ORIGINAL ARTICLE

Parishin C's prevention of $A\beta_{1-42}$-induced inhibition of long-term potentiation is related to NMDA receptors

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Abstract  The rhizome of Gastrodia elata (GE), a herb medicine, has been used for treatment of neuronal disorders in Eastern Asia for hundreds of years. Parishin C is a major ingredient of GE. In this study, the i.c.v. injection of soluble $A\beta_{1-42}$ oligomers model of LTP injury was used. We investigated the effects of parishin C on the improvement of LTP in soluble $A\beta_{1-42}$ oligomer-injected rats and the underlying electrophysiological mechanisms. Parishin C (i.p. or i.c.v.) significantly ameliorated LTP impairment induced by i.c.v. injection of soluble $A\beta_{1-42}$ oligomers. In cultured hippocampal neurons, soluble $A\beta_{1-42}$ oligomers significantly inhibited NMDAR currents while not affecting AMPAR currents and voltage-dependent currents. Pretreatment with parishin C protected NMDA receptor currents from the damage induced by $A\beta$. In summary, parishin C improved LTP deficits induced by soluble $A\beta_{1-42}$ oligomers. The protection by parishin C against $A\beta$-induced LTP damage might be related to NMDA receptors.

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1. Introduction

The rhizome of Gastrodia elata Blume (GE) is a traditional herb medicine which has been commonly used in Eastern Asia for centuries. It is widely used especially for treatment of headache, dizziness, epilepsy, stroke and dementia. GE has been reported to have therapeutic effects on animal models of Alzheimer’s disease (AD), including improvements in spatial memory deficits and Aβ deposit in the hippocampus. In clinical studies, the GE extract has been used to treat vascular dementia and showed improvement on cognition in the patients. Parishin C is known as major component of GE. As shown in Fig. 1, parishin C is bis-gastrodin citrates constituted of two gastrodin molecules esterified with two terminal carboxyl groups of citric acid. Previous studies demonstrated that parishins improved animal performances in a variety of cognitive-behavioral tests, such as step-down test, passive avoidance task and the Morris water maze task. We found that parishin C was more potent than other parishins or gastrodin.

AD is the most common type of dementia in aging adults. Learning and memory declines progressively and can linger for many years. Beta-amyloid (Aβ) is one of the most important pathological features in AD patients and animal models, including Aβ plaques and soluble Aβ oligomers. Recent studies focused on soluble Aβ oligomers, which were thought to correlate with disease progression better than insoluble fibrillar plaques. To mimic symptoms of AD and investigate the effects of the parishin C, the soluble Aβ oligomers-induced cognitive deficits animal model was employed in our study.

Long-term potentiation (LTP) recording is a well-known and widely documented model for investigating the synaptic basis of learning and memory. The induction of LTP is affected by changing activity of ion channels, including voltage-dependent ion channels such as Na⁺, Ca²⁺ and K⁺ channels as well as ionotropic glutamate receptors. Functional N-methyl-D-aspartate receptors (NMDARs) and α-aminooxyhydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are essential for LTP induction.

Therefore, in the present study we focused on the electrophysiological mechanisms of parishin C in protection of LTP after Aβ1–42 injection into the cerebral ventricle of rats. We presently performed LTP in vivo recordings and whole cell patch clamp recordings for voltage-dependent ion channels and NMDA receptors in cultured neurons.

2. Materials and methods

2.1. Animals

Male Wistar rats (240–260 g) were used in the LTP recordings. Rats were housed in a temperature- and light-controlled environment (23 ± 1 °C and 12 h light cycle), with free access to food and water. They arrived at least 3 days before the experiments and were handled carefully. All the experiment procedures were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Care Committee of Peking Union Medical College and Chinese Academy of Medical Sciences.

2.2. Drugs and materials

Parishin C was provided by Professor Jiangong Shi (HPLC purity > 98%, Institute Meteria Medica). NMDA, MK-801, memantine and Aβ1–42 were purchased from Sigma–Aldrich Chemicals Inc. (St Louis, MO, USA). All drugs were dissolved in saline and freshly prepared before use.

2.3. Soluble Aβ1–42 oligomers preparation

Soluble Aβ1–42 oligomers were prepared as reported previously. Aβ1–42 was dissolved to 1 mmol/L with hexafluoroisopropanol (HFIP, Sigma), separated into aliquots, and was stored at −80 °C after evaporation of HFIP. Soluble Aβ1–42 oligomers were prepared freshly before use. The peptide was resuspended in DMSO to 5 mmol/L, and diluted by cold F12 medium to yield a 100 μmol/L stock solution, then incubated at 4 °C for 24 h. The preparation was then centrifuged at 14,000 × g for 10 min at 4 °C. The supernatants were kept at 4 °C and further used for electrophysiological experiments. Solvents processed meanwhile were used as control.

2.4. Hippocampal neuron culture

Rat hippocampal neurons were isolated and cultured from Wistar rats on postnatal day 0–1 according to protocols modified from Kaech and Banker. Hippocampi were isolated and incubated in trypsin (0.1%) for 30 min. Neurons were initially cultured in DMEM with 10% fetal bovine serum and 10% horse serum on poly-D-lysine coated glass coverslips. After 4 h the medium was removed and changed to Neurobasal-A with B27 medium (2%). Then culture medium was half-changed every 3 days. 10 μmol/L cytosine arabinoside (AraC) was added on day in vitro (DIV) 2, and removed on DIV 3. Whole cell recordings were performed between DIV 9–14.

2.5. LTP recordings in vivo

The rats were anesthetized with urethane (20%, 1.3 g/kg, i.p.) and then positioned in a stereotaxic frame. The skull was exposed and two holes were drilled for the placement of electrodes. A stainless bipolar stimulating electrode was placed in the perforant path (7.5 mm posterior to bregma, 4.2 mm lateral to the midline, 2.8–3.5 mm ventral). A recording electrode was placed in the dentate gyrus of the same side (3.8 mm posterior to bregma, 2.0 mm lateral to the midline, 3.0–3.5 mm ventral). A separate hole (1 mm posterior to bregma, 1.2 mm lateral to the midline, 3.5 mm ventral) was drilled to allow a guide cannula for intracerebroventricular (i.e., i.v.) injection of drugs or soluble Aβ1–42 oligomers. Silver wire was fixed to the bone or skin and used as reference and ground. The population spike (PS) was obtained from the dentate gyrus in response to stimulation in the perforant path at a frequency of 0.033 Hz with single constant current pulse (100 μs in duration). The PS was collected and amplified by TDT RA16PA amplifier.
and digitized by TDT RX7-5 processor (Tucker-Davis Technologies, USA). The depth of both electrodes was adjusted until the maximal response was observed. At that point, the intensity of the test stimuli was adjusted until it evoked about 40% of the maximum response of PS amplitude. LTP was induced by high frequency stimuli (HFS) delivered at 100 Hz, 10 stimuli, repeated 10 times at an interval of 300 ms. Pulse width stayed the same with the test stimuli, and intensity doubled.

Stable baseline PS responses were recorded for at least 15–20 min prior to drug applications. Soluble Aβ_{1-42} oligomers (1 nmol/L in 5 μL) or vehicle or drugs were injected i.c.v. at 1 μL/min. After injection, the cannula remained in place for 5 min before starting injections. Thus it took 10 min for injection of one drug. Volume of drugs or soluble Aβ_{1-42} oligomers was calculated based on the volume of rat cerebrospinal fluid, which was estimated about 500 μL according to previous reports.8,19.

2.6. Whole cell patch clamp recordings

Whole cell patch clamp recordings were performed using EPC-10 amplifier (HEKA Elektronik, German) at room temperature.

For sodium currents: the internal solutions contained (in mmol/L): CsF 140, NaCl 10, EGTA 1, HEPES 10 (300–310 mOsm with sucrose, pH 7.25). The external solution contained (in mmol/L): NaCl 140, MgCl_2 1, CaCl_2 1, CdCl_2 0.2, HEPES 10, Glucose 10, 4-aminopyridine (4-AP) 5 (310–330 mOsm with sucrose, pH 7.4). After whole cell recordings were assessed, membrane potential was clamped at −80 mV. Sodium currents were activated by 50 ms pulse from −80 to +40 mV in 10 mV steps.

For potassium currents: the internal solutions contained (in mmol/L): KCl 140, EGTA 10, CaCl_2 1, HEPES 10 (300–310 mOsm with sucrose, pH 7.25). The external solution contained (in mmol/L): Choline Cl 140, KCl 5, MgCl_2 1, CaCl_2 1, HEPES 10, Glucose 10 (310–330 mOsm with sucrose, pH 7.4). After whole cell recordings were assessed, membrane potential was clamped at −70 mV. For potassium currents activation, firstly holding potential was depolarized to −110 mV for 250 ms, then was applied 250 ms pulse from −40 to +60 mV in 10 mV steps.

For calcium currents: the internal solutions contained (in mmol/L): CsCl 130, EGTA 10, HEPES 10, ATP-Mg 5 (300–310 mOsm with sucrose, pH 7.25). The external solution contained (in mmol/L): Choline Cl 130, MgCl_2 1, BaCl_2 10, HEPES 10, Glucose 10, TTX 1, 4-AP 5, 42 oligomers 5 (310–330 mOsm with sucrose, pH 7.4). After whole cell recordings were assessed, membrane potential was clamped at −90 mV. Calcium currents were activated by 200 ms pulse from −50 to +50 mV in 10 mV steps.

For NMDAR and AMPAR currents: the internal solution contained (in mmol/L): K-glutamate 140, NaCl 10, CaCl_2 1, EGTA 10, HEPES 10, ATP-Mg 5, GTP- Na 0.2 (310–320 mOsm with sucrose, pH 7.25 with KOH). The external solution contained (in mmol/L): NaCl 150, KCl 5, CaCl_2 2, MgCl_2 1, Glucose 10, HEPES 10 (320–330 mOsm with sucrose, pH 7.4 with NaOH). 0.1 μmol/L TTX was added when used. Patch pipettes were pulled from glass capillaries with resistance 3–4 MΩ. The junction potential was close to 14 mV and corrected. After whole cell recordings were accessed the membrane potential was clamped at −70 mV. After membrane rupture, 5 min were allowed for equilibration between neuron and the internal solution. Currents were sampled at 10 kHz by Pulse v6.74 (HEKA). Series resistance and capacitance were monitored throughout the experiments. Data were discarded if series resistance and capacitance were changed by >20%. NMDAR and AMPAR currents were evoked by pressure application system. A glass capillary (tip diameter 2–5 μm) containing 100 μmol/L NMDA and 10 μmol/L glycine or 3 μmol/L AMPA was positioned at a distance of 10–20 μm to the somata of the neuron. A picospritzer (PV830, WPI, USA) was used to apply a pressure of 2–5 psi for 2 s duration to elicit currents at an interval of 2 min. Tetrodotoxin (TTX), soluble Aβ_{1-42} oligomers and other drugs were bath perfused.

2.7. Data analysis

For LTP recordings, PS amplitudes were analyzed using Matlab v7.1 (Mathworks) as previously reported. Briefly, every data point was calculated as average of 10 PS amplitude values in 5 min. The baseline was calculated as average of PS amplitude 30 min before HFS, and each time point was calculated as percentage of the baseline values. LTP was induced successfully if PS amplitudes after HFS increased 30% than baseline. LTP values expressed here are those at 55–60 min after HFS, unless stated otherwise. NMDAR currents and AMPAR currents were all normalized to the first current evoked and all results were shown in a relative way. All data were expressed as mean ± SEM. Difference between groups was detected by two-way ANOVA with repeated measures in LTP recordings. Subsequently, the student’s t test or one-way ANOVA was performed in other experiments. The results are statistically significant when P value was less than 0.05.

3. Results

3.1. Soluble Aβ_{1-42} oligomers inhibited NMDAR-dependent LTP induction

The rats were anesthetized and then positioned in a stereotaxic frame. The population spike (PS) was obtained from the dentate gyrus in response to stimulation in the perforant path. LTP was induced by high frequency stimuli (HFS, described before) successfully and it could be abolished by 5 mg/kg MK-801 (i.p., Fig. 2B and 2D). Memantine (10 mg/kg, i.p.), an open channel antagonist of NMDA receptors20. This indicated that the LTP from the perforant pathway to the dentate gyrus (PP-DG) was NMDAR-dependent. Soluble Aβ_{1-42} oligomers were given i.c.v. in rats 60 min before HFS as shown in Fig. 2D, which significantly inhibited LTP induction (184.7 ± 7.6% vs. control 196.7 ± 20.5%, P < 0.05, n = 5, Fig. 2A and 2C). MK-801 is a noncompetitive blocker of NMDA receptors20. This indicated that the LTP from the perforant pathway to the dentate gyrus (PP-DG) was NMDAR-dependent. Soluble Aβ_{1-42} oligomers were given i.c.v. in rats 60 min before HFS as shown in Fig. 2D, which significantly inhibited LTP induction (148.7 ± 6.5% vs. control group 195.1 ± 9.6%, P < 0.05, n = 5, Fig. 2B and 2D). Memantine (10 mg/kg, i.p.), an open channel competitive inhibitor of NMDA receptors19, (clinically used for treatment of AD) given before Aβ injection significantly ameliorated Aβ-induced inhibition of LTP (186.6 ± 12.6% vs. Aβ_{1-42} group 148.7 ± 6.5%, P < 0.05, n = 5, Fig. 2B and 2D). These results suggested that Aβ-induced inhibition of LTP was related to NMDA receptors.

3.2. Parishin C ameliorated the suppression of LTP induced by Aβ_{1-42}

Parishin C 20 mg/kg was given i.p. two days consecutively and then given a third time before the LTP recordings as shown in Fig. 3A and 3B. Attenuation of LTP mediated by soluble Aβ_{1-42} oligomers was significantly ameliorated by parishin C (179.0 ± 8.4% vs. average in the PS recordings).
Figure 2 Memantine rescued inhibition of LTP induced by soluble Aβ1–42 oligomers in rats. (A) and (B) showed original traces of PS before (1) and after (2) HFS. (C) 5 mg/kg MK-801 abolished HFS induced LTP (control group n=4; MK-801 group n=5). (D) 2 μmol/L soluble Aβ1–42 oligomers significantly inhibited LTP (vs. control group, P < 0.05), and 10 mg/kg memantine administrated i.p. in advance rescued this inhibition (control group n=7, Aβ1–42 group n=5, memantine group n=5). Data were shown as mean ± SEM, *P < 0.05 vs. control group, #P < 0.05 vs. Aβ1–42 group.

Figure 3 Effects of parishin C on inhibition of LTP induced by soluble Aβ1–42 oligomers in rats. (A) showed original traces of PS before (1) and after (2) HFS in four groups. (B) Parishin C 20 mg/kg was given i.p. for two days before LTP recordings and was given i.p. 10 min before i.c.v. injection of 2 μmol/L soluble Aβ1–42 oligomers. (C) Parishin C 10 μmol/L was given i.c.v. before 2 μmol/L soluble Aβ1–42 oligomers injection. HFS was performed 60 min after injection of soluble Aβ1–42 oligomers in all experiments. Parishin C improved LTP induction significantly after Aβ treatment (P < 0.05). Data were shown as mean ± SEM, control group n=7, Aβ1–42 group n=5, pC (i.p.) group n=5, pC (i.c.v.) group n=5, *P < 0.05 vs. control group, #P < 0.05 vs. Aβ1–42 group.
Parishin C prevents Aβ_{1-42}-induced inhibition of LTP

3.3. Effects of parishin C on ion channels on normal cultured rat hippocampal neurons

The above results demonstrated that parishin C is a potent compound for improving LTP deficits caused by Aβ. Further mechanisms of parishin C on LTP and relevant receptors were studied. In LTP recordings, PS amplitudes prior to HFS represented the basic synaptic transmission. The data showed that 20 mg/kg parishin C given i.p. did not influence either basic synaptic transmission or LTP induction in normal rats (Fig. 4A).

NMDA receptors and AMPA receptors are the main glutamate receptors responsible for excitatory postsynaptic ion channel currents, and they are essential for basic synaptic transmission and LTP formation. Our results showed that parishin C at 10 μmol/L had no influence on either of these ion channel currents in hippocampal pyramidal neurons (Fig. 4B and C).

The effects of parishin C on voltage-dependent channels were also studied. At 10 μmol/L, parishin C had no effect on the currents of sodium channels, potassium channels or calcium channels of neuronal cells (Fig. 5A–C). The I–V (current and voltage relationship) curves obtained before and after parishin C treatment did not differ for each channel. These results demonstrated that parishin C did not affect the major voltage-dependent ion channels under normal conditions.

3.4. Parishin C rescued attenuation of Aβ_{1-42} on NMDAR currents

The above results showed that Aβ reduced the activity of NMDAR-dependent LTP but had no effects on PS before HFS. Parishin C also had no effects on AMPAR currents and NMDAR currents, while parishin C protected against the effects of Aβ. The results demonstrated that the mechanism of parishin C might be related to interactions between Aβ and NMDA receptors. Then we studied the effects of Aβ on AMPAR and NMDAR currents in primary cultured hippocampal pyramidal neurons as well as the role of parishin C in this system. As shown in Fig. 6A and C, perfusion of 2 μmol/L Aβ_{1-42} oligomers did not influence AMPAR currents. However, 2 μmol/L soluble Aβ_{1-42} oligomers reduced NMDAR currents to 71.0 ± 5.0% of control (vs. before perfusion 93.6 ± 4.0%, n=5, P<0.05, Fig. 6B and D). The currents did not recover during washout, and continued to decline to 44.1 ± 7.1% (vs. Aβ_{1-42} group, P<0.05, Fig. 6D). In the following experiments, parishin C was perfused 10 min in advance and continued during Aβ_{1-42} perfusion (Fig. 6B and D). The NMDAR currents inhibited by Aβ_{1-42} were attenuated by parishin C. Since parishin C had no effects on NMDAR currents (Fig. 4C and 6D), these results suggested that parishin C protected NMDA receptors from the damage of Aβ, and subsequently protected against LTP suppression.
4. Discussion

GE is widely used in China and many Asian countries as a traditional drug and food supplement for improving learning and memory as well as protection of brain function. Recently, it was found that an active fraction isolated from the crude extract of GE contained parishin, parishin B and parishin C, improved the performance of rodents in scopolamine-induced cognitive deficits. In the present study we aimed at the pharmacological effects of parishin C on cognitive disorders and LTP impairment induced by Aβ, as well as the electrophysiological mechanisms related to NMDA receptors.

In accordance with behavioral tests, our results indicated that parishin C is a potent agent for improving learning and memory. Parishin C given i.p. had no effects on normal rats, but it protected LTP deficits induced by soluble Aβ oligomers. Parishin C (10 μmol/L) delivered directly into the brain via i.c.v. also restored the LTP inhibition by soluble Aβ oligomers. This indicated that parishin C might cross brain blood barrier to protect the central nervous system. In our previous studies, we investigated dose-dependent effects of parishin C on scopolamine mediated inhibition of LTP in vivo. In this study, the high dose (20 mg/kg) of parishin C was selected to observe the electrophysiological mechanisms. The patch clamp recordings indicated that the nootropic effects of parishin C might be involved in protection of NMDA receptors from the injury of Aβ.

Although the mechanisms of AD are not well understood, Aβ is believed to be centrally involved. Compared with Aβ plaques, soluble Aβ oligomers correlate more strongly with AD-type dementia. Previous reports demonstrated that soluble Aβ oligomers impaired functions of synapse and both are required in learning and memory procession and LTP formation. NMDA receptor activation needs both glutamate and membrane depolarization, in order to reverse Mg2⁺ block. AMPAR currents are the main form of spontaneous and fast excitatory synaptic transmission, while NMDA receptors are essential for induction of LTP. Thus in our research, firstly, we demonstrated that LTP in our system was NMDA-receptor-dependent, and that the inhibitory effects of Aβ might be related to NMDA receptors. Secondly, Aβ had no effects on baseline PS, but significantly inhibited LTP induction. Thirdly, acute Aβ perfusion had no effects on AMPAR currents, but it irreversibly inhibited NMDAR currents. Results in vivo and in vitro are identical, which indicate that acute Aβ administration modulates NMDA receptors and inhibits LTP induction.

In the following, whether soluble Aβ oligomers–induced impairment of LTP could be prevented by parishin C was investigated. Parishin C neither had effects on basic synaptic transmission, nor on LTP levels in normal rats. It also had no effects on NMDAR currents or AMPAR currents, or voltage-dependent channels in normal cultured hippocampal neurons. However, parishin C attenuated soluble Aβ oligomers–induced inhibition of LTP given via i.p. and i.c.v. Furthermore, it was interesting that the Aβ oligomer–induced reduction of NMDAR currents in primary cultured hippocampal neurons, but did not affect the AMPAR currents at the same concentration. Parishin C obviously prevented Aβ-induced attenuation of NMDAR currents. Therefore we suggested that actions of parishin C might be related to NMDA receptors. It may decrease the toxicity of soluble Aβ oligomers on the receptors. Soluble Aβ oligomers–induced dysfunction

Figure 5 10 μmol/L parishin C had no effects on voltage-dependent currents in cultured hippocampal neurons of rats. (A) parishin C (10 μmol/L) had no effects on voltage-dependent total sodium currents (n = 5), holding potential = -80 mV. (B) parishin C (10 μmol/L) had no effects on voltage-dependent total outward potassium currents (n = 3), holding potential = -70 mV. (C) parishin C (10 μmol/L) had no effects on voltage-dependent total calcium currents (n = 3), holding potential = -90 mV. Data are shown in mean ± SEM.
of NMDA receptors might involve various pathways. For example, Aβ disrupts calcium homeostasis\(^28\), and influences phosphorylation of NMDA receptors, especially on NR2A and NR2B subunits\(^18,29,30\). In addition, Aβ might down-regulate surface expression of NMDA receptors in the synapse\(^31\). NMDA receptors have been suggested as an important target of Aβ\(^32,33\), and they are closely related to the inhibition of LTP\(^34–38\). In our study we used NMDAR antagonist d-APV, NMDA receptor subtype NR2A antagonist NVP-AAM077 and NR2B antagonist Ro 25-6981. Parishin C could not reverse inhibitory effects of APV, NVP-AAM077 or Ro 25-6981 (data not shown). Aβ and parishin C might bind to specific sites on the receptors other than the binding sites of APV, NVP-AAM077 and Ro 25-6981. It is possible that parishin C binds to NMDA receptors and prevents Aβ binding to the receptors.

The effects of AMPA receptors might involve various pathways. For example, Aβ disrupts calcium homeostasis\(^28\), and influences phosphorylation of NMDA receptors, especially on NR2A and NR2B subunits\(^18,29,30\). In addition, Aβ might down-regulate surface expression of NMDA receptors in the synapse\(^31\). NMDA receptors have been suggested as an important target of Aβ\(^32,33\), and they are closely related to the inhibition of LTP\(^34–38\). In our study we used NMDAR antagonist d-APV, NMDA receptor subtype NR2A antagonist NVP-AAM077 and NR2B antagonist Ro 25-6981. Parishin C could not reverse inhibitory effects of APV, NVP-AAM077 or Ro 25-6981 (data not shown). Aβ and parishin C might bind to specific sites on the receptors other than the binding sites of APV, NVP-AAM077 and Ro 25-6981. It is possible that parishin C binds to NMDA receptors and prevents Aβ binding to the receptors.

5. Conclusions

We studied the effects of parishin C on LTP deficits induced by soluble Aβ\(_{1-42}\) oligomers. Inhibition of ion channel currents by Aβ which were mediated by NMDA receptors could be prevented by parishin C. Therefore, parishin C could be a potent compound for treating neuronal degenerative diseases.
Acknowledgments

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