Original Article

Protective effect of Achyranthes bidentata polypeptides on NMDA-mediated injury is developmentally regulated via modulating NR2A and NR2B differentially

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Background: Achyranthes bidentata polypeptides (ABPPs) are a potent intervention for excitotoxicity-related disorders such as Parkinson's disease and ischemic stroke. Previous work suggests that overstimulation of N-methyl-D-aspartate (NMDA) receptors plays a critical role in excitotoxicity, and expression of NR2 subunit variations is developmentally regulated. Our current study focused on neuroprotection of ABPPs on cultured neurons by modulation of NR2A and NR2B differentially.

Methods: Primary cultured neurons were treated with NVP-AAM077, Ro-256981, ABPPs, and then the neurons were exposed to NMDA to induce excitotoxicity. Cellular viability was detected promptly and 24-hour after exposure to NMDA by MTT assay. Patch-clamp recording was applied to evaluate the effect of ABPPs on NMDA-evoked current and the differential modulation of ABPPs on NR2A and NR2B subunits in conjunction with NVP-AAM077 and Ro-256981.

Results: ABPPs (10 μg/mL) blocked neuronal injury by NMDA in mature cultures, and the peptides conferred neuroprotection in immature cultures unless co-applied with NVP-AAM077. Furthermore, ABPPs enhanced NMDA current in mature cultures, while decreasing NMDA current in immature cultures. On the other hand, we showed that ABPPs increased NMDA current when Ro-256981 was present and decreased NMDA current when NVP-AAM007 was present.

Conclusions: Neuroprotection of ABPPs on NMDA-mediated injury differentially in immature and mature cultures involves enhancement of NR2A subunits and prevention of NR2B subunits, indicating that dosage of ABPP should be considered in treatment with patients at different developmental stages.

Keywords: *Achyranthes bidentata* polypeptides (ABPPs); development; neuroprotection; N-methyl-D-aspartate (NMDA) receptor

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Introduction

Glutamate is one of the most prevalent excitatory neurotransmitters in the mammalian central nervous system (CNS), where it is essential for the rapid excitatory signals. During neurotransmission, glutamate is released to synaptic cleft, and some of them is spilled over to extra-synapses (1). Residual glutamate in the extra-synapses is cleared by glutamate uptake transporters, which are expressed, principally, in astrocytes to keep extracellular glutamate at low concentration (2). In the brain, extracellular glutamate level ranges between 0.18 and 2.12 μM to avoid neurotoxicity (3-6). The pathological accumulation of extracellular glutamate, referred to as excitotoxicity, contributes to a broad range of neurological and neuropsychiatric diseases, including ischemic stroke, Parkinson's diseases, epilepsy, Huntington's disease, mood disorders and schizophrenia (2).

N-methyl-D-aspartate receptors (NMDARs), a calcium permeable ionotropic glutamate receptor family, play a fundamental role in the mechanisms of excitotoxicity (7). Typically, NMDAR comprises two obligatory NR1 and two regulatory NR2, which have been identified four variations (NR2A-2D) (8,9). In the hippocampus, NMDARs mainly contain NR1 subunits in combination with NR2A or NR2B subunits, and expression of NR2 subunit variations switches from NR2B to NR2A during development (10-13).

A.bidentata poplypeptides (ABPPs) were obtained by means of classical salting-out sedimentation with ammonium sulfate from the extract of *A.bidentata Blume* root decoction (14). Calcium imaging data showed that ABPPs block NR2B-containing NMDARs, and enhance function of NR2A-containing NMDARs in cultured hippocampal neurons (14). However, during development, the change in the expression of NR2 subunit variations is observed in primary cultured neurons. Immature cultures (7 days *in vitro*) contain mainly NR2B subunits, while mature cultures (14 days *in vitro*) express mostly NR2A subunits (8,15). Therefore, in the current study, we assessed the neuroprotective effect of ABPPs on NMDA-mediated excitotoxicity in immature and mature cultured hippocampal neurons, and clarified the differential modulation of ABPPs on NR2A- and NR2B-containing NMDAR by electrophysiological recording technique. We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/atm-20-581).

Methods

Preparation of ABPPs

A. bidentata Blume root was powdered and soaked in ultrapure water at 80 ℃. ABPPs were subsequently prepared as described by Shen *et al.* (14). Briefly, the decoction was saturated with ammonium sulfate, and the precipitate was collected by centrifugation. After desalination in 1,000 MW cutoff tubing with ultrapure water, the dialysate was lyophilized into a powder of ABPPs, which are soluble in water. The ABPPs were characterized by high performance liquid chromatography (14).

Primary hippocampal neuron culture

The whole study protocol was approved by the Ethics Committee of Nantong University (No. 20080118-001) and all the procedures were followed by the Chinese Guidelines for the Care and Use of Laboratory Animals.

All experiments were implemented on primary cultures of rat hippocampal neurons. Tissues were isolated from embryonic day 18 Sprague-Dawley (SD) rats, and prepared as previously (16). Briefly, the whole fetal brains were quickly dissected out, and the hippocampi were harvested and digested with 0.25% tyrosine (Gibco, New York, NY, USA) at 37 ℃ for 5 min. Neurons were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco), and were plated at a density of [6–8]×10^4 cells/cm^2 onto poly-D-lysine-coated 96-well plates or 8 mm glass coverslips in dishes. After 24 hours, the medium was replaced with Neurobasal medium (Gibco) containing 2% B27 supplement (Gibco) and glutamine (0.5 mM, Gibco). After that, half of the medium was replaced with fresh medium every 3 days.

NMDA-induced neuronal injury model and cell treatment

Neurotoxicity was triggered by exposing primary cultured hippocampal cells to high-concentration NMDA (Sigma, St Louis, MO, USA). Because of the blockage by Mg^{2+} of NMDA receptors, hippocampal cultures, at 7 or 14 days in vitro, were washed with Mg^{2+}-free extracellular solution (ECS) ($CaCl_2$ 2 mM, NaCl 140 mM, KCl 3 mM, HEPES 10 mM, glucose 10 mM, pH 7.2–7.3, Osmotic pressure 290±5 mOsmol/L). After washing, NMDA in Mg^{2+}-free ECS was added to the wells to evoke excitotoxicity. After

NMDA stimulation, cultures were washed three times with Mg^{2+}-free ECS and returned to the original medium. ABPP, Ro-256981, NVP-AAM077, or (+)-5-methyl-10, 11-dihydro-5H-dibenzo (a, d) cyclohepten-5, 10-imine maleate (MK-801, Sigma) was added 12-hour prior to NMDA stimulation until the excitotoxic insult was complete.

Assessment of neuronal cell viability

Cellular viability measurements were performed promptly and after 24 hours of exposure to NMDA using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. The detailed methods were described previously by Shen *et al.* (14). Data were expressed as the percentage value, which was normalized to the control cultures (Elx 800, Bio-TEK Instruments Inc., VT).

Electrophysiological recording

Receptors for NMDA that are ligand-gated ion channels play a critical role in NMDA-induced neurotoxicity (17). The activation of NMDA receptors is identified by the influx of Na^+, Cl^-, Ca^{2+} ions, which are examined by whole-cell patch clamp recording in cultured hippocampal neurons (18). The NMDA-elicited currents were measured in Mg^{2+}-free solution containing Ca^{2+} (2 mM), with 2-minute bath perfusion after NMDA current was recorded. All detailed procedures were described previously by Shen *et al.* (19). The change of amplitude NMDA currents was expressed as the percentage value, which was normalized to the basal level recording in the same hippocampal neuron.

Statistical analysis

All data were expressed as means ± SD. Significance was analyzed by one way analysis of variance (ANOVA) with sigmaplot 13.0 software packages (Systat, San Jose, CA, USA), and differences between two groups were assessed by Student-Newman-Keuls *post hoc* test. The value of $P<0.05$ was considered statistically significant. All data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (20).

Results

Neuroprotective effects of ABPPs on immature cultures

Primary cultured hippocampal neurons (DIV7) were exposed to an increasing concentration of NMDA (10–1,000 μM) for 1hour. NMDA induced a concentration-dependent reduction in cell viability of hippocampal neurons, as detected immediately after NMDA stimulation by MTT assay (*Figure 1A*). Exposure to 300 μM NMDA for 1 hour was used to induce excitotoxic neuronal injury in the ensuing test.

In hippocampus, majority of NMDARs are either NR2B-containing or/and NR2A-containing receptors. Treatment with Ro-256981, a specific NR2B subunit antagonist, or NVP-AAM077, a specific NR2A subunit antagonist, prevented NMDA-induced cell injury in a concentration-dependent manner (*Figure 1B,C*), suggesting that overactivation of NR2B-containing or NR2A-containing NMDA receptors contributes to NMDA-induced neuronal injury in immature cultures. NMDA-induced excitotoxicity was also blocked by 10 μM MK-801, the gold standard of neuroprotective NMDA antagonists (*Figure 1C*). However, treatment with ABPP (0.1–10 μM) did not prevent NMDA-induced cellular viability decrease, which was detected immediately after NMDA stimulation (*Figure 1D*).

Over stimulation of NMDA receptors induced apoptotic cell death, characterized by Hoechst/PI doubled staining and DNA ladder detection at 24-hour after exposure to NMDA, in rat cultured hippocampal neurons (14). NMDA-stimulation resulted in the decrease of cellular viability, which was assessed by MTT at 24-hour after exposure to NMDA, in a concentration dependent way (*Figure 1E*). Thus, NMDA-mediated apoptotic cell viability decrease was detected at 24 hours after exposure to 300 μM NMDA for 1 hour.

To verify that NMDA-induced apoptotic cell injury required NR2B-containing and NR2A-containing receptors, immature cultures were treated with Ro-256981 or NVP-AAM077 for 1 hour before NMDA stimulation. Data showed that NMDA-mediated excitotoxicity was blocked by Ro-256981, NVP-AAM077 or MK-801 (*Figure 1F,G*), proving that overactivation of NR2B-containing or NR2A-containing NMDA receptors contributes to NMDA-induced apoptotic cell injury in primary cultured hippocampal neurons (DIV7). Interestingly, treatment of cultures with ABPP (0.1 μg/mL) blocked NMDA-induced delayed damage, while ABPP (10 μg/mL) treatment could not (*Figure 1H*). However, treatment of neurons with ABPP (10 μg/mL) prevented NMDA-induced apoptotic injury in combination with NVP-AAM077 (5 μM). These data might imply that ABPP (10 μg/mL) could not confer the neuroprotection because of its overactivation on NR2A-containing NMDA receptors.

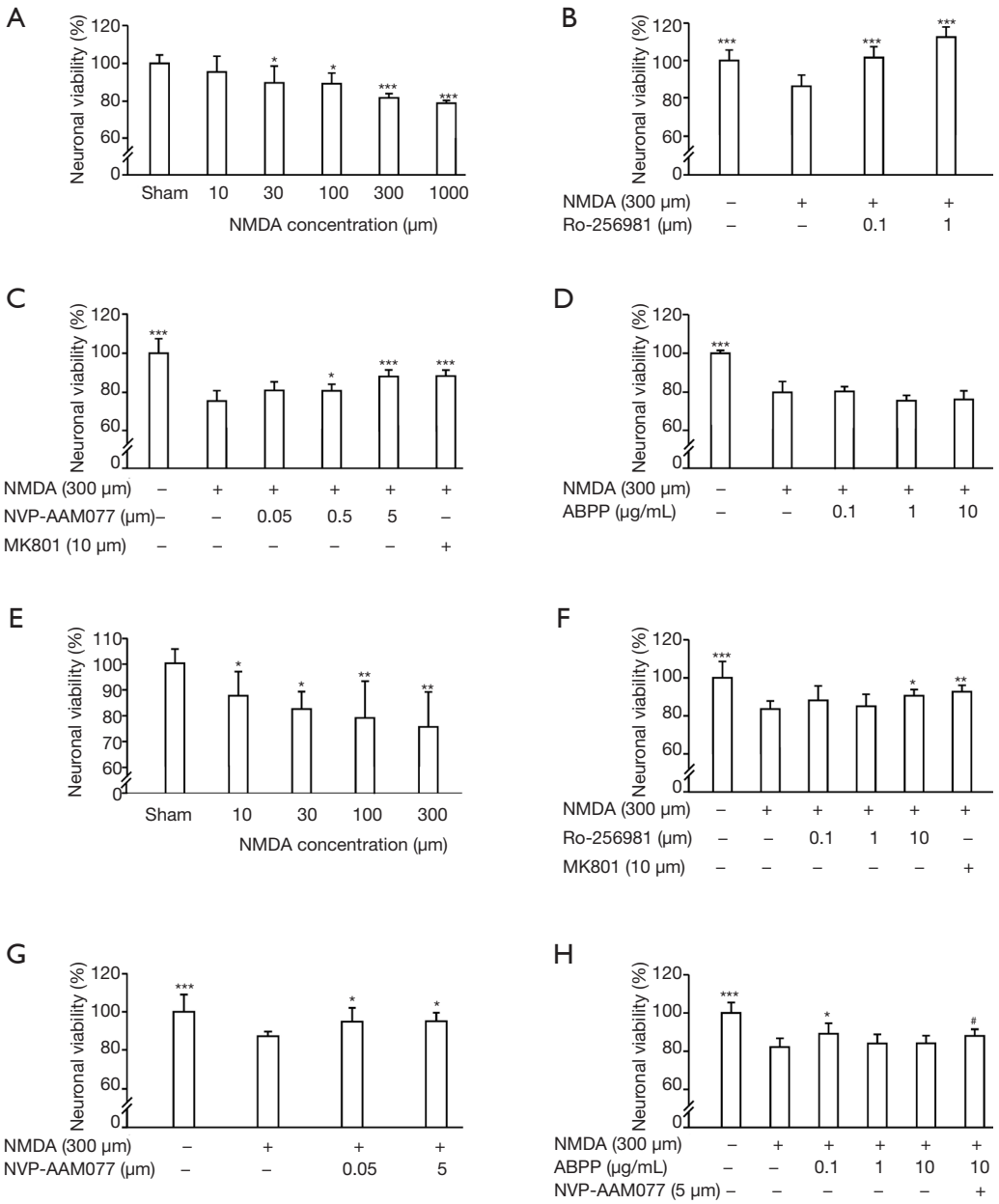

Figure 1 Level of NMDA-induced excitotoxicity was alleviated by ABPP (0.1 μg/mL) in cultured hippocampal neurons at DIV 7. Cell viability was measured after NMDA stimulation (A-D). NMDA induced a concentration-dependent decrease in cell viability of cultured hippocampal neurons (A). Application of Ro-256981 and NVP-AAM077 along with NMDA prevented NMDA-mediated excitotoxicity in a concentration-dependent manner (B,C), while application of ABPP (0.1, 1, 10 μg/mL) with NMDA could not block NMDA-mediated excitotoxicity (D). Cell viability was detected at 24 hours after NMDA stimulation (E-H). NMDA induced a concentration-dependent decrease in cell viability of cultured hippocampal neurons (E). Application of Ro-256981 and NVP-AAM077 with NMDA blocked NMDA-mediated excitotoxicity in a concentration-dependent manner (F,G). Application of ABPP (0.1 μg/mL) prevented NMDA-mediated excitotoxicity, while other concentrations of ABPP (1, 10 μg/mL) could not (H). However, concurrent treatment of ABPP (10 μg/mL) with NVP-AAM (5 μM) blocked NMDA-induced excitotoxicity (H). NMDA-mediated excitotoxicity was also blocked by 10 μM MK-801 (C,F). *P<0.05, **P<0.01, ***P<0.001 *vs.* control group. The NMDA stimulation neurons were referred as control group (A-H). #P<0.05 *vs.* concurrent treatment with ABPP (10 μg/mL) and NMDA group (H). NMDA, N-methyl-D-aspartate; ABPP, achyranthes bidentata polypeptide.

Thus, NR2A-containing and NR2B-containing NMDA receptors have identical function on NMDA-mediated cellular injury in primary cultured hippocampal neurons (DIV7). In addition, ABPP, only in low concentration, confers neuroprotective effect on NMDA-induced apoptotic damage in immature cultures.

Neuroprotective effects of ABPPs on mature cultures

Cultured hippocampal neurons (DIV14) were exposed to an increasing concentration of NMDA (10–1,000 μM) for 1 hour. Data showed that NMDA evoked the decrease of cell viability, which was measured immediately after NMDA stimulation, in a concentration dependent manner (*Figure 2A*). NMDA-mediated neurotoxicity was blocked by Ro-256981, NVP-AAM077, or MK-801 (*Figure 2B,C*), but ABPP (0.1–10 μM) did not prevent the transient decrease of neuronal cell viability by NMDA stimulation (*Figure 2D*).

Neuronal cell viability was assessed by MTT at 24-hour after exposure to an increasing concentration of NMDA (10–1,000 μM) for 1 hour. The results showed that the cellular viability of hippocampal neurons (DIV14) decreased in a concentration dependent manner (*Figure 2E*), and the NMDA-mediated excitotoxicity was prevented by Ro-256981 and MK-801 significantly (*Figure 2F,G*). However, NVP-AAM077, a specific NR2A subunit antagonist, aggravated the NMDA-mediated apoptotic excitotoxicity (*Figure 2G*). Additionally, treatment of neurons with ABPP (0.1–10 μg/mL) prevented NMDA-induced apoptotic injury in primary cultured hippocampal neurons (DIV 14) (*Figure 2H*).

Taken together, NR2A-containing and NR2B-containing NMDA receptors play an identical role on NMDA-mediated transient injury in primary cultured hippocampal neurons (DIV14). However, NR2A-containing and NR2B-containing NMDA receptors have different function on NMDA-induced apoptotic injury, which detected at 24-hour after NMDA stimulation, in mature cultures. Thus, both low and high concentrations of ABPP confer neuroprotective effect on NMDA-induced apoptotic injury in primary cultured hippocampal neurons (DIV 14).

Regulatory effects of ABPPs on NMDA-evoked current (Figure 3)

To investigate the differential effects of ABPPs on immature and mature hippocampal neurons, we examined the NMDA-evoked current by whole-cell patch clamp recording. ABPP (10 μg/mL) did not evoke a significant current (*Figure 3A*). However, NMDA (300 μM) induced a significant current, which was divided into two sections: the peak current (Ip) and the sustained state current (Iss) (*Figure 3B*). The sustained state current was inhibited by ABPPs in a concentration dependent manner (*Figure 3C,E*), while coefficient of desensitization of NMDA currents was promoted by ABPPs in primary cultured hippocampal neurons (DIV 7) (*Figure 3D,E*). In primary cultured hippocampal neurons (DIV 14), ABPPs elevated the amplitude of the peak and sustained state current (*Figure 3F,G*), while coefficient of desensitization of NMDA currents dropped off (*Figure 3G,H*). Since NR2 subunit variations switches from NR2B to NR2A during development in cultured hippocampal neurons (21-24), the results suggested that ABPPs might modulate NR2A-containing and NR2B-containing NMDA receptors in a differential way.

Different regulatory effects of ABPPs on NR2A- and NR2B-containing NMDA receptors (Figure 4)

In primary cultured hippocampal neuron, NMDA (300 μM) evoked a significant current, which was totally blocked by Ro-256981 (0.5 μM) and NVP-AAM077 (0.7 μM) (*Figure 4A*). The addition of Ro-256981 (0.5 μM) alone lowered the amplitude of the peak and the sustained state of NMDA (300 μM)-evoked current, and the simultaneous addition of Ro-256981 (0.5 μM) and ABPPs (1 μg/mL) inversely raised the amplitude of the peak and the sustained state of NMDA (300 μM)-evoked current (*Figure 4B,D*). On the contrary, the addition of NVP-AAM077 (0.7 μM) alone lowered the amplitude of the peak and the sustained state of NMDA (300 μM)-evoked current, and the simultaneous addition of NVP-AAM077 (0.7 μM) and ABPPs (1 μg/mL) further lowered the amplitude of the peak and the sustained state of NMDA (300 μM)-evoked current (*Figure 4E,G*). These data suggested that ABPPs not only enhanced the action of NR2A-containing NMDA receptors, but also inhibit the function of NR2B-containing receptors.

Discussion

In the present study, we originally reported the differential neuroprotective effects of *A.bidentata* polypeptides (ABPPs) on NMDA-induced excitotoxicity in immature and mature cultures, in combined with NR2 subunit specific antagonist. With electrophysiological recording technique, modulation of ABPPs on NMDA receptors was also observed differentially in immature and mature cultured neurons.

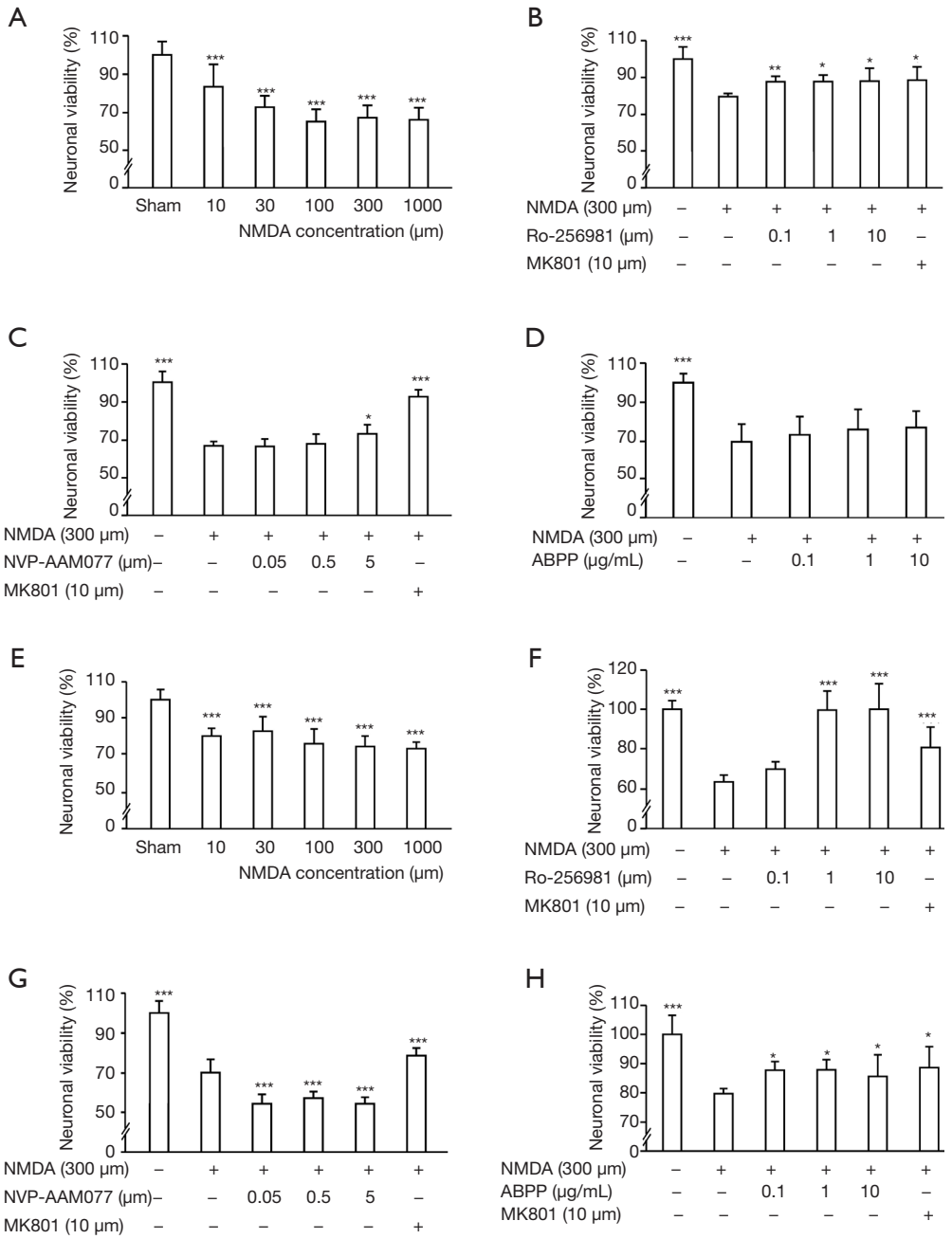

Figure 2 Level of NMDA-induced excitotoxicity was alleviated by ABPP in cultured hippocampal neurons at DIV 14. Cell viability was measured after NMDA stimulation (A-D). A concentration-dependent decrease was induced by NMDA in cell viability of cultured hippocampal neurons (A). Application of Ro-256981 and NVP-AAM077 prevented NMDA-mediated acute excitotoxicity in a concentration-dependent manner (B,C), while application of ABPP (0.1, 1, 10 μg/mL) could not block NMDA-mediated acute excitotoxicity (D). Cell viability was analyzed at 24 hours after NMDA stimulation (E-H). NMDA induced a concentration-dependent decrease in cell viability of cultured hippocampal neurons (E). Application of Ro-256981 and NVP-AAM077 blocked NMDA-mediated excitotoxicity in a concentration-dependent manner (F,G), and application of ABPP (0.1, 1, 10 μg/mL) prevented NMDA-mediated excitotoxicity (H). NMDA-mediated excitotoxicity also was blocked by 10 μM MK-801 (B, C, F, and G). *P<0.05, **P<0.01, ***P<0.001 *vs.* control group. The NMDA stimulation neurons were referred as control group (A-H). NMDA, N-methyl-D-aspartate; ABPP, achyranthes bidentata polypeptide.

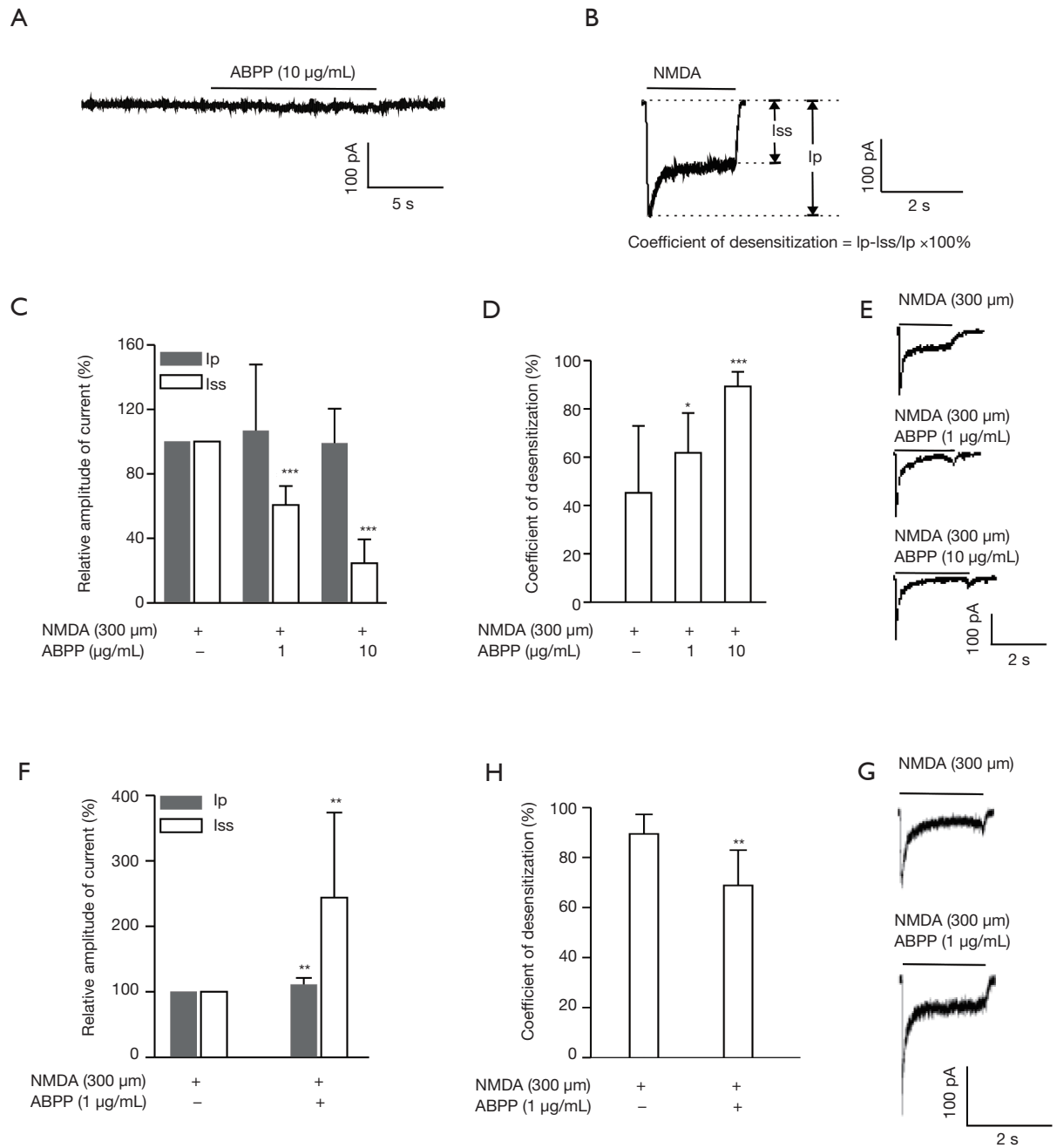

Figure 3 ABPP modulated NMDA-evoked current differentially in cultured hippocampal neurons at DIV 7 and DIV 14. (A,B) The representative traces after addition of ABPP (10 μg/mL) (A), or NMDA (300 μM) (B) in the cultured hippocampal neurons. (C) Mean ± SD of the peak and the sustained state amplitudes of NMDA current in cultured hippocampal neurons at DIV 7 with NMDA (300 μM) or application of ABPP (1, 10 μg/mL) with NMDA (300 μM). (D) Mean ± SD of coefficient of desensitization of NMDA current. (E) The representative traces of current were evoked by NMDA (300 μM) and by NMDA (300 μM) with ABPP (1, 10 μg/mL) concurrent application in the same cultured hippocampal neuron at DIV 7. (F) Mean ± SD of the peak and the sustained state amplitudes of NMDA current in cultured hippocampal neurons at DIV 13-14 with NMDA (300 μM) alone and ABPP (1.0 μg/mL) with NMDA (300 μM). (G) Mean ± SD of coefficient of desensitization of the NMDA current in. (H) The representative traces of current evoked by NMDA (300 μM) and by NMDA (300 μM) with ABPP (1.0 μg/mL) in the same cultured hippocampal neuron at DIV 14. *P<0.05, **P<0.01, ***P<0.001 *vs.* NMDA (300 μM)-evoked current. NMDA, N-methyl-D-aspartate; ABPP, achyranthes bidentata polypeptide.

Furthermore, in conjunction with NR2 subunit specific antagonist, we confirmed that the differential role of ABPPs on NR2A- and NR2B-containing NMDA receptors.

ABPPs are a composition from the decoction of *A. bidentata Blume* roots, and they are analyzed by HPLC with UV spectrophotometry at different wavelengths (14). Application of ABPPs *in vivo* has the potential to be a pharmacotherapy intervention for ischemic stroke and Parkinson's disease (25-28). In the serum of patients with Parkinson's disease, glutamate concentration was higher compared to that of healthy subjects (29). Blood glutamate grabbers have proved to be the protective efficacy in the treatment of patients with ischemic stroke (30). These findings suggested that glutamate excitotoxicity plays a critical role in Parkinson's disease and ischemic stroke.

NMDA receptors are a subtype of ionic glutamate receptors, and are characterized of high permeability to calcium ions (18). Considerable literature has demonstrated that calcium influx via NMDA receptor is the key step in glutamate-induced excitotoxicity. In primary hippocampal neurons, NMDA receptors are mainly NR2A- and NR2B-containing NMDA receptors, which are blocked by NVP-AAM077 and Ro-256981 respectively. Our data confirmed that NR2A- and NR2B-containing receptors contribute to NMDA-evoked current by electrophysiological recording trace in cultured hippocampal neuron (*Figure 4A*). Furthermore, NMDA-evoked decrease of cell viability, which was measured immediately after NMDA stimulation, was inhibited by Ro-256981 or NVP-AAM077 in a concentration dependent manner (*Figures 1B,C,2B,C*). However, treatment of cultures with ABPPs did not reverse the immediate drop of cell viability by NMDA stimulation (*Figures 1D,2D*). There are two possible explanations for the results. One is that ABPPs have no effects on NMDA receptors; the other is that ABPPs confer differentially modulation of NR2A- and NR2B- containing NMDA receptors.

Previous studies showed that NMDA-induced neurotoxicity was closely related to the expression and distribution of NR2 subunits, which are changed with development in primary cultured hippocampal neurons (18,22,24,31). Overstimulation of NMDA receptors resulted in the decrease of cellular viability, which was detected at 24-hour after NMDA stimulation, in a concentration dependent manner (*Figures 1E,2E*). Treatment of neurons with Ro-256981, a specific NR2B subunits antagonist, cut down the decrease of cellular viability by NMDA stimulation in a dose dependent way (*Figures 1F,2F*). However, NVP-AAM077, a specific NR2A subunits antagonist, showed different effects in immature hippocampal neurons (DIV 7) and mature hippocampal neurons (DIV 14) (*Figures 1G,2G*). NVP-AAM077 alleviated cell injury by NMDA stimulation in cultured hippocampal neurons (DIV 7), while it deteriorated cell injury in cultured hippocampal neurons (DIV 14) (*Figures 1G,2G*). Interestingly, ABPPs (10 μg/mL) reversed the decrease of cellular viability by NMDA only in cultured hippocampal neurons (DIV 14), but not in neurons (DIV 7) (*Figures 1H,2H*). However, treatment of neurons (DIV 7) with ABPPs (10 μg/mL) conferred neuroprotective effect only when NVP-AAM077 was present (*Figure 1H*). Thus, ABPPs could not block the prompt decrease of cell viability caused by NMDA stimulation because of its opposite modulation of NR2A- and NR2B-containing NMDA receptors.

NMDA receptors, which are ligand dependent ion channels, can be detected by patch clamp recording in whole-cell model. To verify the differential modulation of ABPPs on NR2A- and NR2B-containing NMDA receptors, NMDA (300 μM)-elicited inward current was recorded in hippocampal neurons (DIV 7) and in neurons (DIV 14). As our previous data from calcium imaging, the sustained state amplitude of NMDA current was inhibited by ABPPs in cultured hippocampal neurons (DIV 7) (*Figure 3C,D,E*). However, NMDA evoked current was enhanced by ABPPs in neurons (DIV 14) (*Figure 3F,G*). The data provide another evidence to support that ABPPs conferred differential modulation of NR2A- and NR2B-containing NMDA receptors since expression of NR2B subunits are replaced by NR2A subunits in neurons (DIV 14).

To clarify the modulation of ABPPs on NR2B- and NR2A- containing NMDA receptors, NMDA-elicited current was recorded in conjunction with two selective inhibitors of NMDA subunits, Ro 25-6981 and NVP-AAM077. When NR2B subunits antagonist Ro 25-6981 was present, the NMDA current was mediated by NR2A-containing NMDA receptors. In this condition, application of ABPPs raised the amplitude of NMDA current (*Figure 4B,C,D*). In contrast, when NR2A subunits antagonist NVP-AAM077 was present, the NMDA current was mediated by NR2B-containing NMDA receptors. Therefore, addition of ABPPs inhibited the amplitude of NMDA current (*Figure 4E,F,G*). Taken together, these data collectively demonstrate that ABPPs inhibited the action of NR2B-containing NMDA receptors, while enhancing the function of NR2A-containing NMDA receptors. Such

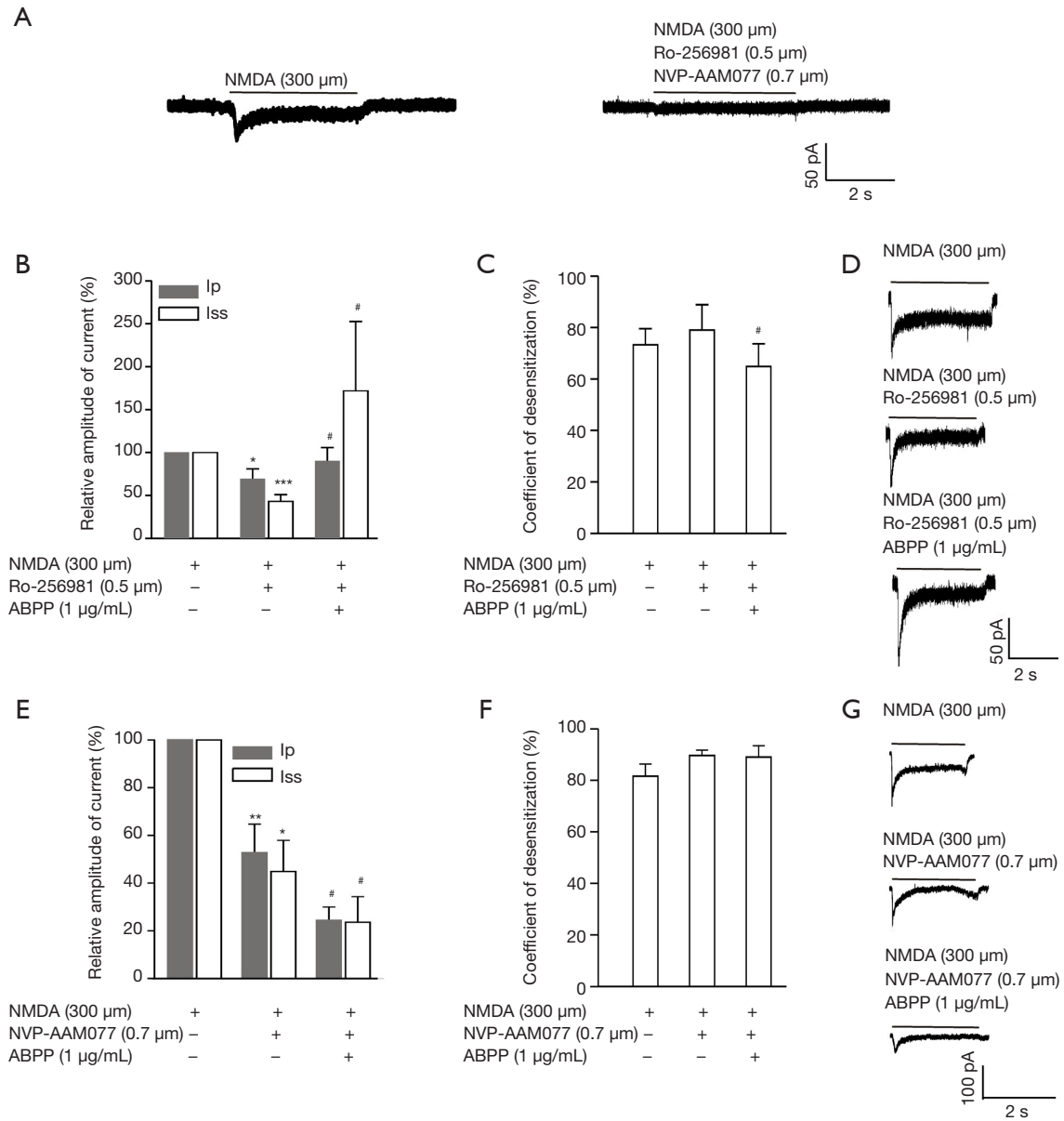

Figure 4 ABPP modulated NR2A- and NR2B-containing NMDA receptors differentially. (A) The representative traces of current evoked by NMDA (300 μM) or concurrent application of NMDA (300 μM), Ro-256981 (0.5 μM) and NVP-AAM077 (0.7 μM). (B) Mean ± SD of the peak and the sustained state amplitudes of NMDA current in cultured hippocampal neurons with NMDA (300 μM), Ro-256981 (0.5 μM) with NMDA (300 μM), or concurrent application of ABPP (1.0 μg/mL), Ro-256981 (0.5 μM) and NMDA (300 μM). (C) Mean ± SD of coefficient of desensitization of the NMDA current. (D) The representative traces of current evoked by NMDA (300 μM), Ro-256981 (0.5 μM) with NMDA (300 μM), or concurrent application of ABPP (1.0 μg/mL), Ro-256981 (0.5 μM) and NMDA (300 μM). (E) Mean ± SD of the peak and the sustained state amplitudes of NMDA current in cultured hippocampal neurons with NMDA (300 μM), NVP-AAM077 (0.7 μM) with NMDA (300 μM), or concurrent application of ABPP (1.0 μg/mL), NVP-AAM077 (0.7 μM) and NMDA (300 μM). (F) Mean ± SD of coefficient of desensitization of the NMDA current. (G) The representative traces of current evoked by NMDA (300 μM), NVP-AAM077 (0.7 μM) with NMDA (300 μM), or concurrent application of ABPP (1.0 μg/mL), NVP-AAM077 (0.7 μM) and NMDA (300 μM). *P<0.05, **P<0.01, ***P<0.001 *vs.* NMDA (300 μM) evoked current. #P<0.05, *vs.* Ro-256981 (0.5 μM) with NMDA (300 μM) evoked current (B,C). #P<0.05, *vs.* NVP-AAM077 (0.7 μM) with NMDA (300 μM) evoked current (E,F). NMDA, N-methyl-D-aspartate; ABPP, achyranthes bidentata polypeptide.

differential modulations result in different neuroprotection effects of ABPPs in neurons (DIV 7) and neurons (DIV 14) on NMDA-mediated injury. Further research is needed to identify the key bioactive component of ABPPs, which modulates NR2A- and NR2B-containing NMDA receptors differentially. Moreover, the structure of the component and its molecular interactions with NR2A- and NR2B subunits will be elucidated to shed light on its recombinant by bioengineering and its clinical application in the future.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The whole study protocol was approved by the Ethics Committee of Nantong University (No. 20080118-001) and all the procedures were followed by the Chinese Guidelines for the Care and Use of Laboratory Animals.

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