

Uncaria rhynchophylla exerts a neuroprotective effect against febrile seizures by inhibiting NMDAR-mediated neuronal excitability

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Abstract

Uncaria rhynchophylla (UR) is a traditional Chinese medicine widely used for disorders of the nervous and cardiovascular systems. Febrile seizures (FS), the most prevalent cause of seizures in young children, remain lacking effective clinical therapies. This study aimed to explore UR's neuroprotective effect against FS-induced neuronal injury and its mechanism. We used animal behavioral to evaluate the severity of FS; hematoxylin-eosin (HE) staining, Nissl staining, and immunohistochemistry (IHC) to assess the neuroprotective effect of UR by detecting neuronal injury; molecular docking and molecular dynamics (MD) simulations to verify target binding sites; the CCK-8 assay to assess UR-mediated neuroprotection and cell viability in SH-SY5Y cells; and patch-clamp technique to analyze neuronal excitability. Results showed that UR significantly reduced the severity of FS, especially the incidence of generalized tonic-clonic seizures, and decreased neuronal necrosis and apoptosis in FS models. It also downregulated myelin formation marker MBP and glial activation marker S100 β , and decreased neuronal excitability in a dose-dependent manner. UR's active components, rhynchophylline (RHY) and isorhynchophylline (IRHY), stably bound to NMDARs, downregulated NMDAR 2A/2B subunits, and inhibited NMDAR-mediated evoked excitatory postsynaptic currents (eEPSCs). In conclusion, UR effectively protects neurons from FS-induced neurotoxicity, holding promising potential as a preventive agent for FS.

1. Introduction

Febrile seizures (FS), the most prevalent type of seizure in pediatric populations, primarily affect children aged 6 months to 5 years, with a global incidence reported between 2%-5%^{[2][3]}. Although most children are going well after getting this disease^[4], however, previous studies have found that FS may be related to later seizures. Some studies have said that children with FS have a 6% chance of developing epilepsy later^[5]. Additionally, another study demonstrated that 2–8% of children with recurrent FS develop temporal lobe epilepsy^[6]. FS is due to the aberrant electrical discharges of neurons in the brain, clinically presenting as convulsive seizures involving either focal or generalized muscle spasms^[7]. Such seizures can alter the excitability and synaptic transmission of central neurons, ultimately resulting in neuronal injury^[8]. Consequently, ignoring the risks associated with FS is unwise, and effective handling of the disorder is crucial.

UR has the functions of calming endogenous wind, tranquilizing the mind, clearing heat, and regulating the liver^[9]. UR, with its key active constituents, alkaloid compounds, has been demonstrated to exhibit significant neuroprotective effects. The active ingredients of UR, rhynchophylline (RHY) and isorhynchophylline (IRHY), have been found to effectively inhibit effects on neuronal cell apoptosis^[10]. The crude alkaloid extract of UR has demonstrated the ability to reduce NMDA-induced hippocampal neuron injury in rats^[11]. It has been reported that the UR exerts a significant inhibitory effect on glutamate (Glu)-induced apoptotic cell death in a dose-dependent manner^[12]. Furthermore, UR exhibited pharmacological effects such as sedation, anticonvulsant, and anti-epileptic properties^[13]. The

effectiveness of UR in treating FS has been established, demonstrating its capacity to decrease their recurrence. However, the specific cellular and molecular mechanisms through which UR exerts its therapeutic effects remain to be fully elucidated.

In the present study, we discovered that UR could stably bind to NMDARs, inhibit the expression of NMDAR 2A/2B subunits and the excitatory synaptic transmission mediated by these subunits, and simultaneously downregulate the expression of pathology-related proteins such as MBP and S100 β . UR alleviated hippocampal neuronal injury in a dose-dependent manner, thereby reducing neuronal excitability and restoring neural network homeostasis. In summary, this study establishes the potential therapeutic effect of UR on FS-induced epileptic seizures and uncovers its core mechanism of action targeting NMDARs.

2. Material and methods

2.1. Animals

All animal experiments were performed in accordance with protocols approved by the Animal Ethics Committee of Chengde Medical University (Approval No. CDMULAC-20211109-026, Chengde, China). Our animal care routines and experimental methods strictly followed the rules detailed in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health, and the ARRIVE guidelines for disseminating animal research findings^[14]. Males and females of the Sprague-Dawley rat strain (P14), weighing 26–40 g, were procured from the Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). The rats were kept in an environment where the lighting alternated between 12-h light/dark cycle. Moreover, they were permitted to free access to food and water.

2.2. Plant material

UR (Miq.) Miq. ex Havil. (Batch No. 355210902, Anguo Jinglongkang Pharmaceutical Co., Ltd.) was verified by Dr. Ma Chunyan of Hebei Institute for Drug and Medical Device Control.

2.3. Preparation of UR water extract

The dried hooks (200 g) were powdered through a 24 mesh sieve and water was soaked in a ratio of 1:10 for 1h, refluxed for 30 min, and finally filtered. The filtrate was prepared into freeze-dried powder under vacuum drying conditions(16.9601g).

2.4. Animal behavioral experiments

Considering that FS occur in childhood, P14 SD rats were selected as the subjects of this study. UR was dissolved in 0.9% NaCl solution and intraperitoneally injected into the rat pups with indicated doses (low dose: 1g/kg, moderate dose: 5g/kg, high dose: 10g/kg, based on the weight of the crude drug) 1h prior to hyperthermic induction. After that the rats were rendered FS by an incubator (45°C). With reference to the

Racine Scale(1972), facial automatisms served as a marker for the onset of FS^[15]. The animals were observed 15 min post-heat in the incubator, which was preheated to 45°C.

2.5. HE staining

Twenty-four hours after the behavioral testing, the animals were anesthetized using 2.5% isoflurane in oxygen. A portion of the hippocampal tissue was promptly dissected and stored at -80°C, while the remaining brain tissue was collected and fixed in 4% paraformaldehyde. Brain tissues were rinsed with running water, then dehydrated via graded ethanol and infiltrated with xylene for transparency. The brain tissues were embedded in paraffin wax and overnight at 37°C. Following that, the brain tissue samples were meticulously sliced into 4-µm-thick sections and stored at 4°C overnight. The tissue specimens were then dewaxed in xylene for 30 min before being hydrated through a series of graded ethanol solutions. Next, the sections were subjected to hematoxylin staining for a brief four minutes, followed by a quick dip in 1% acid alcohol to differentiate the cells, and then a 30-second soak in a diluted ammonia solution to set the color. This was all capped off with a 3-minute eosin stain to highlight the cellular structures. Post-staining, the sections were rehydrated using an ethanol gradation, cleared in xylene for 15 min, and finally mounted using a neutral resin for preservation. Morphological changes in hippocampal neurons were examined using an Olympus light microscope.

2.6. Nissl staining

Paraffin sections at 4 µm were dewaxed in xylene for 30 min and subsequently hydrated through a graded ethanol series, placed in 0.5% toluidine blue solution at 56°C for 10 min, controlled color separation under the microscope in 95% alcohol, dehydrated with gradient ethanol, infiltrated in xylene for 15 min, and finally mounted with neutral balsam. Hippocampal cell morphology was examined using an Olympus light microscope.

2.7. Immunohistochemical staining

Paraffin slices of 4 µm were subjected to xylene dewaxing for 30 min before being hydrated via a gradient ethanol series, repaired by microwave heating in 0.01 mol/L sodium citrate buffer (pH 6.0) for 20 min, and incubated with endogenous peroxidase blocker at 37°C for 30 min. Overnight with appropriately diluted primary antibodies (MBP, S100β, NMDAR 2A, NMDAR 2B) at 4°C. Twenty-four hours post-treatment, the samples were then rewarmed to 37 degrees Celsius for a half-hour, followed by an incubation period in the reaction-enhanced solution at the same temperature for 30 minutes. Subsequently, they were kept in contact with the enhanced enzyme-labeled goat anti-mouse/rabbit IgG polymer at 37 degrees for 30 minutes. The sections were next processed using Diaminobenzidine for 5 minutes, counterstained with hematoxylin for 5 minutes, and differentiated in a 1% acid alcohol solution for a mere 5 seconds. Upon that, the specimens were dehydrated using a graded ethanol series and subsequently cleared in Xylene for a duration of 15 minutes. Finally, they were mounted using neutral resin. The expression of antibody in hippocampus was observed under Olympus optical microscope, and the average absorbance (A) value was analyzed by Image-J Software.

2.8. CCK8 determination

Cell viability was assessed using the CCK-8 assay. SH-SY5Y cells were seeded in 96-well plates at a density of 2×10^4 cells per well and cultured for 24 hours. Then, the cells were treated with different concentrations of Uncaria for 48 hours. After that, 10 μ L of CCK-8 solution was added to each well, followed by incubation for another 2 hours. The absorbance at 450 nm was measured using a microplate reader.

2.9. Electrophysiology experiments

Slices preparation was conducted in accordance with methods described in prior studies. This solution was continuously aerated with a gas mixture containing 95% oxygen and 5% carbon dioxide^[16]. Coronal sections of brain tissue blocks were obtained using a Model 1200 vibratome (Leica, USA). After incubating for 40 minutes, the slices were then stored at 25°C until further use. A differential interference contrast infrared microscope (Model: IR-1000E, DAGE-MTI, Michigan City, IN, USA) was used to observe brain slices, with a 40 $\times$ water-immersion objective lens (Model: D/N 6000 FS; Leica Microsystems, Germany) attached. Signals were recorded using an Axon 700B amplifier and an Axon 1550B digital interface (Molecular Devices, USA). Series resistance was compensated at 70%–80%; data were discarded if R_s variation exceeded 20%. Glass microelectrodes (3–6 M Ω) were fabricated using a P-1000 puller (Sutter Instrument Company, Novato, USA), with filling solutions containing (in mM) 145 K-gluconate, 2 MgCl₂, 2 Na₂ATP, 10 HEPES, and 0.2 EGTA (286 mOsm, pH 7.2) for action potential recording, and 115 Cs-methanesulfonate, 20 CsCl, 2.5 MgCl₂, 10 HEPES, 0.6 EGTA, 4 Na₂ATP, 0.4 Na₃GTP, 5 QX-314, and 0.1 spermine (280–290 mOsm, pH 7.3–7.4) for recording evoked Excitatory Postsynaptic Current (eEPSCs)^[17].

High-frequency activity, clocking in at 100 Hz and with a duration of 100 milliseconds, yielded action potentials and spike discharges in the mode of current clamp. The study utilized a current step protocol, which featured a range of extended (500 ms) positive current increments. These increments commenced at 0 pA and steadily escalated in 20 pA increments, culminating at 200 pA. To measure evoked currents, we placed a concentric tungsten stimulation electrode (Model 30205; FHC Inc., USA) in the stratum radiatum, 200 μ m away from CA1 pyramidal neurons, and applied stimuli at a frequency of 0.1–0.2 Hz with a pulse duration of 0.1 ms using an ISO-Flex stimulator (A.M.P.I., Jerusalem, Israel). The holding potential was maintained at +40 mV to record NMDAR-mediated eEPSCs. The perfusate (ACSF) was supplemented with picrotoxin (PTX, 100 μ M) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX, 10 μ M)^[18]. Subsequently, 1 mg/mL UR was added to the perfusate, and the NMDAR eEPSCs were recorded and analyzed before and after drug administration, respectively.

2.10. Analysis of molecular docking and molecular dynamics simulation

The NMDAR crystal structure (PDB ID: 6IRA) was sourced from the Protein Data Bank (PDB). Ligand structures (RHY and IRHY) were sourced from the PubChem database, with format conversion to mol2

carried out using Open Babel 3.1.1. AutoDock Vina was employed for molecular docking, and conformation with the lowest binding free energy was visualized via PyMOL 2.6.1.

MD simulations lasting 100 nanoseconds were conducted to determine the best docking conformations, utilizing GROMACS 2023.2. The setup incorporated the Amber99SB-ILDN force field and was immersed in a TIP3P water environment. To kick off the simulation, we knocked out any atomic clashes by carrying out energy minimization with the Steepest Descent method. Next, the system underwent two equilibration runs: first under the NVT ensemble for 100,000 steps (100 ps) and then under the NPT ensemble for 100,000 steps (100 ps). Production MD simulations (100 ns) were then executed with a 2-fs integration time step. Trajectories underwent periodic boundary condition correction to yield artifact-free coordinates. Protein Ca backbone RMSD, ligand positional RMSD, and hydrogen bond occupancy were quantified via xmgrace visualization. Analysis was performed using gromacs, where the RMSD of the protein Ca backbone and ligand sites was calculated, and the number of hydrogen bonds as well as their occupancy was statistically analyzed. This was followed by visualization using xmgrace.

2.11. Statistical processing

SPSS 25.0 and Clampfit 10.7 were utilized for statistical analysis, while GraphPad Prism 9.0 was employed for graph creation. Data shown as mean $\pm$ SEM. One-way ANOVA and Kruskal-Wallis test used for non-parametric comparisons. For paired samples, two-way ANOVA, paired t-test, and the Wilcoxon matched-pairs signed-rank test were used. p-values below 0.05 define the cut-off for statistical significance in all analyses.

3. Results

3.1 UR suppressed acute febrile seizures in a heat-induced rat model

Heat-induced rat models (Fig. 1A) were used to simulate clinical FS and the potential impact of UR on seizure activity was investigated. Seizure like episodes were tracked subsequent to heat-induced seizures; hyperthermic induction consistently promoted these events, as previously reported^[19].

The hyperthermic rat model exhibits numerous severe seizure-related symptoms, and subjects in the fever-induced seizure group show reduced activity and prolonged recovery time after seizures. In contrast, the rats treated with UR, had more tolerance to high heat, exhibited prolonged seizure latency, decreased Racine scores, and a reduced incidence of generalized tonic-clonic seizures. Besides, the rats recovered much rapidly than the control model rats. Seizure onset latency exhibited a significant increase beginning from 70.6 ± 4.46 s for model group to 138.6 ± 15.66 s, 175.7 ± 13.58 s and 249.1 ± 23.03 s, for UR treated group at the dose of 1 g/kg, 5 g/kg and 10g/kg, respectively (Fig. 1B). Besides, Racine score were decreased from 4.82 ± 0.12 in model group to 4.54 ± 0.21 in 1 g/kg UR group, 4.36 ± 0.24 in 5 g/kg UR group, and 4.00 ± 0.27 in 5g/kg UR group after UR treatment ($n = 11$, Fig. 1D). UR therapy markedly mitigated the intensity of fever-induced seizures, notably lowering the proportion

escalating to generalized convulsive events. Generalized tonic-clonic seizure incidence decreased from 81.8% (model group) to 63.6%, 54.5%, and 36.4% in the 1 g/kg, 5 g/kg, and 10 g/kg UR groups, respectively (Fig. 1C). The findings collectively suggest that UR markedly diminishes FS through elevating seizure latency, lowering seizure intensity, and decreasing the incidence of generalized tonic-clonic seizures.

3.2 UR reversed pyramidal neuronal injury in the CA1 region of the hippocampus induced by FS

Nissl and HE stains were conducted to gauge the extent of neuronal harm in rats subjected to FS. The HE stains revealed that the neurons in the hippocampal CA1 area of the control group exhibited typical morphology, maintaining their structural integrity and a neat, organized pattern (Fig. 2A). Numerous nuclei within the brain tissue of individuals with FS rats (control group) appeared pyknotic (shrunken and dark) and red neurons appeared, which means acute neuronal necrosis. The UR treated rats exhibited a reduced incidence of pathological changes in brain tissue (Fig. 2A), and the quantity of red neurons were decreased in a dose dependent manner, showing that UR treatment may enhance the typical structure of neurons. Nissl staining is a standard technique for estimating the hippocampal neuron count in unbiased stereoscopic logic. The Nissl bodies disappeared, the cytoplasm was pale and homogeneous, with irregular morphology and fewer hippocampal cells in control group rats compared with negative group rats (Fig. 2B). UR reversed the neuro-deficit, the number of neurons increased, and the neuron arrangement was more regular compared to control group, especially in high-dose group (UR 10 mg/mL). The findings collectively suggest that UR inhibits neuronal necrosis and demonstrates neuroprotection in FS rats.

IHC staining was conducted to quantify and localize the positive expression of MBP and S100 β . The results revealed distinct distribution patterns of these proteins within the rat hippocampus. MBP was predominantly localized in the medullary sheath. Similarly, S100 β was identified in the cytoplasm and nucleus. MBP was predominantly localized in the medullary sheath (Fig. 2C). Similarly, S100 β was identified in the cytoplasm and nucleus. Among the rats in the negative group, MBP positivity within the hippocampus comprised 10.43% of the hippocampal area, whereas in the model group, this percentage notably increased to 41.79%. The levels of MBP/S100 β positive expression decreased to 31.44%, 14.77%, and 12.04% at UR doses of 1 g/kg, 5 g/kg, and 10 g/kg, respectively (Fig. 2D). Similarly, for S100 β , the negative group exhibited 4.08% positive expression, contrasting with 30.55% in the model group. UR treatment at doses of 1 g/kg, 5 g/kg, and 10 g/kg reduced the positive expression rate of S100 β to 24.42%, 17.7%, and 10.65%, respectively (Fig. 2E). UR treatment significantly attenuated the expression of injury markers such as MBP and S100 β in the rat hippocampus. Notably, the magnitude of reduction positively correlated with the dosage administered. In conclusion, FS can significantly increase MBP and S100 β protein expression, which means that FS caused acute neuronal injury, while UR can reduce protein expression, indicating that UR played a protective role in neurons.

3.3 UR attenuated the firing activity of pyramidal neurons in the hippocampal CA1 region

Prior to conducting electrophysiological experiments, the effect of UR on the viability of SH-SY5Y cells was assessed using the CCK-8 assay. As shown in Fig. 3A, the treatment for 48 hours significantly decreased cell viability, with an IC_{50} value calculated to be 1.066 mg/ml. Based on this result, a concentration of 1 mg/ml was selected for subsequent experiments.

Neuronal hyperexcitability usually leads to seizures, so inhibition of neuronal excitability often relieves convulsions. To assess the impact of UR on neuron responsiveness, Whole-cell patch clamp was utilized to measure the CA1 pyramidal neuron membrane potential in the hippocampus, without the use of any synaptic blockers. As shown in Fig. 3B, action potential frequency escalated alongside augmented current injections. Figure 3C plots the curves relating spike count to injected current. The graph demonstrated a reduction in spikes triggered by the specified depolarizing pulses at a UR concentration of 1 mg/mL ($n = 10$; $p < 0.001$). In addition, although there was no significant difference, Rheobase rose from 20.00 ± 4.21 pA to 30.00 ± 3.33 pA ($n = 10$, $p > 0.05$; Fig. 3D) by 1 mg/mL UR. The threshold values in control group was -39.65 ± 0.85 pA and the value rised to -36.17 ± 1.01 pA after treated UR ($n = 10$, $p < 0.001$; Fig. 3E), membrane rest potentials were recorded as -58.03 ± 4.54 mV in the control and -60.77 ± 3.13 mV in the UR extract-treated group ($n = 10$; $p < 0.01$; Fig. 3F). Besides, UR treatment significantly attenuated the amplitude from 95.37 ± 4.54 mV to 88.41 ± 1.82 mV ($n = 10$; $p < 0.01$; Fig. 3G). Increased rheobase and threshold indicated reduced neuronal excitability and required stronger stimulation to excite action potentials, decreased resting membrane potentials also illustrated decreased neuronal excitability. Collectively, these results indicated that UR treatment decreased the excitability of CA1 pyramidal neurons.

3.4 The active ingredient of UR RHY and IRHY establish interactions with the target protein 6IRA stably

Molecular docking results indicated that both RHY and IRHY could form stable binding conformations within the active pocket of 6IRA. RHY binding yielded a dominant conformation with the lowest binding energy of -8.2 kcal/mol (Fig. 4A, left). This conformation was predicted to form two hydrogen bonds and two hydrophobic interactions (Fig. 4A, right). In contrast, IRHY binding exhibited a dominant conformation with the lowest binding energy of -7.3 kcal/mol (Fig. 4B, left). In addition to forming one hydrogen bond and four hydrophobic interactions, its binding mode was predicted to involve two salt bridge interactions (Fig. 4B, right). Based on the comprehensive analysis of binding energy and molecular interactions, both RHY and IRHY achieved stable binding at the active site of 6IRA through complementary molecular forces, hydrogen bonding, hydrophobic forces, and ionic linkages.

MD simulation results confirmed that the RHY-6IRA system reaches equilibrium after 80 ns and enters a stable state, during which the root-mean-square deviation (RMSD) fluctuations of both protein and ligand are maintained below 0.3 nm (Fig. 4C). The system consistently maintained 1–2 hydrogen bond interactions, each with a occupancy rate exceeding 80% (Fig. 4E), indicating a stable binding conformation. The IRHY-6IRA system achieved stability after 70 ns, with equilibrium RMSD fluctuations below 0.25 nm (Fig. 4D). It formed a stable interaction network involving 2–3 high-occupancy hydrogen bonds, among which a core hydrogen bond exhibited an occupancy rate of over 95% (Fig. 4F), further consolidating its binding stability. These results demonstrated that both the RHY-6IRA and IRHY-6IRA

complexes attained stable binding states, characterized by convergent conformational dynamics (low RMSD fluctuations) and persistent hydrogen bond interactions (key hydrogen bond occupancy > 80%).

3.5 UR impacted the expression of NMDAR 2A and 2B subunits and modulated NMDA-eEPSC within the hippocampal CA1 region

IHC staining was conducted to quantify and localize the positive expression of NMDAR 2A and 2B. NMDAR 2A and 2B was identified in the cytoplasm (Fig. 5A). The positive expression of NMDAR 2A in the rat hippocampus accounted for 12.4% of the area in the negative group, and a substantial increase to 27.5% in the model group. These levels decreased to 17.4%, 14.91%, and 12.45% at doses of 1 g/kg, 5 g/kg, and 10 g/kg, respectively (Fig. 5B). Moreover, NMDAR 2B expression was 8.716% in the negative group and increased to 37.17% in the model group. Treatment doses of 1 g/kg, 5 g/kg, and 10 g/kg led to reductions to 22.72%, 18.23%, and 12.83%, respectively (Fig. 5C). The results suggest that FS may exacerbate neuronal hyperexcitability by increasing expression of NMDAR proteins in hippocampal neurons. Expression of both receptor proteins was significantly elevated in the model group, UR dose-dependently decreased their expression. In conclusion, UR can downregulate the FS-induced increase in NMDAR 2A and 2B protein expression, exert a certain therapeutic effect on FS, and this effect exhibits dose dependency.

Utilizing the patch-clamp technique, NMDAR-eEPSC in hippocampal CA1 pyramidal cells SD rat neonates was recorded before and after drug administration. As illustrated in the figure, following UR administration, the NMDAR-eEPSC amplitude was reduced from 143.3 ± 10.68 pA to 111.9 ± 13.56 pA after 10 μ M UR treatment ($n = 9$; $p < 0.05$; Fig. 5D). The findings suggested that UR can reduce neuronal excitability by inhibiting NMDAR-mediated currents.

4. Discussion

FS represent the most common seizures in pediatric patients^[20], studies have indicated that FS can lead to hippocampal neuronal hyperexcitability, which cause ultrastructural modifications and altered protein expression in hippocampal CA1 and CA3^{[21][22]}. UR is part of a group of traditional Chinese medicinal plants with calming and seizure-suppressing properties, employed in the management of epilepsy^[30]. Pharmacological studies have shown that UR has certain guiding significance in the treatment of essential hypertension, epileptic seizures, major depressive disorder, Parkinson's syndrome, and Alzheimer's dementia.^[23] However, the research on the effect of UR in treating FS is less, and the mechanism study is not clarified. Our findings indicated that UR prevented the occurrence of febrile seizures by inhibiting NMDAR-mediated neuronal excitability and exerting a protective effect against neuronal injury.

The hippocampus has been reported to be responsible for generalized seizures^[24], as well as hyperexcitation of CA1 neurons and a change in activity-dependent forms synaptic plasticity^[25]. In order to better simulate FS in children, 14-day-old SD rats served as the experimental subjects in this study.

Rats in the FS group showed increased severity in seizures, escalating to tonic–clonic seizures. Even lower UR doses (1g/kg) could effectively prevent FS and enhance mental processing and societal conduct in rats with minimal discernible adverse effects.

HE and Nissl stains illustrate the hippocampal neuron morphology in the FS control group induced by heating is significantly worse than that of the normal group, indicating that FS cause acute neuronal necrosis. However, UR effectively reduces neuronal death, attenuated the degree of neuron injury and the neuron morphology gradually improved as the dosage of the drug increased, indicating that UR can indeed protect neurons. MBP, a lipoprotein specifically expressed in the nerve myelin sheath and primarily synthesized by oligodendrocytes, functions as a sensitive indicator of central nervous system (CNS) injury, as its expression level correlates with the extent of axonal injury^{[26][27][28]}. Central nervous system's injury may result in increased MBP expression, which is consistent with this study^[29]. UR can reduce the MBP expression. S100 β participates in the process of nerve injury and exhibits high sensitivity to the severity of brain injury, making it a viable indicator for evaluating the extent of nerve injury^[30]. In this study, S100 β expression levels dropped in UR, subsequently inhibiting glial proliferation and lessening neuronal cell death.

Under pathological conditions, the release of MBP and the upregulation of S100 β jointly contribute to the formation of a pro-inflammatory microenvironment. This microenvironment is characterized by the production of cytokines such as TNF- α , IL-1 β , and IL-6, which can subsequently mediate the phosphorylation of the NMDAR 2B subunit via kinases such as SRC. This response enhances NMDAR-mediated Ca²⁺ influx into neurons, ultimately leading to increased neuronal excitability and potentially predisposing to pathological states such as epilepsy^[31]. The results of electrophysiological experiments indicated that UR (1mg/mL) suppressed the spike numbers generated through ramp-up depolarization stimulus pulses. Besides, the rheobase and threshold were increased, meanwhile, the RMP and amplitude were decreased after treated UR. Ionotropic glutamate receptors can regulate neuronal excitability, and inhibiting these receptors can further suppress the generation of action potentials by restricting the excitatory activity of neurons^[32].

Molecular docking results revealed that both RHY and IRHY can bind stably to 6IRA, with favorable binding energies and complementary intermolecular interactions; molecular dynamics simulations further confirmed the stability of their binding, which is characterized by low RMSD fluctuations and high hydrogen bond occupancy. Immunohistochemical results suggested expression of NMDAR 2A and 2B in rat hippocampal CA1 neurons were up-regulated after FS, which displayed that NMDARs are involved in the process of FS, with increased receptor expression leading to increased receptor activity and increased excitatory neuron activity, thus disrupting the excitation-inhibition network balance in the cerebral cortex and ultimately leading to the occurrence of FS. It was observed that after UR treatment, the expression levels of NMDAR 2A and NMDAR 2B decreased, thereby exerting an inhibitory effect on NMDARs and reducing neuronal excitability. This conclusion was further verified in patch-clamp electrophysiological experiments, where UR was found to directly inhibit NMDA-eEPSC currents,

indicating that UR can suppress NMDAR-mediated excitatory synaptic transmission, which in turn confirms that UR has an inhibitory effect on NMDARs.

In conclusion, UR probably act on the NMDAR, inhibit the expression and function of the receptor, and thus reduce the activity of excitatory neurons. Thus, Utilizing UR could serve as a viable approach for addressing FS.

Conclusion

This study confirms that UR exhibits a definite protective effect against neurological injury induced by FS. UR dose-dependently alleviates hippocampal neuronal injury and downregulates the expression of pathology-related proteins such as MBP, S100 β ; it stably binds to NMDARs, inhibits the expression of NMDAR 2A/2B subunits and their mediated excitatory synaptic transmission, thereby reducing neuronal excitability and restoring neural network balance. In conclusion, UR exerts its neuroprotective effects by suppressing the NMDAR pathway, showing promise as a potential therapeutic strategy for FS.

Study Limitations and Future Directions

This study has certain limitations in the in-depth exploration and mechanism verification of brain-penetrant components. Although the quantitative determination of the dosage concentration of UR has been completed, the specific active components in the extract that can cross the blood-brain barrier (BBB), and exert anti-febrile seizure effects remain unclear. This not only makes it difficult to precisely identify the core active monomers mediating the neuroprotective effect, but also directly impairs the accuracy and reliability of translating the research findings into clinical practice. To address this limitation, subsequent studies should focus on identifying BBB-permeable components, screening the active ingredients, and elucidating their mechanisms of action, thereby providing a solid scientific basis for enhancing the clinical translation potential of the research.

Declarations

Conflict of interest statement

The authors have declared that no competing interest exists.

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Author Contribution

Yongzhou Yu, Xiaoyan Cui, Sixian Xiang and Wei Wu designed and conceived the research. Wei Wu and Leyi Hu performed HE staining and Nissl Staining experiments. Jiaojiao Zhao and Zirou Li carried out the immunohistochemical staining experiments. Tianxiang Li and Shuyao Guo conducted the animal experiments. Sixian Xiang, Nvgui Liu and Shuyao Guo performed the electrophysiology experiments. Nan Yang and Jinyu Wang collected and analyzed the data. Sixian Xiang, Wei Wu and Yongzhou Yu wrote and revised the manuscript. All authors approved the final manuscript.

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Footnotes

[1] Abbreviations: UR, uncaria rhynchophylla; FS, febrile seizures; HE, hematoxylin-eosin; IHC, Immunohisto-chemistry; CNS, central nervous system; ANOVA, one-way analysis of variance; MBP, myelin basic protein; S100 β , central nervous system specific protein β ; NMDAR 2A, N-methyl-D-aspartic acid receptor 2A; NMDAR 2B, N-methyl-D-aspartic acid receptor 2B; GFAP, glial fibrillary acidic protein; NSE, neuron-specific enolase; RHY, rhynchophylline; IRHY, isorhynchophyllin; TBST, Tris-buffered saline with 0.1%

Figures

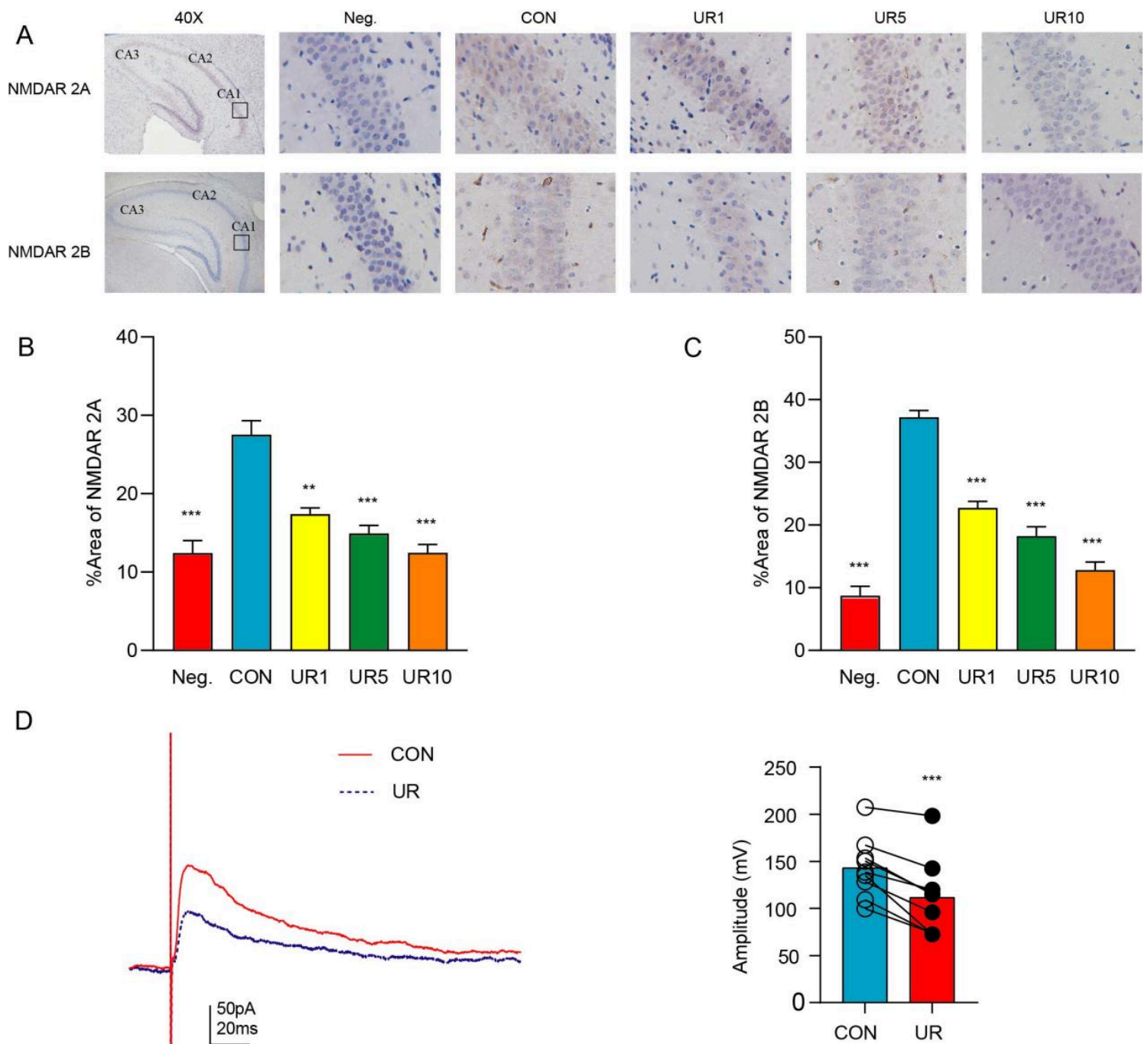


Figure 1

UR elevated seizure threshold in a rat model of acute FS. A. Experimental scheme displaying timing of drug administration and the procedural workflow. B. UR increased FS latency in a dose-dependent manner. C. Treatment with UR resulted in a reduction in seizure severity. D. The frequency of 5-stage seizures decreased in a dose-dependent manner following UR treatment. Results are expressed as mean $\pm$ SEM. Mean values at point B were analyzed using a standard one-way ANOVA with Dunn's multiple comparisons post hoc. Data in panel C were assessed using a nonparametric test followed by Dunn's multiple comparisons. For panel D, Pearson's chi-squared test was employed ($\chi^2 = 4.184$, $*p < 0.05$). Sample size was $n = 11$. Significance levels are indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the control group.

