR subunit. This phenomenon is quite beneficial for neurodegenerative diseases relevant to glutamate excitotoxicity.

The key step in NMDA-induced neuronal cell apoptosis is the overload of intracellular Ca^{2+}, followed by overstimulation of NMDARs [40]. Ca^{2+} overload triggers several downstream lethal reactions, including nitrosative stress, oxidative stress, and mitochondrial dysfunction [41]. In this study, the elevation of Ca^{2+} stimulated by NMDA was inhibited by Vit in cultured cortical neurons, suggesting that the neuroprotective effects of Vit is partially due to the inhibition of calcium influx through the NMDA receptors. In our previous researches, we also reported other components derived from traditional Chinese herbal medicine such as salidroside [42], hydroxysafflor yellow A [43], and hyperoside [44]. FMNT showed comparative neuroprotective effects as compared with other classic components mentioned above.

In conclusion, our results suggest that the neuroprotection of Vit is, at least partially, associated with the down-regulation of NR2B-containing NMDARs. The neuroprotective effects of Vit are closely related to the inhibition of apoptosis and Ca^{2+} overload induced by NR2B-containing NMDARs. We hope that findings of the present study may shed light upon the pharmacological basis for clinical application of Vit.

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Conflict of interest The authors declare no conflict of interest.

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